Systematic and evolutionary studies of the terrestrial gastropods Helicoidea and *Pyramidula*

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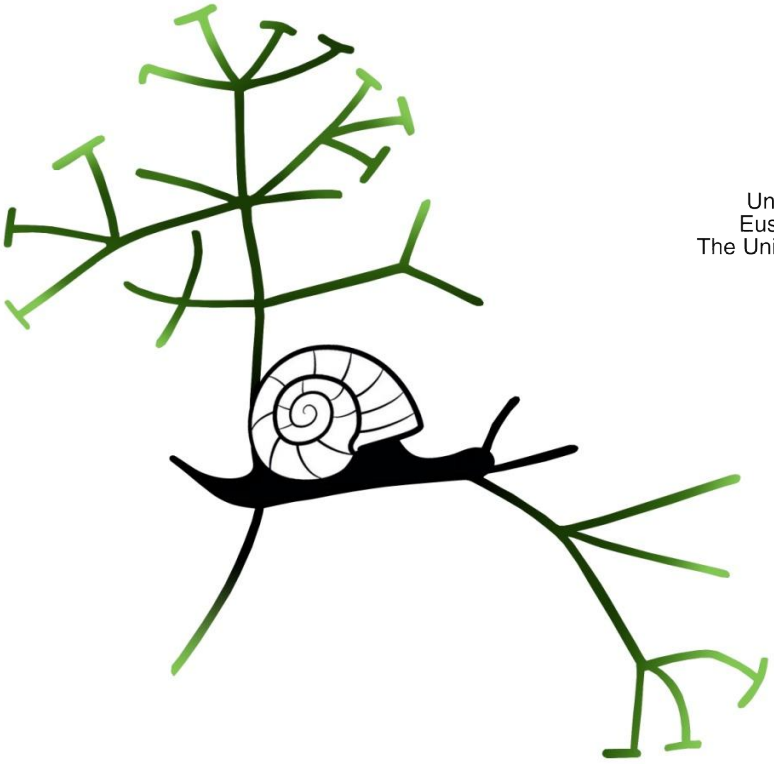

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Universidad del País Vasco
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Systematic and evolutionary studies of the terrestrial gastropods Helicoidea and *Pyramidula*

A thesis submitted by

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for the degree of Doctor of Philosophy,

under the supervision of

Dr. Benjamín Juan Gómez-Moliner and **Dr. María José Madeira**

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ABSTRACT

The clade Stylommatophora contains the vast majority of terrestrial gastropods, probably exceeding 30,000 species. Traditionally, the classification of its taxa has been based on morphology (shell morphology and anatomical characters of the reproductive system), some of them resulting in confusing and conflicting taxonomies. Therefore, phylogenetic relationships of stylommatophoran taxa from superfamily to species rank remain largely unresolved. The development of sequencing technologies allowed using the DNA to study phylogenies, producing large amounts of data to resolve phylogenetic questions for any taxonomic group. Molecular data together with other data such as morphology or ecological niche modeling procedure are being used to address phylogenetic and evolutionary questions. In this thesis these three lines of evidence are applied in order to resolve systematic and evolutionary questions for two stylommatophoran taxa: the worldwide distributed Helicoidea superfamily and the *Pyramidula* genus, the last one comprised by cryptic species.

First, a molecular phylogeny of the western Palaearctic Helicoidea is presented obtained by means of mitochondrial and nuclear rRNA gene fragments. Most of the morphologically-defined families are confirmed, but a revised phylogenetic classification is proposed in which families, subfamilies and tribes are monophyletic. The anatomy of the auxiliary copulatory organs of the reproductive system of families, subfamilies and tribes is highlighted. The origin of the Helicoidea is estimated at the end of the Early Cretaceous and its families as Late-Cretaceous to Paleogene. Western Palaearctic Helicoidea belongs to two different lineages that diverged around 86 Ma ago, both starting their diversification at the end of the Cretaceous (around 73-76 Ma). Radiation of some western Helicoidean families started during the Eocene.

Second, systematic and evolutionary studies are performed for *Pyramidula* in its western Palaearctic distribution range. Species discovery and validation methods are used in an integrative approach to delimit the species of the genus. Species boundaries are inferred from multiple lines of evidence arising from mitochondrial and nuclear DNA sequencing and ecological niche modeling. Nine putative species are identified for the western Palaearctic distribution range. Their phylogenetic relationships are also inferred. So far, descriptions of the morphospecies of this genus have been based exclusively on shell characters. Hence, the present work also explores the variation of shell characters to assess their utility to differentiate the species defined, and to examine whether shell shape in *Pyramidula* is influenced by environmental factors. The results indicate that although shell characters are useful to discriminate between some putative species, they are not sufficient as unique taxonomic characters and other lines of evidence are needed. According to the ecological niche modeling results, shell shape may be influenced by environmental factors.

Besides, restriction site-assocciated DNA sequencing (RADseq) is used to jointly assess unresolved phylogenetic questions, involving interspecific hybridization or incomplete

lineage sorting events and for accurate species delimitations in *Pyramidula*. Therefore, a robust phylogeny is inferred using a matrix of concatenated sequences of almost 1,500,000 bp long, fully resolving the phylogenetic relationships among species and resolving previous incongruences detected between mtDNA and nDNA gene trees. The best species delimitation scenario is tested using 875 unlinked single nucleotide polymorphisms, reaffirming that nine *Pyramidula* species should be distinguished in Europe. Applying D-statistics provide no or weak evidence of ancestral interspecific hybridization among the species of *Pyramidula.* We demonstrate that RADseq is a useful tool to resolve phylogenetic problems in closely related species complexes.

Finally, the evolutionary history of *Pyramidula* is studied using phylogenetic, distributional and morphological information. First, the biogeographical history of the western species of *Pyramidula* is inferred, reconstructing ancestral areas. Then, the individual responses of the species during climatic fluctuations are also evaluated, using the ecological niche modeling procedure. Finally, the evolution of shell shape is explored, by reconstructing ancestral states. The results indicate that different migration patterns shaped the diversification of the species in *Pyramidula*; that individual species responded with diverse range dynamics to climate fluctuations; and that cryptic species and the current variability on shell shape may have been the results of several adaptive and non-adaptive processes.

Given all the exposed above, this thesis aims to combine multiple approaches and methodologies from different disciplines to provide an important contribution to the knowledge on the systematics of terrestrial gastropods, and to help better comprehending the evolutionary history of them. This information is useful in the attempt of reconstructing the tree of life and contributing to complete the biodiversity inventory, which forms the basis for the effective conservation of species and habitats. Furthermore, it highlights the importance of jointly using several lines of evidence in order to explore and resolve phylogenetic and evolutionary questions.

RESUMEN

Capítulo 1

El primer capítulo de la tesis resume el desarrollo histórico, principios y prácticas de la biología evolutiva, enfatizando en las principales dificultades, disciplinas y conceptos que serán tratados a lo largo de la discusión. A continuación se ofrece un breve resumen:

La teoría de la evolución de Darwin y Wallace ha sido una de las ideas más revolucionarias de la historia. Aunque no fueron los primeros en sugerir que los organismos cambian con el tiempo, las dos principales ideas propuestas en *On the origin of the species* en 1859 cambiaron la forma de entender el mundo. Darwin concluyó que todas las especies descendían de uno o unos pocos ancestros comunes, con modificación, dando lugar al concepto darviniano de "descendencia con modificación" y apuntando a la selección natural como el agente causante de la evolución. La unificación de la teoría de Darwin y las leyes de Mendel, a comienzos del siglo XX, dio lugar a la *síntesis moderna de la evolución* donde se establecieron los principios de la evolución. Desde entonces, la biología evolutiva se ha mantenido como un campo activo en el que se han realizado avances muy significativos durante las últimas décadas.

Históricamente, la sistemática y la taxonomía tradicional se han encargado de describir y clasificar las especies, mientras que la filogenética trata, además, de establecer las relaciones evolutivas entre los diferentes grupos de organismos, a través de la determinación de sus filogenias. La filogenética reconstruye los patrones de descendencia, lo que permite comprender los eventos evolutivos que han ocurrido a lo largo de la historia de la vida. La reconstrucción de las filogenias requiere conocer la distribución de los caracteres primitivos y derivados en cada taxón, de forma que los organismos se agruparán de forma más cercana o lejana en el árbol en función de los caracteres evolutivos homólogos (heredados por ascendencia común) que muestren en común.

Tradicionalmente, las clasificaciones y las descripciones de las especies se han basado en caracteres morfológicos, siendo la morfología un pilar importante de la sistemática. Aún así, a menudo se han detectado incongruencias entre las clasificaciones tradicionales y las nuevas filogenias basadas en marcadores moleculares, sugiriendo la existencia de homoplasias (caracteres similares por analogía) para algunos caracteres morfológicos. El reciente avance en las tecnologías de secuenciación, ha incrementado el uso de la información del ADN en los estudios filogenéticos, a la par que se han desarrollado diversos métodos que permiten la reconstrucción de los árboles filogenéticos, los cuales pueden ser clasificados como métodos de distancia (NJ, ME) o métodos discretos (MP, ML, IB). Con el advenimiento de tecnologías de secuenciación genómica de alto rendimiento, el campo de la filogenética está entrando en una nueva era, favorecido por la disminución de los costes económicos y el significativo aumento de la cantidad de datos disponibles. Actualmente, se están desarrollando diferentes estrategias de partición genómica que permiten producir

grandes cantidades de datos filogenéticamente informativos que pueden ser utilizados de manera eficiente para resolver cuestiones filogenéticas prácticamente en cualquier grupo taxonómico.

A pesar de que los estudios en sistemática principalmente han aplicado datos morfológicos y moleculares, en los últimos años se observa un incremento de los trabajos que integran diversos tipos de información o evidencias (como, por ejemplo, morfología, secuencias de ADN y modelos de nichos ecológicos), especialmente en aquellos que tienen como objetivo la delimitación de especies. Las especies constituyen las unidades fundamentales en biología y son consideradas unidades de evolución, por lo que su delimitación es esencial para la biología sistemática, con implicaciones importantes para otras muchas disciplinas como la biogeografía, la ecología, y la biología evolutiva y de la conservación. Una vez definidas las especies y conocidas las relaciones filogenéticas existentes entre linajes o grupos taxonómicos, se puede comenzar a estudiar la historia evolutiva de los mismos, lo que permitirá poder entender y reconocer los procesos que han originado la diversidad morfológica, genética y/o ecológica que existe actualmente en la Tierra.

En esta tesis los estudios de sistemática y evolución se han aplicado a taxones de uno de los grupos animales más diversos, los gasterópodos terrestres. El clado Stylommatophora contiene la amplia mayoría de los gasterópodos terrestres, probablemente excediendo las 30.000 especies. Es un clado altamente diverso, actualmente clasificado en 103 familias. Tradicionalmente, las clasificaciones de sus taxones han sido basadas en la morfología, algunas de ellas generando taxonomías confusas y conflictivas. Por lo tanto, las relaciones filogenéticas de los stylommatophoros continúa presentando incertidumbres desde el rango de superfamilia al de especie. Este trabajo pretende resolver la sistemática e historia evolutiva de dos taxones de Stylommatophora de diferente rango y características, representativos de la fauna europea: la superfamilia Helicoidea con una amplia distribución a nivel mundial y el género *Pyramidula* restringida a la región Paleártica.

Capítulo 2

En el segundo capítulo se definen los objetivos generales y específicos de esta tesis, siendo el principal objetivo *"Incrementar el conocimiento en la sistemática e historia evolutiva de dos taxones de gasterópodos terrestres representativos de la malacofauna europea"*.

Capítulo 3

El tercer capítulo de la tesis incluye el primer artículo:

Artículo I: "**Molecular phylogeny of the western Palaearctic Helicoidea (Gastropoda, Stylommatophora)**" - "Filogenia molecular de Helicoidea del Paleártico occidental (Gastropoda, Stylommatophora)" *Molecular phylogenetics and evolution 83 (2015) 99–117*.

Este artículo ofrece el estudio filogenético y de la historia evolutiva de Helicoidea. La

superfamilia Helicoidea está clasificada en 19 familias distribuidas a nivel mundial, de las cuales ocho habitan en la región del Paleártico occidental. Es el grupo de moluscos terrestres más diverso de esta región, con su centro de distribución localizado en la región mediterránea, donde representan más de la mitad de la biodiversidad de los moluscos. Se han propuesto varios sistemas de clasificación, todos ellos basados en diferentes caracteres anatómicos del aparato reproductor, pero los nuevos datos basados en recientes filogenias moleculares han detectado incongruencias con las clasificaciones tradicionales, sugiriendo la existencia de homoplasias en algunos de sus caracteres anatómicos. De esta manera, la composición y las relaciones filogenéticas de algunas de sus familias, tribus y géneros permanecen sin resolver, evidenciando la necesidad de utilizar nuevos caracteres con suficiente señal filogenética. En este trabajo se presenta una filogenia molecular centrada principalmente en los helicoideos del Paleártico occidental y basada en el análisis del fragmento del gen mitocondrial 16S rRNA y el cluster de genes nucleares rRNA incluyendo el final 3' del gen 5.8S, la región ITS2 completa y el final 5' de 28S. La mayoría de las familias, definidas previamente mediante caracteres morfológicos, se confirman mediante estos nuevos análisis. Se propone una clasificación filogenética revisada, de manera que las familias, subfamilias y tribus sean monofiléticas. Además, se ofrece una diagnosis de cada taxón desde tribu hasta familia, basada en datos anatómicos. La familia Hygromiidae es dividida en tres clados a los que se les da rango de familia: Canariellidae y Geomitridae, que son reconocidas por primera vez como familias, y Hygromiidae (incluyendo *Ciliella* y *Trochulus*). Las subfamilias Ciliellinae, Geomitrinae, Hygromiinae, Monachainae y Trochulinae no se reconocen como grupos monofiléticos. A la familia Cochlicellidae se le da rango de tribu (Cochlicellini) perteneciente a Geomitridae. También se describe una nueva tribu: Plentuisini. Dentro de Helicidae se reconocen tres subfamilias: Ariantinae, Helicinae (incluyendo *Theba*) y Murellinae. Se estima que el origen de Helicoidea está situado al final del Cretácico Inferior y el de sus familias entre el Cretácico Superior y el Paleógeno. Los resultados muestran que los helicoideos del Paleártico occidental pertenecen a dos linajes diferentes que divergieron hace unos 86 millones de año, los cuales comienzan su diversificación al final del Cretácico (hace unos 73-76 millones de años). La radiación de algunas familias de Helicoidea comenzó durante el Eoceno.

Capítulo 4

El cuarto capítulo de la tesis, dividido en tres artículos, engloba los estudios de sistemática e historia evolutiva del género *Pyramidula*. Este género habita en la región Paleártica, mayoritariamente en el centro y sur de Europa, llegando hasta la península ibérica e islas Británicas por el oeste. También se encuentra en el norte de África y alcanza Asia, llegando hasta Japón. Habita únicamente en rocas calizas y puede ocupar zonas desde el nivel del mar hasta los 3000 m de altitud. Se alimenta de líquenes y algas y muestra una buena capacidad de dispersión pasiva, principalmente mediante aves. Varias de las especies del género presentan una distribución simpátrica. Se caracterizan por tener una concha de forma trocoide, aplanada en mayor o menor grado, con un amplio ombligo, y por no

superar los 3 mm de diámetro. Hasta mediados del siglo XX, los taxones de *Pyramidula* eran considerados como formas diferentes de una única especie, *P. rupestris*, pero a finales del siglo XX se propuso la existencia de seis especies en Europa. La identificación de las especies se ha centrado exclusivamente en caracteres conquiológicos, particularmente en la altura y el diámetro de la concha y la anchura del ombligo. Estos caracteres están estrechamente relacionados entre sí y generan dudas a la hora de intentar determinar a qué especie corresponde cada individuo. En varios listados faunísticos europeos no se asigna especie a los ejemplares de *Pyramidula*, ya que los caracteres establecidos no resultan siempre lo suficientemente claros. Además, la morfología del aparato reproductor, carácter muy utilizado en taxonomía de gasterópodos, no sirve, en este caso, como carácter diferenciador. Debido a que los caracteres morfológicos de la concha parecen ser, de alguna manera, subjetivos e insuficientes para identificar las especies, resulta evidente la necesidad de encontrar nuevos caracteres taxonómicos.

Artículo II: "**Species delimitation for cryptic species complexes: case study of *Pyramidula* (Gastropoda, Stylommatophora)**" - "Delimitación de especies para complejos de especies crípticas: caso de estudio de *Pyramidula*" *Cladistics* (en segunda revisión).

En este trabajo se emplean métodos de descubrimiento y validación de especies para poder delimitar las especies crípticas del género *Pyramidula*. Los límites de las especies se infieren utilizando varias fuentes de información, concretamente la información de secuencias de ADN mitocondrial y nuclear, y modelización de nichos ecológicos. Las relaciones filogenéticas de las especies se analizan aplicando los procedimientos de inferencia bayesiana, máxima verosimilitud y máxima parsimonia. El análisis de 211 ejemplares recolectados en la región Paleártica, permite identificar 9 especies para esta región. Teniendo en cuenta la morfología, distribuciones geográficas y la inclusión de topotipos, siempre que ha sido posible, se han podido nominar siete de las nueve especies definidas. Debido a que hasta ahora las descripciones de las morfoespecies se han basado exclusivamente en caracteres de la concha, también se ha estudiado la variabilidad de los mismos para poder determinar si son útiles a la hora de diferenciar las especies definidas o si la forma de la concha puede estar influenciada por factores ambientales. Los resultados indican que los caracteres de la concha sirven para discriminar entre algunas de las especies consideradas, pero no resultan caracteres diagnósticos para todas las especies, por lo que es necesario emplear otras fuentes de información. De acuerdo con los resultados obtenidos para la modelización de nichos ecológicos, se puede concluir que la forma de la concha está influenciada por factores ambientales. Los modelos ecológicos predicen un hábitat más favorable caracterizado por altitudes y precipitaciones más elevadas y temperaturas más bajas para aquellos ejemplares con morfologías de concha más aplanadas; mientras que las condiciones de hábitat caracterizado por un clima mediterráneo resultan más favorables para ejemplares de conchas de mayor altura relativa.

Artículo III: "**Species limits, interspecific hybridization and phylogeny in the cryptic land snail complex *Pyramidula*: the power of RADseq data**" - "Límites de especies, hibridación interespecífica y filogenia en el complejo críptico de caracoles terrestres *Pyramidula*: el poder de RADseq" *Molecular phylogenetics and evolution* (en revisión).

En este trabajo se emplea la novedosa técnica de secuenciación RADseq (Restriction site-assocciated DNA sequencing) para determinar y/o confirmar las relaciones filogenéticas, la presencia de eventos de hibridación interespecífica y la delimitación de las especies en el complejo de especies de *Pyramidula* en su rango de distribución occidental. La filogenia se infiere utilizando una matriz de secuencias concatenadas de aproximadamente 1.500.000 pares de bases de longitud, incluyendo más de 97.000 sitios polimórficos. Los análisis de máxima verosimilitud realizados, permiten resolver las relaciones filogenéticas existentes entre las diferentes especies y mejoran significativamente los árboles filogenéticos obtenidos previamente mediante los análisis de los genes mitocondriales y nucleares (COI, 16S rRNA, 5.8S rRNA, ITS2 y 28S rRNA). Las incongruencias detectadas entre los árboles de genes mitocondriales y nucleares son resueltas con la información ofrecida por RADseq. El mejor escenario de delimitación de especies es testado utilizando 875 SNP (single nucleotide polimorphism) no ligados, demostrando que en Europa deben considerarse nueve especies, de forma concordante con lo propuesto en el anterior trabajo. Los resultados muestran una evidencia prácticamente nula de hibridación interespecífica ancestral entre las especies de *Pyramidula*. Este trabajo demuestra que la técnica RADseq es útil para resolver dificultades filogenéticas en grupos de especies cercanas.

Artículo IV: "**Evolutionary history of the western species of *Pyramidula* (Gastropoda, Stylommatophora)**" - "Historia evolutiva de las especies occidentales de *Pyramidula* (Gastropoda, Stylommatophora)" *En preparación.*

Este último trabajo se centra en el estudio de la historia evolutiva de las especies occidentales del género *Pyramidula*. Su estudio es particularmente interesante, debido a la presencia de especies crípticas que pueden ser definidas mediante la integración de la información genética y de sus requerimientos de nicho ecológico. Por un lado, las especies cercanas que muestran una diferenciación de nichos ecológicos son buenas candidatas para estudiar sus respuestas ante fluctuaciones climáticas. Por otro lado, resulta también muy interesante intentar conocer los procesos que han dado lugar a la evolución fenotípica de estas especies crípticas, en el que pueden estar involucrados fenómenos tanto adaptativos como no adaptativos. Así pues, los objetivos de este trabajo se centran en (1) estudiar la historia biogeográfica de la especies occidentales de *Pyramidula*, reconstruyendo para ello áreas ancestrales; (2) evaluar las respuestas individuales de las especies durante las principales fluctuaciones climáticas (Último Máximo Glaciar, presente y futuro); y (3) explorar la evolución de la forma de la concha reconstruyendo los estados ancestrales y explorando las fuerzas adaptativas involucradas. Las reconstrucciones ancestrales obtenidas sugieren diferentes patrones de migración para la diversificación de los dos clados principales encontrados. Las especies

de uno de los clados presentan un origen y diversificación mediterránea (*P. cephalonica*, *P. chorismenostoma*, *P. jaenensis* y *Pyramidula* sp2), principalmente con direccionalidad de este a oeste; mientras que las especies del segundo clado (*P. rupestris*, *P. saxatilis* y *P. pusilla*) se originaron y diversificaron principalmente a través de Europa Central. Se popone la región del mediterráneo oriental como área ancestral de varias de las especies. Los análisis de modelización de nichos ecológicos demuestran que cada especie de *Pyramidula* responde de manera diferente ante fluctuaciones climáticas: las especies más adaptadas a la humedad y al frío sufrieron contracciones de distribución después de la última glaciación, mientras que las especies más adaptadas a la aridez y calor expandieron sus rangos de distribución. Los análisis sobre la evolución de la concha sugieren que la variabilidad existente hoy en día en la forma de la concha, y la cripticidad de las especies de *Pyramidula* ha podido ser el resultado de varios procesos evolutivos, tanto adaptativos como no adaptativos.

Capítulo 5

El último capítulo recopila las principales conclusiones obtenidas en esta tesis. Esta tesis supone una importante contribución para el conocimiento de la sistemática de los gasterópodos terrestres, y representa un avance significativo en la comprensión de la historia evolutiva de los mismos. Los resultados obtenidos contribuirán a la aportación de nueva información en el proyecto de reconstrucción del árbol de la vida. Además, contribuye significativamente al desarrollo del inventario de la biodiversidad, la base para una conservación efectiva de las especies y sus hábitats. Por último, este trabajo pone de manifiesto la importancia y necesidad de integrar la información derivada de diferentes áreas de conocimiento a la hora de poder llevar a cabo un estudio robusto y completo de los diferentes procesos que determinan la biodiversidad.

LABURPENA

1. kapitulua

Tesiaren lehen kapituluak biologia ebolutiboaren garapen historikoa, haren oinarriak eta praktikak laburtzen ditu, lan honetan kontuan izango diren funtsezko zailtasun, diziplina eta kontzeptuak nabarmentzen dituelarik. Jarraian kapitulu honen laburpena eskaintzen da:

Darwin eta Wallacen eboluzioaren teoria zientziaren historiako ideia iraultzaileenetariko bat izan da. Izaki bizidunak denborarekin aldatzen doazela iradokitzen lehenak izan ez ziren arren, 1859. urtean, *On the origin of the species* liburuan argitaratutako bi ideia nagusiek mundua ulertzeko era aldatu zuten. Darwinek, espezie guztiak arbaso bakar batetik edo arbaso multzo txiki batetik zetozela ondorioztatu zuen, eta hautespen naturala proposatu zuen eboluzioaren eragile bezala. XX. mendearen hasieran, Darwinen teoria eta Mendelen legeak bateratzearen ondorioz, *eboluzioaren teoria sintetikoa* sortu zen, non eboluzioaren oinarriak adostu ziren. Harrezkero, biologia ebolutiboak ikerketa eremu bizi bat izaten jarraitu du eta aurrerapen esanguratsuak egin dira sintesiaren sorreratik.

Historikoki, sistematika eta taxonomia espezieak sailkatu eta deskribatzeaz arduratu dira. Filogenetikak, aldiz, izaki bizidunen arteko ahaidetasun eta ondorengotzak eraiki eta ulertzea du helburu, filogenien azterketaren bidez. Horrek bizitzaren historian emandako gertaera ebolutiboak ulertzea ahalbidetzen du. Filogenien eraikuntzak organismoek aurkezten dituzten karaktere ebolutibo homologoen (arbaso beretik eratorritakoen) informazioa behar du, non karaktere homologo berak partekatzen dituzten organismoak zuhaitz filogenetikoan gertuago elkartuko diren.

Tradizionalki, organismoen sailkapenak eta espezieen deskribapenak karaktere morfologikoetan oinarritu dira, morfologia sistematika osoaren oinarri garrantzitsu bihurtuz. Hala ere, sailkapen tradizionalen eta informazio genetikoan oinarritutako sailkapen berrien artean batzuetan inkongruentziak aurkitu dira, eta horrek karaktere morfologiko batzuk homoplasikoak direla iradokitzen du (karaktere ez homologoak). Sekuentziazio teknologietan emandako aurrerapenen ondorioz, teknika molekularren erabilera hedatu egin da, filogenien azterketan DNA bezalako molekulen informazioa erabiltzea baimenduz. Aldi berean, zuhaitz filogenetikoak eraiki ahal izateko metodo konputazional ugari sortu dira, distantzia-metodo eta metodo diskretuetan sailkatu daitezkeenak. Berriki, genomaren etekin altuko sekuentziazio teknologien etorrerarekin batera, filogenetika garai berri batean sartu da, gastuak jaitsi eta datu erabilgarriaren kantitatea handitzen doalarik. Genomak zatitzeko estrategia ezberdinak garatzen ari dira, eta modu horretan, informazio ugari lortu daiteke era eraginkorrean ia edozein talde taxonomikoren galdera filogenetikoak erantzuteko.

Ikerketa sistematikoak, gehienbat, datu morfologiko eta molekularretan oinarritu badira ere, mota ezberdineko informazioa erabili eta bateratzen dituzten lanak garrantzia irabazten ari

dira (adibidez, morfologia, DNA sekuentzia eta nitxo ekologikoen informazioa bateratzen dutenak), batez ere, espezieak mugatzea helburu duten lanetan. Izan ere, espezieak biologiako oinarrizko unitateak dira, eta hauen mugaketa funtsezkoa da sistematikan, beste hainbat diziplinatan ere inplikazioak dituelarik, hala nola, biogeografia, ekologia edota kontserbazio-biologian. Behin espezieak mugatuta daudela eta leinu edo talde taxonomikoen arteko ahaidetasun filogenetikoak ezagunak direla, beren historia ebolutiboa aztertzen hasi daiteke, modu horretan gaur egun Lurrean agertzen den dibertsitate morfologiko, ekologiko edota genetikoa gauzatu duten prozesuak ulertu eta aztertu ahal izateko.

Tesi honetan egindako sistematika eta eboluzioari buruzko ikerketak animalia talde anitzenetariko batean burutu dira, gastropodo lurtarretan. Stylommatophora kladoak gastropodo lurtar gehienak hartzen ditu bere barne, 30.000 espezieak gainditu ditzakeelarik. Klado anitza da, 103 familiaz osatua. Tradizionalki, bere taxonen sailkapenak morfologian oinarritu dira, hauetako batzuk zalantzakoak eta nahasgarriak direlarik. Hori dela eta, stylommatophoroen ahaidetasun filogenetikoek ebatzi gabe diraute, superfamilia mailatik espezie mailara. Honela bada, lan honen helburua Europako faunan esanguratsuak diren bi Stylommatophora taxonen sistematika eta historia ebolutiboa ebaztea da: alde batetik, mundu mailan banaturik dagoen Helicoidea superfamiliarena, eta bestetik, Paleartiko mendebaldeko *Pyramidula* generoarena.

2. kapitulua

Bigarren kapituluan, tesi honen helburu orokor eta espezifikoak definitzen dira. Helburu orokorra "*Europako fauna malakologikoan esanguratsuak diren gastropodo lurtarren sistematika eta historia ebolutiboaren jakintza handitzea*" da.

3. kapitulua

Hirugarren kapituluan lehen artikulua dago:

I. artikulua: "**Molecular phylogeny of the western Palaearctic Helicoidea (Gastropoda, Stylommatophora)**" - "Paleartiko mendebaldeko Helicoidearen (Gastropoda, Stylommatophora) filogenia molekularra" *Molecular phylogenetics and evolution 83 (2015) 99–117*.

Artikulu honetan Helicoidearen filogenia molekularra eta historia ebolutiboa aztertzen dira. Helicoidea superfamilia 19 familiatan sailkatuta dago, horietatik zortzi eremu Paleartikoaren mendebaldean aurkitzen direlarik. Eremu horretako molusku lurtarren talde anitzena da, moluskuen biodibertsitatearen erdia baino gehiago suposatzen duelarik. Bere hedapen tokiaren erdigunea eremu mediterraneoan dago. Ugalketa-sistemako karaktere anatomikoetan oinarritutako hainbat sailkapen proposatu dira, baina filogenia molekular berriek hainbat inkongruentzia aurkitu dituzte sailkapen tradizionalekiko, karaktere anatomiko batzuentzat homoplasiak iradokiz. Hori dela eta, familia, tribu eta generoen konposaketa eta ahaidetasun filogenetikoek ebatzi gabe jarraitzen dute, seinale filogenetikoa

duten karaktereen erabilera beharrezkoa dela agerian utziz. Lan honetan, bereziki, Paleartiko mendebaldeko helicoideoen filogenia molekularra aurkezten dugu eta horretarako, hainbat gene zati analizatu dira: 16S rRNA mitokondria-genearen zatia, eta 5.8S genearen 3' amaierak, ITS2 osoak eta 28S genearen 5' bukaerak osatzen duten gene nuklearren elkarketa. Morfologikoki definituta zeuden familien gehiengoa baieztatua izan da. Familia, subfamilia eta tribuak monofiletikoak diren sailkapen filogenetiko bat proposatzen dugu. Hygromiidae familia, familia maila ematen zaien hiru kladotan banatzen da: Canariellidae eta Geomitridae, familia bezala lehen aldiz onartuak, eta Hygromiidae (*Ciliella* eta *Trochulus* barne dituela). Ciliellinae, Geomitrinae, Hygromiinae, Monachainae eta Trochulinaek ez dituzte talde monofiletikorik eratu. Cochlicellidae familiari tribu maila ezartzen zaio (Cochlicellini), Geomitridae-ren barruan kokatuta. Tribu berri bat ere deskribatzen da: Plentuisini. Helicidae-ren barruan hiru subfamilia antzematen dira: Ariantinae, Helicinae (*Theba* barne) eta Murellinae. Helicoidearen jatorria Behe Kretaziar amaieran estimatu da eta bere familiena Goi Kretaziarretik Paleogenora. Paleartiko mendebaldeko helicoideoak bi leinu ezberdinetan banatzen dira, zeintzuk elkarrengandik duela 86 millioi bat urte aldendu ziren, bien dibertsifikazio prozesua Kretaziar amaieran (duela 73-76 millioi urte) gertatu zelarik. Helicoideako familia batzuen dibertsifikazio prozesua Eozenoan hasi zen.

4. kapitulua

Laugarren kapitulua hiru artikulutan banatuta dago, eta *Pyramidula* generoaren sistematika eta eboluzioari buruzko ikerlanak hartzen ditu bere barne. Genero hau eremu Paleartikoan aurkitzen da, batez ere Europa erdialde eta hegoaldean. Iberiar penintsula eta Britainiar irletaraino iristen da mendebaldetik eta ipar Afrikan eta Asian aurkitzen da, Japoneraino iritsiz. Kareharrizko arroketan bizi da eta itsaso mailatik 3000 metroko altuera bitartean topatu daiteke. Likenez eta algaz elikatzen da eta pasiboki dispertsatzeko ahalmena du, batez ere hegazti bidez. Generoko hainbat espeziek banaketa simpatrikoa dute. Espezieen maskorrak forma trokoideoa du eta garai samarretik zapalera alda daiteke, zila zabala aurkezten duelarik, eta diametroan ez dituzte 3 mm-ak gainditzen. XX. mendearen erdialdean *Pyramidula* taxonak espezie bakar bateko, *P. rupestris,* forma ezberdinak zirela kontsideratzen zen. Mende horren amaieran Europan sei morfoespezie proposatu ziren, beraien identifikazioa ezaugarri konkiologikoetan oinarrituz, zehazki, maskorraren altuera eta diametroa eta zilaren diametroa. Ezaugarri horiek estuki erlazionaturik daude elkarrekin eta generoaren taxonomia ebazteko ez dira nahikoak. Europako fauna zerrendetan ez zaie espezie izenik ematen *Pyramidula* banakoei, karaktere konkiologikoak beti ere ez direlako argiak. Horrez gain, ugalketa-aparatuaren morfologiak, gastropodoen taxonomian oso erabilia den karaktereak, ez du espezieak ezberdintzen laguntzen. Hori guztia dela eta, ezaugarri osagarrien erabileraren beharrak ezinbestekoa dirudi.

II. artikulua: "**Species delimitation for cryptic species complexes: case study of *Pyramidula* (Gastropoda, Stylommatophora)**" - "Espezieen mugaketa espezie talde kriptikoentzat: *Pyramidula* (Gastropoda, Stylommatophora) ikerketa kasu" *Cladistics* (berrikusten).

Lan honetan espezieen aurkikuntza eta balioztatze metodoak erabili dira *Pyramidula* generoko espezie kriptikoak mugatu ahal izateko. Espezieen mugak informazio ezberdinetatik eratorritako datuekin ondorioztatu dira, zehazki, mitokondria-DNA eta DNA nuklearraren sekuentzia, eta nitxo ekologikoen modelizaziotik eratorritakoak. Espezieen arteko ahaidetasun filogenetikoak hainbat metodo konputazional erabiliz ondorioztatu dira: inferentzia bayesiarra, probabilitate maximoa eta parsimonia maximoa. Paleartiko mendebaldean zehar bildutako 211 banako aztertuz, bederatzi espezie identifikatu dira eremu honetarako. Morfologia, banaketa geografikoa eta topotipoen informazioa kontuan hartuz, bederatzi espezie horietatik zazpi izendatzea lortu da. Orain arte espezieak oskolaren karaktereetan oinarrituta definitu eta deskribatu izan direnez, karaktere horien aldakortasuna aztertu da, karaktere horiek lan honetan mugatu diren espezieak definitzeko baliagarriak diren ala ez ebazteko, edota oskolaren forma ingurune-faktoreengatik eraginda dagoen jakiteko. Lortutako emaitzen arabera, oskolaren karaktereak espezieetako batzuk desberdintzeko balio badute ere, ez dira baliagarriak karaktere taxonomiko bakar bezala erabiltzeko, eta beraz, beste motako informazioa beharrezkoa da. Nitxo ekologikoaren modelizazioak emandako emaitzen arabera, oskolaren forma ingurune-faktoreengatik eraginda egon daitekeela ondorioztatzen dugu. Modelo ekologikoen arabera, oskol zapalenak aurkezten dituzten banakoen habitat aproposenak altitude eta prezipitazio altuak aurkezten dituzte; oskol samarragoak dituzten banakoentzat aldiz, kontrako ezaugarri klimatikoak aurkezten dituzten habitatak (klima mediterraneoak aurkezten dituen ezaugarriak dituztenak, alegia) dira aproposagoak.

III. artikulua: "**Species limits, interspecific hybridization and phylogeny in the cryptic land snail complex *Pyramidula*: the power of RADseq data**" - "Espezieen mugak, espezieen arteko hibridazioa eta filogenia *Pyramidula* barraskilo lurtar talde kriptikoan: RADseq informazioaren boterea" *Molecular phylogenetics and evolution* (berrikusten).

Hirugarren artikuluan, RADseq (Restriction site-assocciated DNA sequencing) teknika berria erabili da mendebaldeko *Pyramidula* espezieen arteko ahaidetasun filogenetikoak, hibridazioa eta mugaketa ebatzi edota baieztatzeko. Lortutako filogenia sendoa 1.500.000 nukleotido base eta 97.000 leku aldakor barneratzen dituen sekuentzia datu base batetik ebatzi da. Probabilitate maximoaren bidez lortutako filogeniak guztiz ebatzi ditu espezieen arteko ahaidetasun filogenetikoak eta aurrez erabilitako gene zatiek (COI, 16S rRNA, 5.8S rRNA, ITS2 eta 28S rRNA) lortutako filogeniekin alderatuta hobekuntza nabarmena eman da. Mitokondria-geneetan eta gene nuklearretan oinarritutako zuhaitzen artean aurretik aurkitu ziren inkongruentziak ebatzita geratu dira RADseq-ek eskainitako informazioarekin. Espezieen mugaketaren eskenatoki hoberena loturik ez dauden 875 SNP (single nucleotide polimorphism) erabiliz ikertu da. Honen arabera, Europan bederatzi espezie identifikatu beharko genituzke, aurreko artikuluan proposatu dugun bezala. Estatistikoen aplikazioak ebidentzia ahul edo nulua eskaini du *Pyramidula*-ren espezieen arteko antzinako hibridazioarentzat. Lan honek agerian uzten du RADseq teknika baliogarria dela gertuko espezieen zailtasun filogenetikoak ebazteko orduan.

IV. artikulua: "**Evolutionary patterns of the western species of *Pyramidula* (Gastropoda, Stylommatophora)**" - "Mendebaldeko *Pyramidula* (Gastropoda, Stylommatophora) espezieen historia ebolutiboa" *Prestatzen.*

Azken artikuluan mendebaldeko *Pyramidula* espezieen historia ebolutiboa ikertzen da. *Pyramidula*-ren espezieak kriptikoak direnez, eta DNA-k eta nitxo ekologikoek eskaintzen duten informazioan oinarrituz mugatu daitezkeenez, honek guztiak bereiziki interesgarria egiten du generoaren historia ebolutiboaren ikerketa. Alde batetik, nitxo ekologiko ezberdina duten gertuko espezieak hautagai egokiak dira klima aldaketen aurrean bakoitzak duen erantzuna ikertzeko. Beste alde batetik, espezie kriptikoen eboluzio fenotipikoa gauzatu duten prozesuak ezagutzea interesgarria da, prozesu moldagarri edo ez-moldagarriak izan daitezkeelako eragileak. Beraz, lan honen helburuak ondorengoak dira: (1) mendebaldeko *Pyramidula* espezieen historia biogeografikoa ikertzea, horretarako arbaso- areak eraikiz; (2) klima aldaketen aurrean banakako espezieen erantzunak ikertzea; eta (3) oskol-formaren eboluzioa ikertzea, horretarako arbaso-egoerak eraikiz eta moldaera-indarrak aztertuz. Arbaso-areen eraikuntzan lortutako emaitzen arabera, ezaugarri ezberdineko migrazioak eman ziren aurkitutako bi klado filogenetiko nagusietan. Lehen kladoko espezieen jatorri eta dibertsifikazio prozesua eremu mediterraneoan eman zen, bereziki ekialdetik mendebaldera. Bigarren kladoko espezieak, aldiz, Europa Erdialdean sortu eta dibertsifikatu ziren. Mediterraneo ekialdeko eremua hainbat *Pyramidula* espezieren jatorri bezala proposatua izan da. Nitxo ekologikoaren modelizazioan oinarritutako analisiek erakutsi dutenez, *Pyramidula*-ren espezie bakoitzak modu ezberdinean erantzun izan dute klima aldaketen aurrean: hezetasun eta hotzera moldatuta dauden espezieek beren banaketa eremuak murriztu zituzten azken glaziazioaren ondoren; aldiz, lehorte eta berora egokituta dauden espezieek beren banaketa eremuak zabaldu zituzten. Oskolaren eboluzioari buruz egindako ikerketaren arabera, gaur egun aurkitzen den forma aldakortasuna, hainbat prozesuren ondorio izan daitekeela dirudi, tartean prozesu moldagarri edo ez-moldagarriak egon daitezkeelarik.

5. kapitulua

Azken kapituluan tesi honetan aurkitutako ondorio nagusiak biltzen dira. Tesiak gastropodo lurtarren sistematikaren jakintzan ekarpen handia suposatzen du eta beren historia ebolutiboa hobeto ulertzen laguntzen du. Lortutako emaitzek informazio berria eskaintzen diote "bizitzako zuhaitza"-ren eraikuntzaren proiektuari. Gainera, biodibertsitatearen inbentarioaren garapenean era esanguratsuan laguntzen du, espezieen eta beren habitaten kontserbazioarentzat oinarrizkoa dena. Azkenik, lan honek agerian uzten du galdera filogenetiko eta ebolutiboak ebazteko orduan oso lagungarria dela mota ezberdineko informazioa bateratzea.

PREFACE

This thesis is divided into five chapters.

The present work deals with multiple aspects of evolutionary biology, thus in **Chapter 1** the historical development, principles and practices of evolutionary biology are summarized. Special emphasis is placed on the existing challenges and problematic issues that will be addressed in the dissertation. The disciplines and most important concepts applied or discussed in this thesis are also briefly described. The evolutionary studies were applied to two taxonomic groups of terrestrial gastropods and therefore, terrestrial gastropods are also described in chapter 1, and the aspects that make the two selected taxa interesting for the study of their systematic and evolutionary histories are mentioned.

The general and specific goals of the thesis are reported in **Chapter 2**.

In **Chapter 3** the molecular phylogeny and time divergence of the superfamily Helicoidea (Gastropoda, Stylommatophora) is investigated. The study is summarized in Paper I.

Chapter 4 comprises systematic and evolutionary studies of the genus *Pyramidula* (Gastropoda, Stylommatophora) and it is subdivided into three papers (Papers II-IV).

In **Chapter 5** the main conclusions that can be drawn from this PhD thesis are exposed.

1

CHAPTER 1

INTRODUCTION

1. Evolutionary biology

1.1. Evolutionary theory

The theory of biological evolution of Darwin and Wallace has been one of the most revolutionary facts in science. Although others had previously suggested that organisms change over time, the two main ideas presented in *On the Origin of Species* in 1859 challenged the prevailing world view. The first fundamental insight focused on the common ancestry of all living things. Darwin hypothesized that all species have descended from one or a few common ancestors, with modifications. The second thesis explained the causal agents of how the characteristics of organisms change over time and of the suitability of organisms to their environments: the process of natural selection. Thus, Darwin proposed that descendants of a common ancestor evolve different features because they adapted under different life conditions.

The main objection to Darwin's theory was that it lacked a theory of the mechanism of heredity. However, during the second decade of the twentieth century, research on Mendelian genetics became relevant and independent works about populations genetics performed by R. A. Fisher, J. B. S. Haldane, and S. Wright showed that natural selection could operate with the laws of Mendelian inheritance. The synthesis of this statement established what is known as the *modern evolutionary synthesis* (Huxley, 1942). The reconciliation between Mendelism and Darwinism encouraged new research from several branches of biology (Dobzhansky, 1937; Mayr, 1942; Simpson, 1944) reflecting the consensus about how evolution proceeds and establishing the principles of evolution. As molecular methods developed and became more available, new fields of evolutionary studies arose, especially those using the DNA. These studies lead to the formulation of the *neutral theory of molecular evolution*, developed specially by Kimura (1984). He hypothesized that most of the variation of DNA sequences occurs by random drift rather than by natural selection and he predicted a molecular evolutionary clock, according to which DNA sequences evolve and diverge at a constant rate.

Evolutionary biology remains an active field and indeed, significant advances have been made since the foundation of the *modern evolutionary synthesis,* including new disciplines such as developmental biology, genomics, epigenetics, ecology or social science, among others, where novel concepts have been elaborated (Laland et al., 2014). This extension and updating of the *modern evolutionary synthesis* is also known as the *extended evolutionary synthesis* (Pigliucci and Müller, 2010).

1.2. Classification and phylogeny

Historically, the fields of systematics and taxonomy have been in charge of the classification and naming of organisms. Linnaeus, whose universal binomial nomenclature system still endures, realized that organisms could be arranged in a hierarchical classification without having a theoretical basis for it. Darwin (1859) provided the answer with his theory of

evolution recognizing that an evolutionary process of branching descent with modification would produce nested hierarchies as the result of the phylogenetic history. He described it using the metaphor of a tree of life, the historical relationships that connect all living things. Henning (1996) established the modern classification approach of phylogenetic systematic emphasizing that phylogenies allow classifying organisms according to their evolutionary histories. A phylogeny is a tree containing nodes that are connected by branches. Each branch represents the persistence of a genetic lineage through time, and each node represents the birth of a new lineage (Yang and Rannala, 2012). The study of the phylogeny (phylogenetics) aims to reconstruct and understand patterns of descent, in order to comprehend the evolutionary events occurred throughout the history of life (Bergstrom and Dugatkin, 2012). It requires the information of characters (inheritable traits) displayed by organisms, and those lineages displaying more characters in common will branch closer in the tree. Characters can be morphological, behavioural or molecular, among others; nevertheless, nowadays with the advent of molecular genetics, DNA sequences are most commonly used, in which nucleotide bases at particular sites are considered characters. The latter can be classified as homologous or analogous: homologous characters are found in two or more lineages because they were inherited from a common ancestor; while analogous characters are shared by two or more lineages because they were acquired independently during their evolutionary history, probably due to natural selection but not because of a common ancestry (Fitch, 2000). Given that the reconstruction of phylogenetic trees is based on the theory of the common ancestry, it requires homologous characters. Thus, the distinction between homologous and analogous characters is crucial in phylogenetics.

1.3. Species

Species problem

Species are considered fundamental units in biology (Mayr, 1982) and their delimitation is essential for systematic biology, with implications for biogeography, ecology, and evolution and conservation biology (Avise, 2000; Goldstein et al., 2000; Hey et al., 2003). However, since Linnaeus formalized the taxonomic rank of species, the debate on how we should identify them and how we should define the term "species" has been an endless discussion. Mayr (1942) pointed out that, biologists have different ways of identifying species and hence, they actually have different species concepts. Indeed, more than 22 species concepts can be found in the literature (Mayden, 1997). Most of them can be subsumed under four major categories: Biological species concept (BSC), Phenetic species concept (PSC), Ecological species concept (ESC) and Phylogenetic species concept (PhSC).

De Queiroz (1998) considered that all these definitions (operational criteria) describe variants of a single general concept of species (General Lineage Concept of Species), where the only property of species is that they are separately evolving metapopulation lineages. Operational criteria (BSC, PSC, ESC, PhSC...) are now considered as different lines of evidence relevant to assess lineage separation, and hence, species boundaries. In this way,

species conceptualization and delimitation issues are not confused (De Queiroz, 2007).

Evolutionary history of species

Once species boundaries are established, it is possible to study their evolutionary history in order to understand and recognize the processes that originate the morphological, genetic or ecological diversity existing nowadays. Evolutionary radiations have been well documented (Irschick et al., 1997; Albertson et al., 1999; Brusatte et al., 2008), adaptive radiation being considered as the main force producing phenotypic and ecological diversity as species adapt to different niches (Gavrilets and Losos, 2009). Phenotypic plasticity, known as the ability of organisms to change its behaviour, morphology and physiology (that may or may not be permanent) induced by different environments (Price et al., 2003), is also responsible for the variability of lineages. However, other evolutionary processes tend to maintain traits similarity between or within species over time. For example, parallel evolution involves similar developmental modifications that evolved independently (often in closely related taxa) (Futuyma, 2005). Also "constraint" in evolution is understood as "a property of a trait that, although possibly adaptive in the environment in which it originally evolved, acts to place limits on the production of new phenotypic variants" (Blomberg and Garland, 2002), which retains similar traits over longer periods of time.

Using information on phylogenetic relationships and present distribution data of species, it is possible to infer ancestral geographic ranges and past biogeographic events in order to study the evolutionary history of species (Lomolino et al., 2010). The field which focuses on the geographic distributions of organisms it is known as biogeography, and when distributions are inferred over evolutionary time scales is known as historical biogeography (Crisci et al., 2003). The study of biogeography is intimately related to geology, palaeontology, systematics and ecology. Ecology is being particularly widely used nowadays to identify refugia and expansion/contraction patterns of species since the Last Glacial Maximum, by applying the ecological niche modeling approach (Marske et al., 2011; Hidalgo-Galiana et al., 2014; Anadón et al., 2015; Feuda et al., 2015). Certainly, it is well established that the climatic fluctuations of the Quaternary have had a crucial influence in shaping current species distributions (Hewitt, 2000, 2004; Hofreiter and Stewart, 2009). Following this approach, it is also feasible to inspect how current climate change may affect species' distributions under different climate models for the future (Bystriakova et al., 2014).

1.4. Characters and methods

In systematics, in order to either study phylogenetic relationships or to delimit species we need to apply information of homologous characters as previously explained. Although several types of characters can be used, the two most applied ones are morphological and molecular characters.

Morphological characters

For centuries, traditional classifications and species have been, in practice, recognized by

phenotypic characters. Undeniably, the fundamental observation of biology is morphology, morphological data being the basis of virtually all systematic description (MacLeod and Forey, 2003). However, incongruences have been detected between some traditional classifications and new molecular-based phylogenies, suggesting high levels of homoplasy for some morphological traits (Manuel et al., 2003; Mueller et al., 2004; Ranker et al., 2004). Homoplasic characters are those that are similar in two or more taxa even though they were not present in their common ancestor, which can be misleading to reconstruct phylogenies. Moreover, species delimitations using morphological traits may also be confounded by polymorphism, phenotypic plasticity or cryptic species (Agapow et al., 2004). Polymorphism is the occurrence of more than one recognizable form in a population; indeed, the individuals of a natural population will vary for almost any morphological character (Ridley, 1996). Phenotypic plasticity, as previously stated, is the capacity of organism to develop several phenotypic states induced by different environments. Finally, cryptic species are two or more distinct species classified as a single species because they are morphologically very similar. During the last decades, molecular studies have led to the detection of many morphologically cryptic lineages (Bickford et al., 2007) revealing that they are far more common than previously estimated.

Molecular characters

When Watson and Crick first identified the structure of the DNA in 1953, they demonstrated a physical basis for the theory of evolution. With the posterior advent of sequencing technologies, the expansion of molecular techniques allowed using molecules as characters to study phylogenies. This discipline is known as molecular phylogenetics. Molecular phylogenetic trees are generated from character datasets that consist of fragments of DNA, RNA or amino acids, called molecular markers. The most commonly used is the DNA, in which each nucleotide at a specific location represents a discrete character. The alignment of sequences is a crucial step so as to identify homologies between characters (Lio and Goldman, 1998). Phylogenetic trees can be reconstructed using many different computational methods, which can be classified as distance-based or character-based methods. Distance-based methods calculate the distance between every pair of sequences, generating a distance matrix, which is used to reconstruct the tree. Character-based methods examine each character in the alignment separately to calculate a score for each tree. After comparing all possible trees, the tree with the best score should be identified, but since an exhaustive search among all possible trees is not computationally feasible, heuristic tree search algorithms are used. The most commonly used distance-based method is neighbour joining, while the most common character-based methods include maximum parsimony, maximum likelihood and Bayesian inference (Yang and Rannala, 2012). Maximum parsimony method searches for the phylogenetic tree that requires the minimum number of evolutionary changes (Fitch, 1971; Hartigan, 1973); maximum likelihood method searches for statistically the most probable tree (Felsenstein, 1981); and Bayesian inference searches for the tree with the highest posterior probability (Huelsenbeck

and Ronquist, 2001).

Genes accumulate change overtime and therefore, the genetic distance between two taxa, measured by the number of changes accumulated, will be proportional to the time of their divergence. Branch lengths on ultrametric molecular phylogenetic trees represent the amount of evolutionary change that has occurred. In order to date when different lineages diverged, the amount of change observed can be converted into absolute time using additional data. For example, information of fossil data, that records the divergence time of known taxa in the tree, can be used to determine the rate of a molecular clock. This rate is subsequently applied to the phylogenetic tree so as to estimate the divergence times of other taxa that have not left fossil records. Recently developed methods that allow variation in rates of substitution among lineages make it possible to relax the assumption of a global molecular clock providing more accurate estimates of divergence times (Heath, 2012).

In the late twentieth century, the advent of powerful computers, PCR, and Sanger sequencing have lead to an exponential growth of molecular phylogenies. However, they are not free of problems. Phylogenetic inferences can be challenging if DNA sequences contain insufficient phylogenetic signal. If divergence events are ancient in time, terminal branches tend to be long and with high levels of homoplasy. The effect of this non-phylogenetic signal can be the long branch attraction artefact (Philippe et al., 2011). Instead, the phylogenetic reconstruction among recently diverged closely related taxa may show conflicting topologies due to interspecific gene flow or incomplete lineage sorting (Maddison, 1997; Wendel and Doyle, 1998; Degnan and Rosenberg, 2009).

With the advent of high-throughput genomic sequencing, the field of phylogenetics is entering in a new era by decreasing the cost and increasing the quantity and rate of data collection. Several genomic partitioning strategies have been developed in order to overcome the high cost of whole-genome sequencing and assembly. They can produce large amounts of informative data that can now be efficiently collected for virtually any taxonomic group to resolve phylogenetic questions from shallow to deep timescales (Lemmon and Lemmon, 2013). In particular, reduced representation genotyping approaches do not require previous knowledge of the genome and hence, they are becoming particularly popular for systematic studies since they are applicable to non-model species with no reference genome sequenced. Reduced representation genotyping such as restriction-site associated DNA sequencing (RADseq; Baird et al., 2008; Davey and Blaxter, 2010) can identify and score thousands of genetic markers, randomly distributed across the genome in many individuals. It reduces the representation of the genome by subsampling only at specific sites defined by restriction enzymes (Davey and Blaxter, 2010). The RADseq technique was initially designed and successfully applied for population genomic studies (Emerson et al., 2010; Hohenlohe et al., 2010, 2011; Reitzel et al., 2013) and seems to be a promising tool to assess species limits and phylogenetic relationships in closely related taxa for which traditional DNA sequence approaches have failed to provide well-supported solutions.

Other lines of evidence

Although systematic studies have mainly used morphological or molecular characters, the works demonstrating the benefits of integrating multiple lines of evidence are becoming increasingly common, especially for species delimitation approaches (Padial et al., 2010; Andújar et al., 2014; Hamilton et al., 2014; Melville et al., 2014; Zhang et al., 2014). Frontiers between the disciplines are diffuse, and a comprehensive study of ecological and evolutionary processes requires integration across a vast array of disciplines, including ecology and evolutionary biology, biogeography, systematics and phylogenetic (Ladle and Whittaker, 2011). In fact, this need for integration has resulted in the recent emergence of multiple hybrid disciplines and concepts such as integrative taxonomy.

Indeed, integrative taxonomy has been proposed as a framework that bears on what species are, how they can be discovered, and how much diversity is on Earth (Padial et al., 2010). Integrating ecological information with molecular phylogenies is becoming particularly interesting since the development of the ecological niche modeling (ENM) approach (Raxworthy et al., 2007; Rissler and Apodaca, 2007; Leaché et al., 2009; Hawlitschek et al., 2011; Hidalgo-Galiana et al., 2014). ENM is a powerful tool to predict the habitat suitability of a species by combining information of environmental variables and species occurrence sites (Graham and Hijmans, 2006; Warren et al., 2008). This approach has become interesting for species delimitation because it enables statistically comparing niche models between species (Warren et al. 2008). Therefore, ecological species concept can be used as additional line of evidence in order to establish species boundaries. Besides, ENM can also be applied to evolutionary studies by inspecting how different climatic conditions affect species' distributions. In this way, niche models can be projected onto historical landscapes (e.g. Last Glacial Maximum or Last Inter Glacial) (Marske et al., 2011; Hidalgo-Galiana et al., 2014; Anadón et al., 2015; Feuda et al., 2015) or future conditions (Bystriakova et al., 2014) offering the possibility to assess the main factors determining broad scale distributions of biodiversity from a global and phylogenetically controlled perspective.

2. Terrestrial gastropods as case study

2.1. Terrestrial gastropods

Gastropoda is the largest molluscan class and the second animal class with most species (Aktipis et al., 2008). Estimates of the number of extant species range widely, from 40,000 to 150,000 (Lindberg et al., 2004). Primitively, gastropods were marine animals, but several lineages have made the adaptive shift from aquatic to terrestrial ecosystem (Ponder and Lindberg, 1997). Terrestrial gastropods, conformed by lands snails and slugs, have been estimated to include 35,000 extant species (Solem, 1984; Van Bruggen, 1995). The vast majority of terrestrial gastropods are stylommatophoran pulmonates, probably exceeding 30,000 species (Barker, 2001).

The clade Stylommatophora is highly diverse, classified into 103 families (Bouchet and Rocroi, 2005). Although they are more common in moist environments, they can occupy a large range of habitats almost worldwide. They can be both ground dwellers and arboreal. Anatomically the Stylommatophora is a highly cohesive group and they can be defined by three synapomorphies: the ability to retract the cephalic tentacles, the presence of a membrane covering the pedal gland, and the acquisition of a secondary ureter (Dayrat and Tillier, 2002). They present two pairs of retractile tentacles, with eyes on the tips of the posterior, cephalic pair; and the reproductive system is hermaphroditic and monotrematous, with a single genital pore (Mordan and Wade, 2008).

The morphological, physiological and behavioural adaptations that allow regulation of hydration have been critical for the success of the group (Barker, 2001). The same as for all pulmonates, a major adaptive feature is the enclosed and heavily vascularised, air-breathing lung, together with the contractile pneumostoma, which reduces the exposition to the evaporative environment. Most Stylommatophora also developed a secondary ectotermal ureter, important for water resorption (Mordan and Wade, 2008). Most land snails face unfavourable environmental conditions retracting the animal into the shell and sealing the aperture with epiphragms or cemented to the substrate.

Unravelling the earliest evolutionary phases of Stylommatophora is challenging, because the available fossil records are irregular. Indeed, their shells are formed of aragonite, which does not allow a good preservation, and besides, many of them are slugs which lack the shell (Mordan and Wade, 2008). Moreover, the interpretation of fossil data is difficult because it is based on anatomical characterization. Although Solem and Yochelson (1979) proposed that the initial stylommatophoran radiation occurred in the Carboniferous (320 Mya), the fossil attribution was highly controversial. The first undoubted stylommatophoran fossil records belong to the Late Cretaceous (Bandel and Riedel, 1994), and Tillier et al. (1996) proposed the origin for the group in the Upper Jurassic (150 Mya) with a period of radiation during the Palaeocene (65-55 Mya).

Traditionally, based on the morphology of the kidney and ureter, the Stylommatophora

has been divided into four divisions: the Orthurethra, Heturethra, Sigmurethra, and Mesurethra. Of these, only Orthurethra has remained recognized (Madeira, 2010). According to the taxonomy of the Gastropoda by Bouchet and Rocroi (2005) the clade Stylommatophora contains the clades Elasmognatha, Orthuretra and the informal group Sigmurethra. However, phylogenetic relationships of stylommatophoran taxa from superfamily to species rank remain largely unresolved. Most of the classifications are based on morphology and some of them resulted in confusing and conflicting taxonomies.

The low dispersal capacity, the plasticity of the shell characters, the presence of a high number of endemism and the existence of cryptic species make the land snails very suitable models for studies on mechanism of evolution, for exploring the effects of ecology on evolutionary change and for biogeographical studies (Wade et al., 2001).

2.2. Helicoidea and *Pyramidula*

The Helicoidea superfamily is included in the informal group Sigmurethra. According to Bouchet and Rocroi (2005) there are 19 families worldwide distributed within it and eight of them inhabit the western Palaearctic region. It is the most diverse group of the terrestrial molluscs of this region, with its distribution centre being located in the Mediterranean region where it locally can represents more than half of the molluscs' biodiversity. Several classification systems have been proposed mainly based on anatomical characters of the reproductive system. However, new molecular phylogenies found incongruences with traditional classifications, suggesting the existence of homoplasy in anatomical characters. Several questions regarding the composition of families, tribes, genera and their phylogenetic relationships remain unresolved. Therefore, an exhaustive phylogenetic reconstruction is needed for the western Palaearctic Helicoidea, based on new characters showing sufficient phylogenetic signal. The estimation of divergence times on the phylogenetic tree would also be interesting in order to infer the evolutionary history of Helicoidea.

The genus *Pyramidula* belongs to the superfamily Pupilloidea which is included in the clade Orthuretra. The distribution of *Pyramidula* extends to almost all Europe, Mediterranean area, Central Asia and Japan. Species have a trochoid shell, from high to flattened, with a broad umbilicus. They are small, not exceeding 3 mm in diameter and they inhabit limestone rocks. Previous studies (Gittenberger and Bank, 1996) found six morphospecies in the European and adjacent distribution range. Until now, the identification of the species has been based exclusively on shell characters, particularly shell heigth and diameter and umbilicus width. These characters are highly correlated and they could be not enough to resolve the taxonomy. The crypticity of the species in *Pyramidula* makes the species delimitation challenging, and hence, the usage of different lines of evidence would be needed to establish species boundaries. Cryptic species of *Pyramidula* are also interesting to study the phenotypic evolution of the shell in order to elucidate whether adaptive or non-adaptive processes have been responsible for the resultant phenotypic similarity between species. Moreover, being closely related species, they are good models

to evaluate individual responses during climatic fluctuations. The application of reduced representation genotyping approaches such as RADseq may be a promising tool to resolve phylogenetic difficulties in *Pyramidula*, because it does not require a reference genome and it can identify thousands of genetic markers.

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CHAPTER 2

AIMS OF THE THESIS

The main aim of the thesis is to gain insights into the systematics and evolutionary histories of two land snail taxa, which are representative of the European molluscan fauna, through the application of molecular, morphological and ecological data. The general and specific goals, reported below, are to:

1. Revise the phylogeny of the Helicoidea

1.1. Construct phylogenetic relationships for the Helicoidea based on both mitochondrial and nuclear rRNA gene sequences

1.2. Compare the obtained molecular phylogeny with other existing classifications

2. Infer the evolutionary history of the Helicoidea

2.1. Estimate divergence times by fossil calibration from a molecular phylogeny

3. Reconstruct the phylogeny of *Pyramidula* in the western Palaearctic

3.1. Construct phylogenetic relationships for *Pyramidula* based on both mitochondrial and nuclear rRNA gene sequences

4. Delimit species of *Pyramidula* in the western Palaearctic

4.1. Use *species discovery* methods by means of the mitochondrial gene sequences to obtain candidate species

4.2. Use *species validation* methods by means of the mitochondrial and nuclear gene sequences to validate candidate species

4.2. Use *species validation* methods by means of ecological niche modeling procedure to validate candidate species

5. Explore the utility of traditionally used shell characters to differentiate the species delimited for *Pyramidula*

5.1. Compare statistically the delimited species by examining differences in character means

6. Explore whether shell shape in *Pyramidula* is influenced by environmental factors

6.1. Combine morphological and ecological data to explore whether differences exist between the niches of different morphogroups

7. Review the taxonomy of *Pyramidula*

7.1. Describe and name the delimited species

8. Apply RADseq technology to address unresolved phylogenetic questions in *Pyramidula*

8.1. Reconstruct phylogenetic relationships using RADseq data

8.2. Re-assess the species delimitation previously proposed, using RADseq data

8.3. Test for ancestral hybridization between species using RADseq data

9. Infer the evolutionary history of *Pyramidula*

9.1. Study the historical biogeography of the species of *Pyramidula* in the western Palaearctic

9.2. Evaluate individual responses of the species during climatic fluctuations (Last Glacial Maximum, present and future)

9.3. Study the evolution of shell shape, reconstructing ancestral states and exploring adaptive processes

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CHAPTER 3

Helicoidea

PAPER I

Molecular phylogeny of the western Palaearctic Helicoidea (Gastropoda, Stylommatophora)

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Abstract

The Helicoidea is one of the most diverse superfamilies of terrestrial land snails. In this study we present a molecular phylogeny of the western Palaearctic Helicoidea obtained by means of neighbor joining, maximum likelihood and Bayesian analysis of the mitochondrial 16S rRNA gene fragment and the nuclear rRNA gene cluster including the 3'end of the 5.8S gene, the complete ITS2 region and 5'end of the large subunit 28S. Most of the morphologically-defined families were confirmed. We propose a revised phylogenetic classification so that families, subfamilies and tribes are monophyletic. The family Hygromiidae sensu Hausdorf and Bouchet (2005) is divided into three clades which are here given familial rank: Canariellidae and Geomitridae, which are recognized for the first time at familial rank, and Hygromiidae s.str. (including *Ciliella* and *Trochulus*) that is here restricted. The subfamilies Ciliellinae, Geomitrinae, Hygromiinae, Monachainae and Trochulinae recognized in current classifications were not recovered as monophyletic groups. The familily Cochlicellidae is here given tribe rank (Cochlicellini) belonging to the Geomitridae. We describe a new tribe, Plentuisini. Three subfamilies are recognized within Helicidae: Ariantinae, Helicinae (including *Theba*) and Murellinae. New classification indicates that free right ommatophore retractor muscle arose only once within Geomitridae. The anatomy of the auxiliary copulatory organs of the reproductive system of families, subfamilies and tribes is highlighted. We estimate the origin of the Helicoidea at the end of the Early Cretaceous and its families as Late-Cretaceous to Paleogene. Western Palaearctic Helicoidea belongs to two different lineages that diverged around 86 Ma ago, both starting their diversification at the end of the Cretaceous (around 73-76 Ma). Radiation of some western Helicoidean families started during the Eocene.

Keywords

Helicoidea, mitochondrial rRNA, nuclear rRNA, molecular phylogeny, divergence time, stimulatory system

1. Introduction

After arthropods, molluscs are perhaps the most diverse group of metazoans with over 118,000 species (Zhang, 2013). The clade Stylommatophora (Gastropoda: Pulmonata) accounts for around 80% of 30,000-35,000 extant terrestrial molluscs (Solem, 1984) classified into 103 families (Bouchet and Rocroi, 2005). Inferred phylogenetic relationships among the Stylommatophora have been much disputed (Schileyko, 1979; Nordsieck, 1985; Tillier, 1989), and the suborder is currently under revision according to the results of molecular techniques (Tillier et al., 1996; Wade et al., 2001; 2006; Madeira et al., 2010). Within the Stylommatophora the superfamily Helicoidea Rafinesque, 1815 is one of the most diverse groups of land snails and includes a number of large species of commercial value, as well as many microendemisms adapted to specific habitat conditions. The Helicoidea shows an almost worldwide distribution absent only from most of sub-Saharan Africa, and some islands of the South Pacific (Scott, 1997). Ecological, morphological and systematic studies have led to the proposal of several classification systems (Nordsieck, 1987; Schileyko, 1989). The importance of the reproductive system in the classification of the Helicoidea was initiated during the XIX century (Moquin-Tandon, 1855; Pilsbry, 1893-1895) and highlighted by posterior authors (Hesse, 1931; 1934; Zilch, 1960). Recent reviews of the helicoidean classification have since focussed on anatomical characters (e.g. Nordsieck, 1987; Schileyko, 1991; Puente, 1994), particularly the presence of several appendages (diverticulum of the stalk of bursa copulatrix, flagellum, and penis caecum) and the number and morphology of organs comprising accessory copulatory organs, also referred to as stimulatory organs (dart sac, accessory sac, atrial appendages and glands). However, incongruences detected between new molecular-based phylogenies and traditional classifications point to high levels of homoplasy in genital characters (Mejia and Zuñiga, 2007; Hirano et al., 2014). As a result, the composition of the Helicoidea has remained controversial, and several questions remain largely unresolved including its phylogenetic position within the Stylommatophora, or the number and composition of its families and/or subfamilies. Schileyko (2004; 2006a; 2006b) proposed subdividing the Helicoidea into five different superfamilies: Helicoidea *s.str.*, Xanthonychoidea, Camaenoidea, Polygyroidea and Hygromioidea (Figure 1). Nevertheless, recent molecular data obtained for the Stylommatophora (Wade et al., 2001; 2006) support the monophyly of the Helicoidea, indicating that subdivisions into separate superfamilies are not justified. According to the classification of Hausdorf and Bouchet (in Bouchet and Rocroi, 2005: 269-270), there are 19 families within the Helicoidea (see Figure 1), eight of which inhabit the western Palaearctic region. The families Cochlicellidae, Elonidae, Helicodontidae, Sphincterochilidae and Trissexodontidae are restricted to the western Palaearctic; the Helicidae are found across the western Palaearctic and adjacent Arabia, while the range of the highly diverse Hygromiidae sensu Hausdorf and Bouchet (2005) (herein designated Hygromiidae s.l.) extends throughout the western Palaearctic, central Asia, northeastern Africa and Arabia (Falkner et al., 2001; Schileyko, 2004; 2006a; 2006b). The Asian family Bradybaenidae is represented with one species in Europe. Within the past decade, several

authors have used molecular methods as rigorous tests of evolutionary relationships among helicoidean species. Some species have often been included in larger phylogenetic studies of gastropods (Wade et al., 2001; 2006; 2007; Davison et al., 2005; 2009; Holznagel et al., 2010; Dayrat et al., 2011). Recent helicoidean phylogenies based on molecular information have been extended to include representatives of several families and polytypic genera (Koene and Schulenburg, 2005: Helicidae, Hygromiidae, Helminthogliptidae and Bradybaenidae; Mejia and Zuñiga, 2007: *Humboldtiana*, Humboldtianidae; Wade et al., 2007: Camaenidae; Elejalde et al., 2008: *Iberus*, Helicidae; Elejalde et al., 2009: *Pyrenaearia*, Hygromiidae; Fiorentino et al., 2010: *Marmorana* group, Helicidae; Greve et al., 2010: *Theba*, Helicidae; Hugall and Stanisic, 2011: Camaenidae; Kotsakiozi et al., 2012: *Codringtonia*, Helicidae; Groenenberg et al., 2011 and Cadahia et al., 2014: Ariantinae, Helicidae; Hirano et al., 2014: Bradybaenidae). In addition, two studies have examined the molecular phylogeny of the western Palaearctic Helicoidea (Steinke et al., 2004 and Manganelli et al., 2005), but unfortunately, the results of the former study have been questioned due to errors, methodological weaknesses and taxonomic misidentifications (critically reviewed in Groenenberg et al., 2011). Manganelli et al. (2005) included the widest representation of western helicoidean taxa but their study was restricted to the analysis of the mitochondrial 16S rRNA region. According to the studies of Wade et al. (2006, 2007) based on nuclear 28S rRNA gene sequences, the Helicidae (9 genera sequenced) is monophyletic. The monophyly of the Hygromiidae (only 4 genera sequenced) was also proposed by Wade et al. (2006, 2007) but without statistical support. Moreover, the Hygromiidae still emerged as monophyletic when shorter sequences of nine additional hygromiid genera published by Koene and Schulenburg (2005) were incorporated in the analysis (Wade et al., 2007). Recently, Gómez-Moliner et al. (2013) confirmed in a molecular phylogeny of Helicodontidae and Trissexodontidae (23 species included in the study together with other Helicoidea taxa using mitochondrial and nuclear DNA sequences) the monophyly of these two families.

In the present study, we reconstruct the most exhaustive phylogeny of the western Palaearctic Helicoidea to date, based on molecular markers from the nuclear ribosomal RNA (rRNA) gene cluster (partial 5.8S, complete ITS2 and partial 28S sequences) and one mitochondrial gene fragment (16S rRNA). The nuclear DNA fragment was homologous to that used by Wade et al. (2001; 2006; 2007), thus allowing direct comparisons with their results. The mitochondrial gene fragment examined also enabled comparisons with the results of Manganelli et al. (2005) and Groenenberg et al. (2011), who both also focused their work on the western Palaearctic Helicoidea. The introduction of additional taxa can often resolve basal nodes and ambiguities, or increase support values in phylogenetic trees. With this goal in mind, we included sequences of 76 species belonging to 45 genera for the 16S analysis and 67 species of 44 genera for the nuclear rRNA tests along with GenBank sequences from various studies. This paper therefore revisits the phylogeny of the Helicoidea, with the following aims: (i) to construct a phylogenetic hypothesis for the Helicoidea based on both mitochondrial and nuclear rRNA gene sequences; (ii) to test the classifications proposed

BOUCHET & ROCROI					SCHILEYKO			
.oidea	.idae	.inae	T	Type genus	T	.inae	.idae	.oidea
Helic.	Hygromi.	Hygromi.	Leptaxini	***Leptaxis***	Hygromiini	Hygromi.	Hygromi.	Hygromi.
			Hygromiini	***Hygromia* +3**				
				***Cernuella* +1**	Cernuellini			
			Trochulini	***Trochulus***	Trochulini	Trochul.		
			Helicellini	***Helicella* +2**	Helicellini			
			Archaicini	*Archaica*		Archaic.		
			Metafruticicolini	*Metafruticicola*		Metafruticicol.		
		Monacha.		***Monacha* +5**		Monacha.		
				Hesseola		Hesseol.		
		Geomitr.	Geomitrini	*Geomitra** **+3**	Geomitrini	Geomitrin.		
			Trochoideini	***Trochoidea***	Trochoideini			
			Paedhoplitini	*Paedhoplita*		Paedhoplit.		
		Ciliell.		***Canariella***		Canariell.		
				Ciliella		Ciliell.	Ciliell.	
		Ponentin.		***Ponentina***		Ponentin.		
	Halolimnohelic.			*Halolimnohelix*		Halolimnohelic.		
				Vicariihelix		Vicariihelic.		
	Trissexodont.			***Trissexodon***	Trissexodontini	Trissexodont.	Helicodont.	
				Mastigophallus	Mastigophallini			
				***Caracollina* +3**		Caracollin.		
				Oestophora		Oestophor.		
				Gittenbergeria		Gittenbergeri.		
	Helicodont.	Helicodont.		***Helicodonta***		Helicodont.		
				Drepanostoma		Drepanostomat.		
		Lindholmiol.		***Lindholmiola* +1**		Lindholmiol.		
	Helic.	Helic.	Helicini	***Helix* +16**		Helic.	Helic.	Helic.
				Theba	Thebini	Ariant.		
				Murella	Ariantini			
			Murellini	***Arianta* +2**				
		Ariant.		***Campylaea* +1**	Campylaeini			
				Cylindrus	Cylindruini			
				Lampadia	Lampadiini			
	Cochlicell.			***Cochlicella***			Cochlicell.	Xanthonych.
	Elon.			***Elona***			Elon.	
	Sphincterochil.			***Sphincterochila***			Sphincterochil.	
	Epiphragmophor.			*Epiphragmophora*			Epiphragmophor.	
	Monadeni.			***Monadenia***			Monadeni.	
	Humboldtian.	Bunny.		*Bunnya*		Bunny.	Humboldtian.	
		Humboldtian.		***Humboldtiana***		Humboldtian.		
	Xanthonych.	Lysinoe.		*Lysinoe*		Lysinoe.	Humboldtian.	
				Leptarionta		Leptariont.		
				Trionigens		Trionigen.		
				Semiconchula		Semiconchul.		
		Xanthonych.		*Xanthonyx*	Xanthonychini	Xanthonych.	Xanthonych.	
		Trichodisc.	Trichodiscini	*Trichodiscina*	Trichodiscini			
			Miraverellini	*Miraverellia*	Miraverellini			
		Metos trac.		*Metostracon*	Metostracini			
	Helmintho - glypt.	Sonorell.		*Sonorella*		Sonorell.	Xanthonych.	
		Helmintho - glypt.	Helminthoglyptini	*Micrarionta*		Micrariont.		
				Helminthoglypta		Helminthoglypt.	Helminthoglypt.	
				Eremarionta		Eremariont.		
			Sonorelicini	*Sonorelix*				
	Cepol.			***Cepolis***		Cepol.		
	Bradybaen.	Bradybaen.	Bradybaenini	***Bradybaena* +2**		Bradybaen.	Bradybaen.	
			Aegistini	***Aegista* +1**		Aegist.		
			Euhadrini	***Euhadra***				
		Helicostyl.		*Helicostyla*		Helicostyl.		
	Camaen.	Sinumelont.		*Sinumelon*		Xanthomelont.	Camaen.	Camaen.
		Camaen.		*Xanthomelon*				
				Papuina		Papuin.		
				Cristovala		Cristoval.		
				Camaena		Camaen.		
		Rhagad.		*Rhagada*				
Acav.	Megomphic.			*Ammonitella*			Ammonitell.	Polygyr.
Punct.	Oreohelic.			*Oreohelix*			Oreohelic.	
	Thysanophor.			*Thysanophora*			Thysanophor.	
	Pleuront.			*Discolepis*		Discolep.	Pleuront.	
				Gonostomopsis		Gonostomops.		
				Pleurodonte		Pleuront.		
				Polydontes		Polydont.		
				Solaropsis		Solarops.		
	Polygyr.	Polygyr.		*Allogona*	Allogonini	Polygyr.	Polygyr.	
				Ashmunella	Ashmunellini			
				Mesodon	Mesodontini			
				Polygyra	Polygyrini			
				Stenotrema	Stenotremini			
				Vespericola	Vespericolini			
		Triodops.		*Triodopsis*		Triodops.		

Figure 1: Comparison between the systems proposed by Hausdorf and Bouchet (in Bouchet and Rocroi, 2005) for Helicoidea (left columns) and Schileyko (2004-2006) for the corresponding taxa (right columns). Both systems are separated by a central column indicating the type genus for each suprageneric taxon. Columns (superfamily, family, subfamily and tribe) appear in the opposite order to Schileyko's classification scheme. Genera in bold type indicate that one or more species were included in this study; asterisk indicates that a non-type genus was analysed; +no. indicates the number of additional genera included in the analysis for each suprageneric taxon.

by Hausdorf and Bouchet (in Bouchet and Rocroi, 2005) and Schileyko (2004 2006a, 2006b) and to evaluate the systematic genera arrangements of the European helicoideans adopted by the CLECOM system: Check List of European Continental Mollusca (Bank et al., 2001; Bank, 2011), and (iii) to estimate divergence times by fossil calibration of the resultant phylogeny using the program BEAST.

2. Materials and methods

2.1. Specimens

Representatives were included of the eight helicoidean families living in the western Palaearctic region (*sensu* Bouchet and Rocroi, 2005). We obtained 173 new sequences from 99 specimens covering 76 species. New sequences were restricted to the families Helicidae and Hygromiidae s.l., the most diverse families of the western Palaearctic region (see Supplementary material S1). We also included recently published sequences from the work of Gómez-Moliner et al. (2013) mainly focused on Trissexodontidae and Helicodontidae. Published sequences of other representative taxa were obtained from GenBank, including some non-western helicoidean families to obtain a more complete phylogeny of the entire group (Supplementary material S1). Considering both the new and Genbank sequences, we here examine data for 15 out of 20 families of Helicoidea, including all families living in the western Palaearctic. The locations of voucher material are provided in Supplementary material S1.

2.2. DNA extraction, PCR amplification and sequencing

The material examined was preserved in 96% ethanol, and total genomic DNA was extracted from foot muscle using the DNAeasy Tissue kit (Qiagen, Valencia, CA, USA). Four gene fragments were selected for multi-locus analyses: one mitochondrial marker, around 430 bp of the 16S ribosomal RNA gene; and three nuclear fragments, approximately 1445 bp of the rRNA gene cluster, including the 3' end of the 5.8 S gene (-50 bp), the complete ITS2 region (-600 bp) and the 5' end (-840 bp) of the large subunit rRNA (LSU; 28S) gene. General PCR cycling conditions used for DNA amplification were 1 min at 96ºC, [30 s at 94ºC, 30 s at 47–55ºC (depending on the annealing temperature of the primer pair used), 1 min at 72ºC] (repeated for 35 cycles) and 10 min at 72ºC. Amplicons were sequenced using the dRhodamine Terminator Cycler Sequencing Ready reaction Kit (Applied Biosystems, Foster City, CA) run on an ABI PRISM model 3100 Avant Genetic Analyzer and using the same primers as for PCR (see Table 1 for the primers used). The resulting forward and reverse sequences were assembled using Sequencher v4.10.1 (Gene Codes Corporation) and checked for errors/ambiguities. New nucleotide sequences for 16S rRNA and the rRNA gene cluster (5.8S, ITS2 and 28S) were obtained as part of this study. 5.8S fragment is partial and short (-50 bp); therefore we considered 5.8S-ITS2 as a single partition. Consequently, nuclear rRNA gene cluster was divided into two partitions: 5S-ITS2 and 28S. These sequences have been deposited in GenBank under accession numbers KJ458481-KJ458653 (Supplementary material S1).

Table 1: List of primers used for amplification and sequencing.

Gene	Primer	Sequence	Reference
16S rRNA	16sar (5')	5' CGCCTGTTTATCAAAAACAT 3'	Palumbi et al. (1991)
	16sbr (3')	5' CCGGTCTGAACTCAGATCACGT 3'	Palumbi et al. (1991)
5.8S-ITS2	LSU-1 (5')	5' CTAGCTGCGAGAATTAATGTGA 3'	Wade et al. (2006)
	LSU-3 (3')	5' ACTTTCCCTCACGGTACTTG 3'	Wade et al. (2006)
28S	LSU-2 (5')	5' GGGTTGTTTGGGAATGCAGC 3'	Wade et al. (2006)
	LSU-2mod (5')	5' TCTCAGGAGTCGGGTTGTTT 3'	This work
	LSU-5 (3')	5' GTTAGACTCCTTGGTCCGTG 3'	Wade et al. (2006)

2.3. Phylogenetic analyses

Sequences were aligned with Mafft v7 online version (Katoh et al., 2002) as it has been described to perform better than alternative pairwise alignment methods (Golubchik et al., 2007). We used the Q-INS-i algorithm and default values for the rest of the parameters for the alignment of each gene (Katoh and Toh, 2008).

We aligned our sequences of the 16S rRNA and nuclear rRNA cluster genes with sequences published in GenBank. Several sequences were obtained for all genes considered in this work, but some sequences from GenBank belonging to taxa of interest for this study are provided only for one or two of the genes considered. For this reason, each data set was analyzed separately to cover the maximum information possible and to compare the different topologies obtained. Next, we analyzed three different data sets: the first included the mitochondrial marker 16S rRNA, the second incorporated the nuclear rRNA gene cluster and the third data set was a combined matrix for all genes.

Phylogenetic signal for each gene region analyzed was accessed using the parsimony-based method of Steel el al. (1993) and the entropy-based information method of Xia et al. (2003) and Xia and Lemey (2009), both implemented in Dambe v5.2.38 (Xia, 2001; Xia and Xie, 2001).

Phylogenetic inference was based on Bayesian (BI), maximum likelihood (ML) and neighbour joining (NJ) inference. We used MrBayes v3.2.2 (Ronquist and Huelsenbeck, 2003) to estimate the topology shown in this work. The evolutionary model considered was GTR + I + G, estimated independently for each of the gene partitions using jModelTest v2.1.1 (Darriba et al., 2012) applying Akaike weights as selection criterion. MrBayes was run for $20x10^6$ generations using default values and saving trees each 100 generations. Convergence between runs and the choice of an appropriate burn-in value were assessed by comparing the traces using Tracer v1.5 (Rambaut and Drummond, 2007). Maximum likelihood phylogenies were inferred with RAxML v7.2.8 (Stamatakis, 2006) through the Cipres Science Gateway (Miller et al., 2010) (which includes an estimation of bootstrap node support) using a GTRGAMMA model of evolution and 1000 bootstrapping replicates. Due to the different evolutionary rates of markers considered for this study, in both ML and Bayesian analyses, characters within combined sequence sets were partitioned

by gene, allowing different evolution rates for each partition. NJ was performed in PAUP* v4.0b10 (Swofford, 2002). For each data set, we used the available substitution and rate heterogeneity model with the closest match to that selected by jModelTest (Darriba et al., 2012). Statistical support for the resulting topologies was assessed by bootstrapping with 5000 pseudoreplicates (Felsenstein, 1985). For the different topologies obtained, we interpreted as significant statistical support values above 70% for bootstrapping procedures in the ML and NJ analyses and 95% for Posterior Probability (PP) in the BI analysis.

2.4. Divergence time analyses

The use of several calibration nodes has been shown to improve estimates of divergence times and rate estimates (Yang, 2004; Porter et al., 2005; Pérez-Losada et al., 2008). For this study, divergence times were therefore estimated using different genes and multiple fossil calibration points. Time analyses were restricted to the nuclear dataset which allows us to employ more families for the estimation. A relaxed-clock MCMC approach using the uncorrelated lognormal model was implemented in BEAST v1.8 (http://beast.bio.ed.ac.uk/), using $3x10^7$ generations, sampling every 1000th generation and with a burning value of 10%. Independent analyses were performed for the two partitions into which nuclear sequence data were divided. Models of sequence evolution for each nucleotide sequence partition were determined using the corrected Akaike information criterion in jModelTest (Darriba et al., 2012). The Yule model was chosen as the speciation prior for all three data sets. Information in the set of post-burnin trees was summarized using Tracer v1.5 and TreeAnnotator v1.8 not allowing ESS values < 200. The maximum clade credibility tree and clade BPPs were obtained through TreeAnnotator v1.8. Mean values and 95% HPD intervals for the ages of clades and of the stems leading to these clades were calculated using Tracer v1.5.

Calibration was based on fossil evidence and we based divergence time estimates on the age of six fossils attributed to related taxa by Nordsieck (2014) (Table 2). A log-normal prior distribution was assumed for the calibration points (see Ho and Phillips, 2009). Dates of fossils ranged from the Early Eocene (47.8 Ma) to the Late Oligocene (23.03 Ma). Since fossils were ascribed to a geological period, we used the upper limit of each period for divergence time estimates. The age of a fossil represents the minimum age of the

Table 2: Summary of node age constraints (minimum ages) used for the divergence time estimates based on fossil evidence.

Node	Groups of the crown group	Fossil genus	Fossil Age	Upper limit	Source
A	Elonidae	*Megalocochlea*	Middle Eocene	38.0 Ma	Nordsieck, 2014
B	Helicinae	*Parachloraea*	Late Eocene	33.9 Ma	Nordsieck, 2014
C	Helicodontidae	*Protodrepanostoma*	Early Oligocene	28.1 Ma	Nordsieck, 2014
D	Hygromiidae	*Loganiopharynx*	Early Eocene	47.8 Ma	Nordsieck, 2014
E	Sphincterochilidae	*Dentellocaracolus*	Middle Eocene	38.0 Ma	Nordsieck, 2014
F	Trissexodontidae	*Praeoestophorella*	Late Oligocene	23.03 Ma	Nordsieck, 2014

group. Hence, it is more appropriate to present a node within a time interval rather than a fixed time (Norell, 1992). According to Tillier et al (1996), we considered the origin of the Stylommatophora in the Upper Jurassic (150 Ma) and a normal prior distribution was assumed for the calibration of this point. Calibrations were plotted on the node prior to the basal node of the clade of interest.

3. Results

The sequence data obtained are provided in Table 3. Data matrices included 189 sequences for 16S, 136 for the nuclear rRNA gene cluster covering 15 families of the Helicoidea, and 120 for the combined data set covering 11 families. Alignment lengths were 517 base pairs (bp) for 16S, 960 bp for 5.8S-ITS2, and 854 bp for 28S. The length of the mitochondrial/nuclear combined alignment was 2,331 bp. The 5.8-ITS2 gene fragment was the most variable, with 67 bp variable sites (46 positions phylogenetically informative PI), the 16S fragment showed 63 bp variable sites (55 positions PI), and the 28S sequence featured 41 bp variable sites (27 positions PI).

Phylogenetic signal analyses based on substitution saturation showed that all three molecular markers should possess enough information to infer phylogenetic relationships among the families considered (Supplementary material S2).

The phylogenetic reconstruction obtained by the concatenated-gene analyses (nuclear rRNA gene cluster + 16S rRNA) is shown in Figure 2. Unless otherwise indicated, the topology and support of this tree will be referred to in the results section. Other single gene trees (16S rRNA or nuclear rRNA) have been included as supplementary material (Supplementary material S3, S4). These topologies include a higher number of taxa because for some species, only sequences of one or two gene fragments were available, especially for taxa represented exclusively by GenBank sequences. Concatenated-gene analyses were better resolved than single-gene analyses, and thus more accurately represent relationships among taxa. Accordingly, the results of single-gene analyses will not be discussed, except when referring to taxa not represented in the concatenated-gene analyses. It should be noted that no single-gene trees featured any well-supported clades in conflict with the concatenated-gene trees discussed.

Table 3: Lengths of the sequenced fragments (maximum and minimum) before and after alignment, number of informative sites and evolutionary model selected using the Akaike information criteria implemented in jModeltest for the different partitions.

Partition	Maximum lenght	Minimum lenght	Aligned length	No. informative sites	Optimal AIC model
16S rRNA	430	211	517	55	GTR+I+G
5.8S-ITS2	607	374	960	46	GTR+I+G
28S	839	315	854	27	GTR+I+G
5.8S-ITS2-28S	1444	834	1814	73	GTR+I+G
Total	1852	1242	2331	185	GTR+I+G

3.1. Family-level classification

All taxa included in our study were grouped into two main clades, with *Cepolis* as sister group in the nuclear rRNA analysis (Supplementary material S4). The first clade (Figure 2) included Hygromiidae *sensu* Hausdorf and Bouchet (2005) and Cochlicellidae (PP = 0.94; ML = 70%; NJ = 79%). The second clade grouped together all remaining Helicoidea families considered and its monophyly was well supported by ML and NJ in the concatenated-gene tree (PP = 0.9; ML = 71%; NJ = 78%). Clades corresponding to Bradybaenidae, Camaenidae, Cochlicellidae, Elonidae, Helicidae, Helicodontidae, Humboldtianidae, and Sphincterochilidae (represented only by *Sphincterochila candidissima*), were strongly supported (PP = 1.00; ML ≥ 95%; NJ ≥ 99%) in the concatenated-gene tree. The monophyly of Trissexodontidae was only recovered by BI and ML analyses (PP = 1.00; ML = 99%). The Trissexodontidae also constituted a monophyletic group in the NJ analysis when only nuclear rRNA was considered (NJ= 90%), with *Gittenbergeria* appearing as a separate lineage in the concatenated-gene tree. Nuclear rRNA analysis (Supplementary material S4) also recovered Monadenidae (represented only by *Monadenia fidelis*), Pleurodontidae (PP = 1.00; ML = 100%; NJ = 100 %) and Polygyridae (PP = 1.00; ML = 100%; NJ = 100%) with full support.

Relationships among families were not fully resolved even in the concatenated-gene analyses. Nevertheless, the family Cochlicellidae appeared as a derived group within a clade containing all the Hygromiidae *s.l.* genera considered in this study. This relationship among Cochlicellidae and Hygromiidae *s.l.* was recovered with high support in nuclear rRNA and concatenated-gene trees. BI analysis of nuclear rRNA revealed Polygyridae as the sister group (PP = 0.99) of Bradybaenidae (Bradybaeninae + Aegistinae) and Camaenidae, although this sister relationship was not supported by ML and NJ analyses. The nuclear rRNA tree also recovered Monadenidae as the sister group of Humboldtianidae (PP = 0.94; ML = 92%; NJ = 93%), which together with Pleurodontidae form a monophyletic clade supported by BI (PP = 0.99) but not by ML and NJ. Helicodontidae was recovered as the sister group of a clade containing Elonidae, Helicidae, Humboldtianidae, Sphincterochilidae and Trissexodontidae, but this relationship was only supported by the BI analysis in the nuclear rRNA tree (PP = 1.00). Relationships among Elonidae, Helicidae, Humboldtianidae, Sphincterochilidae and Trissexodontidae were not resolved. The sister relationship between Helicidae and Trissexodontidae was recovered only by the nuclear rRNA BI analysis but without significant support (PP = 0.90). Cepolidae (represented by *Cepolis streatori* nuclear rRNA) was recovered as the sister clade of the group joining together all the other families considered in this study.

3.2. Genera arrangements within the highly diverse families Helicidae and Hygromiidae

Nuclear rRNA analysis recovered the monophyly of the helicid subfamilies Ariantinae (PP = 1.00; ML = 100%; NJ = 100%) and Helicinae (containing representatives of the

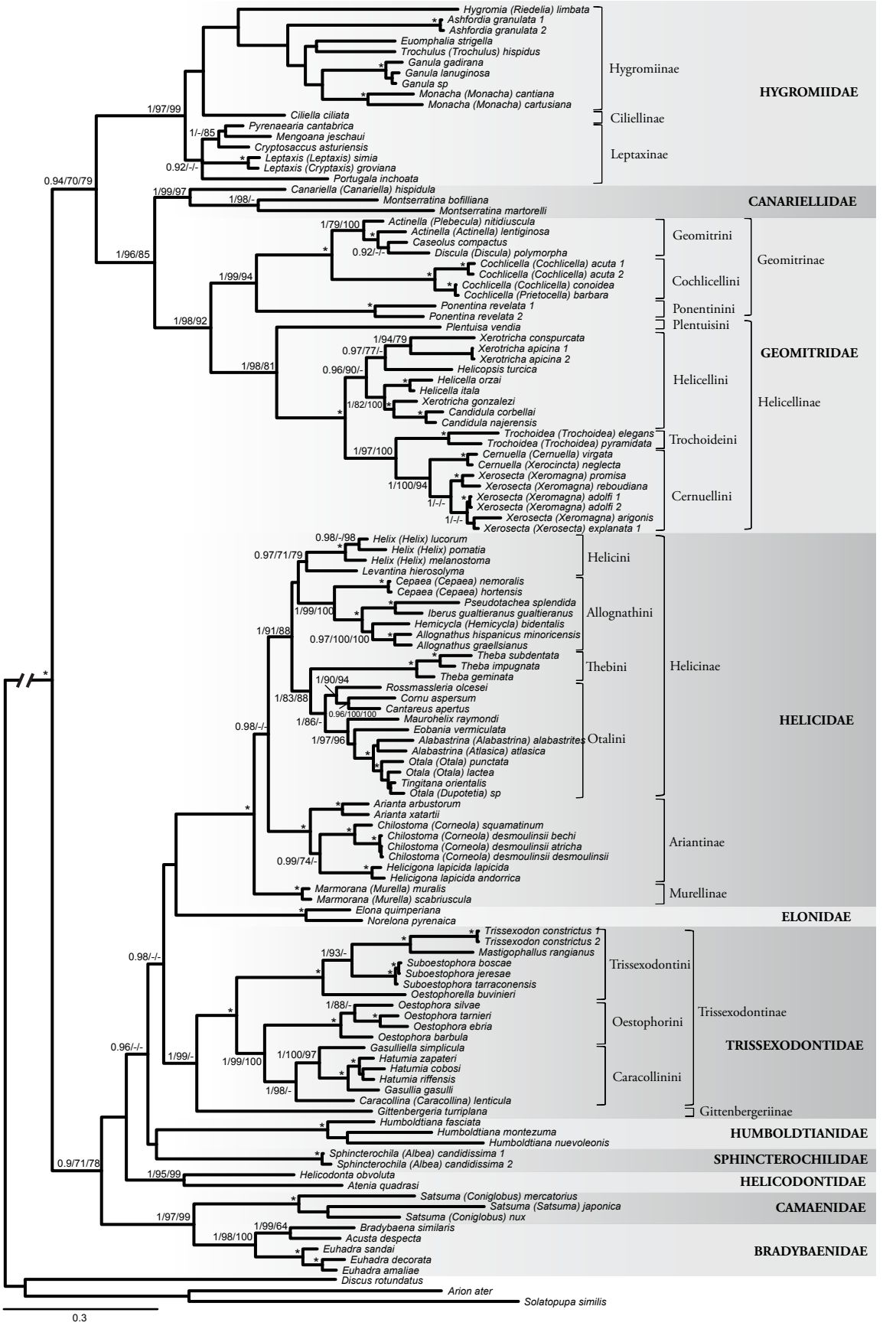

Figure 2: Phylogenetic tree of the Helicoidea based on Bayesian inference (BI), maximum likelihood (ML) and neighbor joining (NJ) analyses of the concatenated data set including 16S rRNA and nuclear 5.8S, ITS2 and 28S sequences. Numbers correspond to BI posterior probabilities, ML bootstrap values and NJ bootstrap, respectively. Asterisks (*) indicate full support of nodes: BI posterior probabilities = 1.00 ML bootstrap values = 100% and NJ bootstrap = 100%.

three tribes: Helicini, Murellini and Thebini) although the monophyly of the Helicinae was weakly supported (PP = 0.78; NJ = 78 %). In contrast, the concatenated-gene tree recovered Murellini as the sister group of Ariantinae and Helicini + Thebini with strong BI support (PP = 0.98). Nevertheless, ML and NJ analyses recovered Ariantinae, Murellini and Helicini as three different lineages in the concatenated-gene tree. Thebini emerged as a different lineage in the BI and ML analysis of the 16S gene fragment, being the sister group of the other helicids. In contrast, Thebini was included with strong support within the Helicini in the nuclear rRNA (PP = 1.00; ML = 97%; NJ = 79%) and concatenated-gene trees (PP = 1.00; ML = 91%; NJ = 88%), as the sister clade of a group containing the genera *Alabastrina*, *Cantareus, Cornu*, *Eobania*, *Maurohelix, Otala, Rossmassleria* and *Tingitana* (PP = 1.00; ML = 83%; NJ = 88% in the concatenated-gene tree; PP = 1.00; ML = 96%; NJ = 96% in the nuclear rRNA tree). This group formed a polytomy with another two lineages of Helicini in the BI analysis. One of these lineages grouped *Allognathus*, *Hemicycla*, *Iberus* and *Pseudotachea* as the sister group of *Cepaea* (PP = 1.00; ML = 0.99%; NJ = 100%). The other clade grouped *Helix* and *Levantina* genera (and *Eremina* in nuclear rRNA analysis) (PP = 0.97 %; ML = 71%; NJ = 79%).

None of the polytypic subfamilies of the Hygromiidae *s.l.* (Ciliellinae, Geomitrinae, Hygromiinae and Monachainae) were recovered as monophyletic lineages. Three main clades were obtained within the Hygromiidae *s.l.* The first main clade was highly resolved (PP = 1.00; ML = 97%; NJ = 99%) and grouped Euomphaliini, Monachaini, Hygromiini (without *Cernuella*), Leptaxini and Trochulini, although relationships among these taxa were not fully resolved. *Ciliella*, *Cryptosaccus*, *Ganula* and *Pyrenaearia* also clustered within this clade. The second hygromiid clade grouped *Canariella* and *Montserratina* (PP = 1.00; ML = 99%; NJ = 97%). The third hygromiid clade grouped Cochlicellidae with Geomitrini and Trochoideini (Geomitrinae), Helicellini (Hygromiinae) and Ponentininae (PP = 1.00; ML = 98%; NJ = 92%). Within this third clade, the genus *Plentuisa* was recovered as the sister group of Helicellini + Trochoideini + *Cernuella* (Hygromiini) (PP = 1.00; ML = 98%; NJ = 81%) and grouped with a clade formed by Ponentininae and Geomitrini + Cochlicellidae (PP = 1.0; ML = 99%; NJ = 94%). The third hygromiid clade was recovered as the sister group of the second hygromiid clade (PP = 1.00; ML = 96%; NJ = 85%). The sister relationship of the first hygromiid clade with the second + third hygromiid clades was supported in the concatenated-gene tree (PP = 0.94; ML = 70%; NJ = 79%) and by BI and ML phylogenetic analyses in the nuclear rRNA tree (PP = 1.00; ML = 79%; NJ = 68%).

3.3. Timing of diversification and biogeographic patterns

Divergence time estimates using BEAST rendered a well-resolved maximum clade credibility tree (see Figure 3). This tree represents the estimated divergence time chronogram using the Maximum Clade Credibility consensus BMCMC tree based on the nuclear rRNA gene cluster matrix, and six fossil calibrations. Divergence times and 95% highest posterior density (HPD) intervals are given in Table 4. Multiple independent

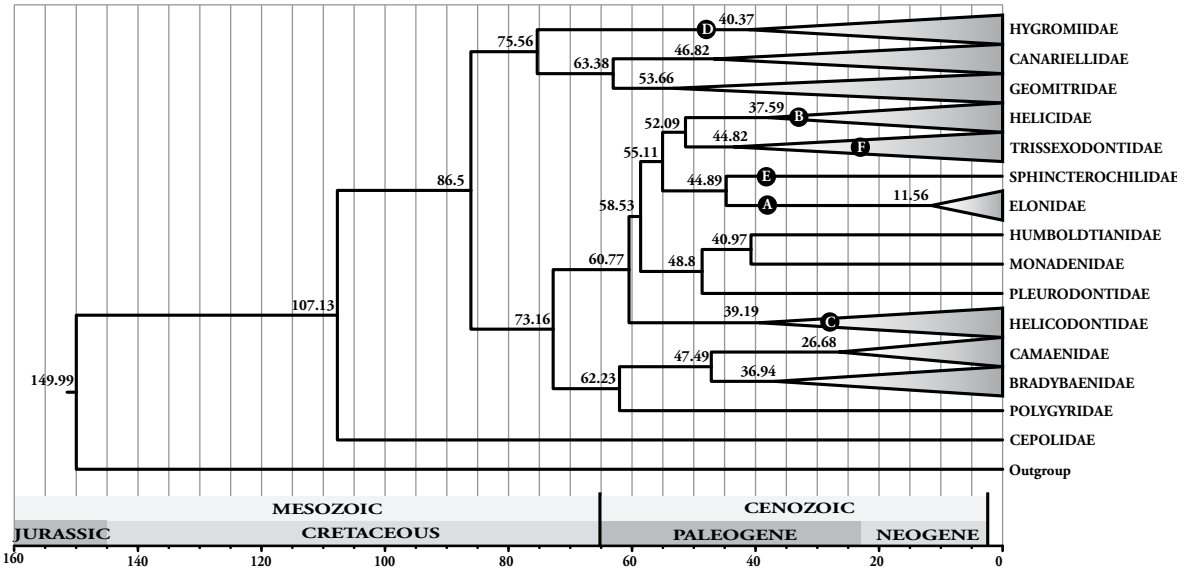

Figure 3: Maximum clade credibility tree generated by the BEAST analysis for the 5.8S-ITS2-28S concatenated data set. Node numbers indicate estimated mean ages provided by the BEAST analysis. The bar below provides ages in millions of years (My).

Table 4: Posterior characteristics of nodes of Helicoidea and all families: estimated mean ages and 95% highest probability density (HPD) intervals of the MRCA and posterior probability.

Group	Mean age MRCA in Ma	95% HPD interval in Ma	Posterior prob.
Helicoidea	107.13	82.65 – 137.99	0.95
Bradybaenidae	36.94	25.13 – 51.29	1.00
Camaenidae	26.68	15.73 – 38.97	1.00
Canariellidae	46.82	28.83 – 65.73	1.00
Elonidae	11.56	4.27 – 21.61	1.00
Geomitridae	53.66	40.51 – 67.98	1.00
Helicidae	37.59	34.44 – 42.53	1.00
Helicodontidae	39.19	20.72 – 57.53	1.00
Hygromiidae	40.37	26.78 – 59.32	1.00
Trissexodontidae	44.82	34.98 – 55.06	1.00

Bayesian runs produced large effective sample sizes and convergence statistics in Tracer that indicated the convergence of all analyses. Variation in the rate of evolution across lineages was evident. According to our divergence time analyses (Table 4), the origin of Helicoidea dates back to 107.13 Ma, in the Early Creataceous. The two main clades obtained share a similarly aged MRCA, 75.56 Ma for Hygromiidae *s.l.* (Hygromiidae *s.str*. Canariellidae, Geomitridae) and 73.16 Ma for the clade grouping the rest of the families considered (Bradybaenidae, Camaenidae, Elonidae, Helicidae, Helicodontidae, Humboldtianidae, Monadenidae, Pleurodontidae, Polygyridae, Sphincterochilidae and Trissexodontidae). According to our reconstruction of the Hygromiidae *s.l.* clade, the three European families considered started their diversification in the Eocene (33.9-56.0 Ma), with ages ranging between 40.37 Ma (Hygromiidae *s.str.*) and 53.66 Ma (Geomitridae). The second main

clade diverged into two groups. The first group started its diversification 62.23 Ma ago and includes families distributed outside the western Palaearctic region: Brabybaenidae (36.94 Ma) living in Asia, Camaenidae (26.68 Ma) in SE Asia and Australia and Polygyridae in North America. The second cluster dates back to 60.77 Ma and joins families of the western Palaearctic region [Elonidae (11.56 Ma), Helicidae (37.59 Ma), Helicodontidae (39.19 Ma), Sphincterochilidae and Trissexodontidae (44.82 Ma)] with the Humboldtianidae, Monadenidae and Pleurodontidae distributed across North and South America.

4. Discussion

Recent molecular studies are gradually improving our knowledge of relationships among the Helicoidea (Manganelli et al., 2005; Wade et al., 2006; Groenenberg et al., 2011). The present study mainly focuses on the helicoidean families that exist in the western Palaearctic region, although we also included representatives of other phylogenetically closely related helicoidean families (after Wade et al., 2006; 2007). Compared to prior studies, the increased number of taxa sampled and the use of mitochondrial and nuclear genes yielded some new insights into relationships, and allowed for direct comparisons with earlier investigations examining families of land snails.

4.1. Congruence of phylogenetic clades with current classification

All the families considered in our study were grouped into two main clades. Thus, Bradybaenidae, Camaenidae, Elonidae, Helicidae, Helicodontidae, Humboldtianidae, Monadenidae, Pleurodontidae, Polygyridae, Sphincterochilidae and Trissexodontidae clustered together as the sister group of Hygomiidae *s.l.* This basal dichotomy between Hygromiidae *s.l.* and the other helicoidean families included in our study is consistent with the classification by Koene and Schulenburg (2005), who grouped Helicidae, Helminthoglyptidae (including Humboldtianidae and Monadenidae) and Bradybaenidae (including Camaenidae) as the sister group of Hygromiidae *s.l.* In contrast, Wade et al. (2007) clustered, although without support, the Hygromiidae *s.l.* with the pleurodontids *Pleurodonte sinuata* and *Theliodomus asper*, as the sister group of a large clade including the other helicoidean taxa, excluding the pleurodontids *Polydontes* and *Zachrysia*. Here, we included the nuclear sequences for *Pleurodonte sinuata* and *Theliodomus asper* of Wade et al. (2007), but both taxa grouped with Monadenidae and Humboldtianidae, confirming the monophyly of the non-Hygromiidae *s.l.* basal clade. Manganelli et al. (2005) clustered the Helicodontidae within the Hygromiidae *s.l.* However, our results located this family outside the Hygromiidae.

Eleven helicoidean families were identified in the concatenated-gene analysis and four additional families appeared in the nuclear rRNA tree, with Cepolidae as the sister group of the other Helicoidea. The family status was confirmed for Bradybaenidae, Camaenidae, Elonidae, Helicidae, Helicodontidae, Humboldtianidae, Monadenidae, Pleurodontidae, Polygyridae, Sphincterochilidae and Trissexodontidae. All these families, defined on the basis of morphological characters (reviewed in Nordsieck, 2010), were

also considered in the classification of Hausdorf and Bouchet (2005). In the helicoidean classification by Schileyko (2004, 2006a, 2006b), Cepolidae was ascribed to a subfamily of Helminthoglyptidae (a family not included in our study), while Trissexodontidae were distributed among four subfamilies within the Helicodontidae. Gómez-Moliner et al. (2013) also recovered the families Trissexodontidae and Helicodontidae as separated lineages. The family Hygromiidae *sensu* Hausdorf and Bouchet (2005) was divided into three clades which are here given familial rank. Hygromiidae *s.str*., Canariellidae and Geomitridae are here shown to be distinct clades with high support. Nevertheless, we should mention that we did not examine some suprageneric hygromiid taxa restricted to the eastern Mediterranean and Caucasus regions (Hesseolinae Schileyko, 1991 and Metafruticicolinae Schileyko, 1972), Tien-Shan mountains in Central Asia (Archaicinae Schileyko, 1978 and Paedhoplitinae Schileyko, 1978), and Tropical Africa (Halolimnohelicinae Nordsieck, 1986 and Vicariihelicinae Schileyko, 1991). The monophyly of all above mentioned 14 families examined was highly supported by the different phylogenetic analyses. However, the family Cochlicellidae *sensu* Hausdorf and Bouchet (2005) and Schileyko (2004) was not recovered as a distinct clade of familial rank, but was included within the Geomitridae. Sister relationships between Helicidae and Trissexodontidae recovered by some but not all the analyses performed require confirmation. The monophyly of the Hygromiidae *s.l.* (Hygromiidae *s.str.* + Canariellidae + Geomitridae, present work) was not observed by Manganelli et al. (2005). Koene and Schulenburg (2005) did recover the monophyly of the Hygromiidae *s.l.* but without statistical support. Wade et al. (2007) also recovered this monophyly, but only supported by NJ analysis. In the present study, the monophyly of the clade grouping Hygromiidae *s.str*., Canariellidae and Geomitridae was supported by ML and NJ analyses in the concatenated-gene tree and by BI, ML and NJ in the nuclear rRNA tree. Besides, the sister relationship between Canariellidae and Geomitridae was highly supported by all phylogenetic analyses.

Relationships among families outside the western Palaearctic region were congruent with the data of Wade et al. (2006; 2007) clustering Brabybaenidae, Camaenidae and Polygyridae with high support. Nuclear rRNA analysis also grouped together Humboldtianidae, Monadenidae and Pleurodontidae with strong support. *Satsuma* and *Coniglobus* classified by Schileyko (2004) within the Aegystinae (Bradybaenidae) were ascribed to the Camaenidae according to Vaught (1989) and Wade et al. (2007).

4.1.1. Family Hygromiidae s.str.

Twelve genera found to cluster within this clade appeared within three main groups here considered subfamilies: Ciliellinae, Leptaxinae and Hygromiinae. The genus *Hygromia* was grouped with *Ashfordia*, *Euomphalia* and *Monacha*, (Monachainae), *Trochulus* (Trochulinae: Trochulini) and *Ganula* (Hygromiinae: Hygromiini). This indicates that neither Trochulinae Lindholm, 1927 *sensu* Schileyko nor Hygromiinae *sensu auctores* are monophyletic groups. All the Monachainae genera included in the present study were assigned to this group, consistent with the data of Koene and Schulenburg (2005) relating

Monacha to the mesophilic Hygromiidae *s.l.*, but not with the scheme of Steinke et al. (2004), who clustered *Monacha* with the xerophilic Hygromiidae *s.l.* (Geomitridae in the present study).

The phylogenetic relationships of the genera included within this family were not fully resolved. Besides, some clusters were not statistically supported. Thus, before suggesting any further subdivisions, more work is needed on this family including the study of more taxa and/or more gene fragments. *Cryptosaccus* and *Pyrenaearia*, considered Hygromiini by Schileyko (2006b), and *Mengoana* (Monachainae *sensu* Schileyko) were grouped with high support values by BI and NJ analyses in the concatenated-gene tree. The relationship of *Trochulus* with the Monachainae, although not supported, was also reported by Koene and Schulenburg (2005) and Wade et al. (2006; 2007). According to Koene and Schulenburg (2005), *Monachoides*, *Perforatella* and *Pseudotrichia* also belong to this family; *Pseudotrichia* being grouped with *Trochulus* and the other two genera being closely related to *Hygromia* and *Ashfordia* based only on the 28S gene fragment (data not shown).

Ciliellinae, only represented by *Ciliella ciliata,* was diagnosed by its simplified genital system with a long free oviduct, a thick bursa copulatrix duct and a short flagellum, and no signs of stimulatory organs (Figure 4). Schileyko (2006b) also included *Schileykiella*, *Cilliellopsis* and *Tyrrheniellina* (all from Tyrrhenian Islands) within the Ciliellinae.

Leptaxinae, considered a tribe by Hausdorf and Bouchet (2005) and represented in our study by five genera (*Cryptosaccus*, *Leptaxis*, *Mengoana*, *Portugala* and *Pyrenaearia*), shows no neat diagnostic differences with respect to the Hygromiinae with its single stimulatory apparatus (Figure 4), as may be seen by examining the drawings of Schileyko (2006b). Leptaxinae shows a reduction trend and even loss of the accessory sac (*Cryptosaccus*, *Leptaxis* and *Portugala*), or dart sac (*Mengoana*). Although our study was centred on western European taxa and we lacked representatives from Central and Eastern Europe, we suggest this clade could have originated in the Iberian Peninsula: four genera are endemic to the northwestern and western Iberian Peninsula, *Leptaxis* being endemic to Macaronesia.

The subfamily Hygromiinae includes six genera: *Hygromia*, *Ashfordia*, *Euomphalia*, *Trochulus*, *Ganula* and *Monacha*. This group is anatomically very diverse and specimens have both a double or single stimulatory system consisting of dart-sac, accessory sac and mucous glands, or these are transformed to vaginal appendiculata (Figure 4): *Monacha* is the only hygromiid genus with a free right ommatophoral retractor (r.o.r.) (but see Hausdorf (2000) for some exceptions), *Euomphalia* and *Trochulus* have a double stimulatory apparatus, while *Ashfordia* has no stimulatory organ. Only *Hygromia* and *Ganula* have the single stimulatory system comprising dart-sac, accessory sac and mucous glands.

4.1.2. Family Canariellidae Schileyko, 1991

Our data recovered a close relationship between *Canariella* and *Montserratina*, which were grouped within a separated clade that was highly supported by nuclear rRNA and all-gene

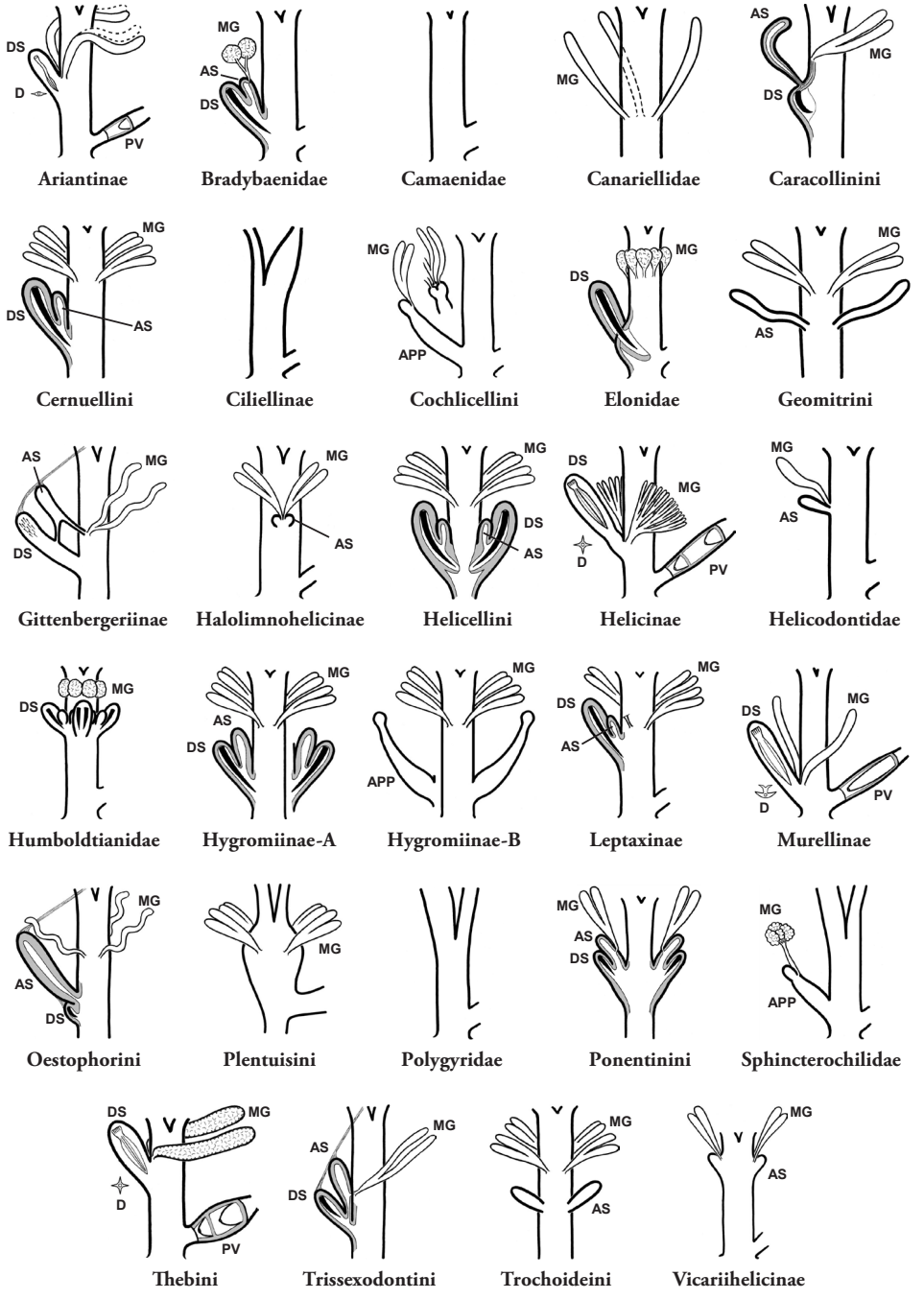

Figure 4: Diagram illustrating the diagnostic features of the stimulatory apparatus of the genital system for the families, subfamilies and tribes of the superfamily Helicoidea defined in this study. The inner structure of the dart and accessory sacs are indicated when necessary; the inner structure of the penis and cross section of the dart are included only for the subfamilies of Helicidae. Shortness of the vagina is indicated by a lengthened oviducal/spermathecal fork. Oligotypical taxa are represented by their type or better-known genera; polytypical taxa are represented in general terms (defective genital systems are not indicated). The great variation within Hygromiinae is represented by two schemes, each showing a single/double stimulatory apparatus (AS: accessory sac; APP: appendicula; D: cross-section of dart; DS: dart sac; MG: mucous glands; PP: penial verge).

concatenated analyses. Schileyko (1991) created the monogeneric subfamily Canariellinae within the Hygromiidae. Hausdorf and Bouchet (2005) and Bank et al. (2001) included *Canariella* within the Ciliellinae. However, Schileyko (2006a) assigned *Montserratina* to the Monachainae Wenz, 1930. Our analyses revealed that both genera were neither related to *Ciliella* nor *Monacha*. On the basis of our results, the subfamily Canariellinae (including *Montserratina*) should be elevated to family rank. The close relationship between *Canariella* and *Montserratina* was indicated by Ibáñez et al. (1995) based on shell microsculpture and anatomical similarities. Anatomically, this family is characterized by having a short flagellum and a stimulatory apparatus (Figure 4) comprised only of one to three single mucous glands (lacking dart or accessory sacs).

4.1.3. Family Geomitridae Boettger, 1909

Both classification systems (Figure 1) ascribed Geomitrini and Trochoideini Nordsieck, 1987 to the Geomitrinae. Besides, Hausdorf and Bouchet (2005) included the Asian Paedhoplitini Schileyko, 1978 within Geomitrinae (considered a separate subfamily by Schileyko, 2006b). We had no Paedhoplitini representatives available for our study.

Two main clades were recovered within the Geomitridae, here considered of subfamily rank: Geomitrinae and Helicellinae. Geomitrinae *sensu* Schileyko (2006b) and Hausdorf and Bouchet (2005) including Geomitrini and Trochoideini emerged here as polyphyletic and both tribes were assigned to different subfamilies: Geomitrini to the Geomitrinae, and Trochoideini to the Helicellinae. The Macaronesian genera *Actinella*, *Caseolus* and *Discula* (Geomitrini) were grouped with *Cochlicella* and its allies (Cochlicellini in our classification), with *Ponentina* (Ponentinini in our proposal) as their sister group.

The Helicellinae grouped together another four clades, that are here ranked as tribes. One clade contained *Cernuella* (Hygromiinae: Cernuellini *sensu* Schileyko, 2006b) as the sister group of *Xerosecta* (Hygromiinae: Hygromiini). Because *Hygromia* was clustered within a different group in our analyses, the name Cernuellini Schileyko, 1991 should be applied here. The second clade, Trochoideini Nordsieck, 1987, was recovered as the sister group of Cernuellini. The other two clades were grouped together and designated Helicellini Ihering, 1909 (grouping *Candidula*, *Helicella* and *Xerotricha*) and Plentuisini new tribe, monotypic for *Plentuisa vendia*. In agreement with Manganelli et al. (2005) *Ichnusomunda sacchii* and *Polloneriella contermina* grouped with Cernuellini in our 16S tree (Supplementary material S3).

The most characteristic feature of the anatomy of geomitrids is that they have a free r.o.r. (passing outside the peni-oviducal angle) and a double stimulatory apparatus. However, there are some exceptions and one subclade has a crossing r.o.r. (*Ponentina*) and four subclades have a single stimulatory apparatus. A free r.o.r. has been linked to adaptation to xeric habitats (Schileyko 1978, 1991; Giusti and Manganelli 1987, Nordsieck 1987). Nordsieck (1993) suggested that this feature arose independently several times, allowing hygromiids *s.l.* to have independently colonized xeric habitats on several occasions. Our

results indicate that all helicoidean genera with a free r.o.r. (except *Monacha*) belong to this clade, suggesting that the free r.o.r. arose only once within the Geomitridae, since this family thrives in prevailing xeric habitats.

Geomitrinae. Although Schileyko (2006b) stated that the r.o.r. passes through the peni-oviducal angle in Geomitrinae, it is certainly free in this subfamily as in other geomitrids (Mandahl-Barth, 1950). The stimulatory apparatus of the Geomitrini (Figure 4) consists of one accessory sac and one bifurcate mucous gland, and may be single (in *Discula* and in other genera not included in our study) or double (in *Actinella* and *Caseolus*). Geomitrini is endemic to Macaronesia (Azores and Madeira). The stimulatory apparatus of the monotypic Ponentinini is double (Figure 4); it is composed of two small dart sacs with two accessory sacs attached to these, and two bifurcate mucous glands joined to the accessory sacs, although this apparatus may be somewhat reduced (Holyoak and Holyoak, 2012).

Helicellinae. We cannot define the Helicellinae based on genital structure because this subfamily fulfils the general criterion for the r.o.r. and stimulatory apparatus common to geomitrids. Nevertheless, our phylogenetic tree reveals its uniqueness and its division into four clades. The tribe Helicellini is characterized by having a double stimulatory apparatus (secondarily single in *Candidula*) with each unit consisting of one accessory sac opening into a large dart sac and two bifurcate mucous glands connected to the vagina (Figure 4). The close relationship of *Candidula* and *Helicella* was already been recognized by Hausdorf (1988) based on morphological characters. The tribe Trochoideini has two small accessory sacs (without dart sacs) and four bifurcate mucous glands (Figure 4). Unlike other Helicellinae, the tribe Cernuellini has a single stimulatory apparatus very similar to each unit of the Helicellini (Figure 4). Other genera ascribed by Schileyko (2006b) to the Cernuellini have a similar stimulatory system, and probably belong to this phylogroup. Nevertheless, Schileyko (2006b) also included *Candidula* within the Cernuellini, a genus that according to our results belongs to the Helicellini. Plentuisini new tribe is characterized by a defective double stimulatory apparatus, with neither a dart sac nor accessory sac, but with four bifurcate mucous glands (Figure 4). Although *Plentuisa* has a free r.o.r., Puente and Prieto (1992) claimed it showed a close relationship with Trochulinae and Schileyko (2006b) ascribed it to the Monachainae. Our data allocate *Plentuisa* to the Geomitridae, being neither related to *Trochulus* nor to *Monacha*.

Plentuisini new tribe. Shell minute, depressed, umbilicate, hairy. Right ommatophore retractor independent of genital system. Penis short, with rudimentary flagellum. Vagina short and wide, with inner lumen occupied by four double longitudinal folds. Stimulatory apparatus lacking dart and accessory sacs, having only two pairs of forked mucous glands.

Tribe Cochlicellini Schileyko, 1972

One of the most surprising results of our study was the phylogenetic position recovered for the cochlicellids *Cochlicella* and *Prietocella*. These two subgenera, together with *Monilearia* and *Obelus* (not included in this study) were considered a separate family in the

classifications of Hausdorf and Bouchet (2005) and Schileyko (2004).

The structure and position of the stimulatory apparatus in cochicellids (Figure 4) is so peculiar that interrelations between this taxon and other Helicoidea families have been largely controversial. The apparatus, inserted in the atrium, consists of one long appendage with one or several bifurcate mucous gland(s) opening at the base of a small apical thickening (Schileyko and Menkhorst, 1997). The apical thickening of the stimulatory apparatus can be single (subgen. *Cochlicella*, *Obelus*) or multiple (*Monilearia*, subgen. *Prietocella*) (Schileyko, 2004). Schileyko (1991) and Schileyko and Menkhorst (1997) suggested that cochlicellids should be assigned to a separate family (Cochlicellidae) and believed that they could have originated from the Aegistinae (Bradybaenidae), including Cochlicellidae within the Xanthonychoidea (Schileyko and Menkhorst, 1997; Schileyko, 2004). In contrast, Ibáñez et al. (2006) mentioned the similarity of the stimulatory apparatus of cochlicellids to the penis appendage of the Orthurethra.

According to partial 16S rRNA gene sequences, Manganelli et al. (2005) recovered *Cochlicella* as the sister group of *Sphincterochila* within the Helicellinae (Hygromiidae *s.l.*). Steinke et al. (2004) also recovered the sister relationship of these two genera, closely related to the Helicellinae using sequences of two mitochondrial and two nuclear DNA gene fragments. Groenenberg et al. (2011), using the 16S sequences published by Manganelli et al. (2005), also recovered the sister relationship between *Cochlicella* and *Sphincterochila* but grouped them with Monachainae and Bradybaenidae. The close relationship between *Cochlicella* and *Sphincterochila* was nevertheless not statistically supported in any of these works. *Cochlicella* did form a derived group within the Helicoidea when nuclear rRNA sequences were included. This genus was recovered as the sister group of the geomitrid genera *Actinella*, *Caseolus* and *Discula* with full support (BI = 1.00; ML = 100%; NJ = 100%) in both nuclear rRNA and all-gene concatenated analyses. Consequently, our data strongly suggest that *Cochlicella* should be assigned to the Geomitridae. This interpretation is consistent with the findings of Wade et al. (2006; 2007) who recovered *Cochlicella acuta* within the Hygromiidae *s.l.*, but as the sister group of *Cernuella virgata*, and of Steinke et al. (2004) and Manganelli et al. (2005), who recovered a close relationship between *Cochlicella* and the Helicellinae. The phylogenetic relationships observed between cochlicellids and geomitrids suggest that cochlicellids might be given the rank of tribe (Cochlicellini) being recovered as an ingroup of Geomitrinae. Accordingly, the peculiar stimulatory apparatus probably arose from the accessory sac with its attached mucous glands that elongated and shifted down to the atrium.

4.1.4. Family Sphincterochilidae

Forcart (1972) recognized the morphological singularity of *Sphincterochila*, proposing a superfamily (Sphincterochiloidea) for this genus, which Schileyko (1991) considered related to the infraorder Zonitinia rather than Helixinia. This interpretation was justified by the oxygnathous mandible and the tripartite sole of the foot, both of which are absent in other

Helicoidean taxa. Schileyko (2004) and Hausdorf and Bouchet (2005) assigned family rank to this taxon, although the former author ascribed it to the Xanthonychoidea. Recent molecular studies by Steinke et al. (2004), Manganelli et al. (2005) and Groenenberg et al. (2011) recovered a sister relationship between *Sphincterochila* and *Cochlicella*, grouping them within the Hygromiidae *s.l.* (Canariellidae, Geomitridae and Hygromiidae, present work) although without statistical support (see discussion on the Cochlicellini). This relationship was highly inconsistent with classifications based on morphology (Nordsieck, 1987; Schileyko, 1991; 2004). In the present study, *Sphincterochila* was recovered in nuclear rRNA and concatenated-gene trees as a separate lineage within a clade grouping Elonidae, Helicidae, Humboldtianidae and Trissexodontidae, not related to *Cochlicella*.

4.1.5. Family Trissexodontidae

Schileyko (1991, 2006b) grouped the Helicodontidae and Trissexodontidae within a single family Helicodontidae comprising seven subfamilies. According to Gómez-Moliner et al. (2013) and the present results, however, they should be treated as two separate families as in the classification of Hausdorf and Bouchet (2005). The monophyly of the Trissexodontidae was not supported in the study by Gómez-Moliner et al. (2013) using the same DNA gene fragments as in the present study, together with the COI gene fragment. This monophyly was strongly supported by our BI and ML analyses in the concatenated-gene tree as well as by BI, ML and NJ analyses in the nuclear rRNA gene tree, suggesting that the mitochondrial COI gene fragment has no phylogenetic signal at the family level in this group. Consequently, the present data strongly confirm the inclusion of *Gittenbergeria* in the family Trissexodontidae. A further two differences from the results obtained by Gómez-Moliner et al. (2013) should be highlighted. The genus *Oestophorella* was here recovered with high support as the sister group of the other genera included in the Trissexodontini (*Trissexodon*, *Mastigophallus* and *Suboestophora*). However, Gómez-Moliner et al. (2013) did not resolve the position of *Oestophorella* within this tribe, and assigned it as the sister group of *Suboestophora*, but without statistical support. On the other hand, our results confirmed the monophyly of *Hatumia*, which was recovered as a paraphyletic group by Gómez-Moliner et al. (2013).

4.1.6. Family Helicidae Rafinesque, 1815

We included 23 Helicidae taxa of generic rank in our concatenated-gene analysis, but neither *Lampadia* (Lampadiini Schileyko, 2006) nor *Cylindrus* (Cylindruini Schileyko, 2006) were included in our study. Groenenberg et al. (2011) and Cadahia et al., 2014 showed that *Cylindrus* is the sister group of *Arianta*, and thus, Cylindruini should be included in the synonymy of Arinatinae. All helicid taxa clustered within three main groups that were here considered of subfamily rank: Ariantinae Mörch, 1864, Helicinae and Murellinae Hesse, 1918. The basal relationships of these three subfamilies were not resolved in the concatenated-gene tree. BI and ML analysis recovered *Theba* (Thebini *sensu* Hausdorf and Bouchet, 2005 and Schileyko, 2006a) as the sister group of the other Helicidae genera in

the 16S tree, but it appeared as a derived group within the Helicinae when nuclear rRNA was included in the analysis. This is consistent with the results of Wade et al. (2007) who recovered *Theba pisana* within the Helicinae. *Theba* also appeared close to several Helicinae genera (*Cantareus*, *Cornu*, *Eobania*, *Otala*) by Koene and Schulenburg (2005), but these authors also recovered Ariantinae and Murellinae within the Helicinae. The consideration of Thebini as a tribe within the Helicinae implies the consideration of a further three taxa with the same rank: Helicini, and the revalidated Allognathini Westerlund, 1902 and Otalini Pfeffer, 1930. The monophyly of these four tribes was supported by BI and ML analyses in the nuclear rRNA and the all-gene concatenated analyses. Only Otalini in the concatenated-gene tree and Helicini in the nuclear rRNA tree were not supported by NJ analysis. The monophyly of the Ariantinae was confirmed, although only three genera were here included, and *Helicigona* and *Chilostoma* (subgen. *Corneola*) were recovered as sister groups, closely related to *Arianta*. These results are in agreement with the phylogeny obtained by Groenenberg et al. (2011) in their extensive work on the Ariantinae.

The classification of *Murella* is controversial. It was considered a tribe of Helicinae by Nordsieck (1987) and Hausdorf and Bouchet (2005), while Schileyko (2006a) placed it within the Ariantinae. Koene and Schulenburg (2005) and Manganelli et al. (2005) described *Murella* as the sister group of the Ariantinae within the Helicinae. However, we recovered *Murella* as a separate lineage in our nuclear rRNA and concatenated-gene analyses. This indicates that *Murella* should be classified within a separate subfamily, the Murellinae. *Marmorana* and *Tyrrheniberus* (no nuclear rRNA sequences available) also belong to the Murellinae since they clustered with *Murella* in a clade highly supported by all the 16S phylogenetic analyses.

Chromosome number seems to be relevant for the diagnosis of subfamilies and tribes within the Helicidae (reviewed in Aparicio, 1981). The ancestral number for Helicidae seems to be n = 30 and this number also exists in the Ariantinae (ranging from 29 to 31), Murellinae and Thebini (Helicinae). Chromosome number in the remaining Helicinae tribes varies from 22 to 27, suggesting their derived nature, although Rainer (1967) reported n=30 for the helicine *Caucasotachea leucoranea* (Mousson, 1863).

The presence of two verges inside the penis (Figure 4) is a synapomorphy of Helicinae (Schileyko, 2004). This subfamily is also characterized by a calcareous dart with four blades along its axis (Figure 4), forming a cross in transverse section (Koene and Schulenburg, 2005), a feature also present in the Murellinae suggesting a closer phylogenetic relationship between both subfamilies. Allognathini could be characterized by a reproductive apparatus with a long vagina, a dart sac near the atrium, mucous glands with 2-4 branches, and a flagellum of medium size; chromosome number, n = 22-25. Helicini have a long vagina, a dart sac positioned near the atrium, two mucous glands from bifurcate to multiramous, and a very long flagellum; chromosome number, n = 27. Otalini have a long vagina, one dart sac near the atrium, two multirramous mucous glands, and a flagellum of medium size; chromosome number, n = 25-27. Thebini have a short vagina, a dart sac in the upper

vagina and broadly joined to the vagina wall, two simple, inflated and alveolar mucous glands, and a rudimentary flagellum (Figure 4); chromosome number, n = 30.

Based on their current distribution, the three subfamilies (Ariantinae, Helicinae and Murellinae) probably originated and diversified within Europe. Subsequently, the Helicinae would have colonized North Africa, giving rise to the North African tribes Thebini and Otalini.

4.2. Divergence time

The data obtained by BEAST analysis should be considered a first approach to deciphering the diversification time of the Helicoidea. Fossil records are critical to interpret the history of a group and it is important to accurately establish the age of each lineage. However, many taxa representing the families under study are lacking in the fossil record or there is no accurate information about their age, so it is difficult to compare inferred results with the palaeontological record. The main problem is that the fossil record for land snails in the Cretaceous is very scarce, and it is completely absent for European helicoideans (Nordsieck, 2014). Since the appearance of some uncertain stylommatophoran families assigned to the Carboniferous (300 Ma) (Solem and Yochelson, 1979; Benton, 1993), there is a gap of over 180 Myr before land snails started to be represented in the fossils of the Cretaceous, probably due to a very low probability of fossilization (Naggs and Raheem, 2005). For this study, we used six fossil calibrations at the family level. The use of the uncorrelated relaxed molecular clock (see Figure 3; Table 4) dated the origin of Helicoidea (107 Ma) to the end of the Early Cretaceous. Thus, these data supported the general hypothesis that considered the Helicoidea to be essentially of Laurasian origin (Nordsieck, 1986a; Tillier, 1989).

Our molecular tree showed a main division of Helicoidea into two principal clades diverging around 86 Ma ago that started their radiation nearly simultaneously in the Late Cretaceous.

The group radiating 75.56 Ma ago includes only elements belonging to the Hygromiidae *s.l.* (Hygromiidae *s.str.*, Canariellidae, Geomitridae). Currently, the Hygromiidae *s.l.* are mainly distributed throughout the western Palaearctic region (Schileyko, 2006b) with some suprageneric taxa extending towards Central Asia (Archaicinae and Paedhoplitinae) and Tropical Africa (Halolimnohelicidae). Our results are consistent with the hypothesis that the Hygromiidae *s.l.* originated in the western Palaearctic. Although the Central Atlantic Ocean started to open at around 195 Ma ago (Smith, 1994; Torvsik et al., 2012) breaking up Pangaea into Laurasia and Gondwana, and the South Atlantic opened at around 130 Ma, Europe and North America were still connected to each other 100 Ma ago (Torvsik et al., 2012). According to Sanmartín et al. (2001), the opening of the North Atlantic occurred during the Late Cretaceous (90 Ma). Thus, the vicariant event prompting the origin of the Hygromiidae *s.l.* could have been the opening of the North Atlantic Ocean, and they were confined to the landmasses that were to subsequently give rise to current Eurasia.

The second basal clade split around 73 Ma ago into two lineages: one American-Asian-Australian lineage (Polygyridae, Bradybaenidae and Camaenidae) that could have colonized Asia via the Bering land bridge and given rise to the Bradybaenidae-Camaenidae complex (see Wade et al., 2007 for the polyphyly of both families) that subsequently extended to Australia, according to the scenario proposed by Solem (1997) and Hugall and Stanisic (2011); and an American-European lineage joining the rest of the Helicoidea families distributed in the western Palaearctic (excluding the Hygromiidae *s.l.*), together with the American Humboldtianidae, Monadenidae and Pleurodontidae. It is difficult to propose a biogeographic scenario for this American-European group. A plausible explanation, supported by the presence of North American representatives in both lineages, is that this second basal clade could have started its diversification in North America, and spread to the East to give rise to families confined to the western Palaearctic (Elonidae, Helicidae, Helicodontidae, Sphincterochilidae and Trissexodontidae). The presence of the Caribbean family Cepolidae as the basal taxon of the families considered in our study supports a Nearctic origin of this lineage. The history of the Holarctic realm is complicated (Sanmartin et al., 2001) by the repeated connection/disconnection by land bridges between Europe and North America that persisted at least until the Early Eocene (50 Ma) (McKenna, 1983; Tiffney, 1985). The Thulean Bridge, that connected southern Europe to Greenland through the British Isles, is considered to have been the most important bridge for exchange of temperate taxa during the earliest part or the Early Eocene (55 Ma), when the climate became markedly warmer (McKenna, 1983). It could also have been the route for the colonization of Europe by the ancestors of the European representatives of the American-European lineage. The split between Neartic and Palaearctic helicoidean lineages of this second clade is dated around 54-60 Ma ago, but their basal relationships were not reliably resolved. It does not allow to state if the origin of the European families occurred once or several times by dispersive events from North America or by a vicariant process as a result of the breakdown of the Thulean bridge. Nevertheless, other large-scale passive dispersal events (winds, drifting floats, birds etc.) can not been discarded as it has been confirmed for other taxa (Rees, 1965; Vagvolgyi, 1975; Gittenberger, 1984; Kirchner et al., 1997).

At the family level, divergence time analysis showed that major diversification processes within the Hygromiidae *s.l.* clade started after the Cretaceous-Paleogene boundary (53.66 Ma for Geomitridae, 46.82 Ma for Canariellidae and 40.37 Ma for Hygromiidae *s.str.*). It is widely known that the Cretaceous-Tertiary boundary had a major impact on terrestrial biotas, including several terrestrial gastropod families thought to have disappeared during this period (Benton, 1995; Vajda et al., 2001; McCleod, 2004). However, this event opened a new opportunity for posterior radiation events during the early Cenozoic as is also well documented for many taxa, including different groups of stylommatophoran land snails (Wade et al., 2006; Rowson et al., 2010; Uit de Weerd and Gittenberger, 2013). The current presence of some Hygromiidae *s.l.* taxa in Central Asia and Tropical Africa can be explained by posterior dispersal events as described for other invertebrate taxa. As an example, the subfamily Phalangiinae (Phalangiidae, Opiliones, Arachnida), with a Holarctic

range (Giribet and Kury, 2007), is represented also in the Ethiopian region by 9 endemic genera out of 26 genera that could be the result of dispersive expansion via a land bridge across the 'Mediterranean' sea (Starega, 1984). With regard to the endemic subfamilies of Hygromiidae (Paedhoplitinae and Archaicinae) from the Tien Shan mountains, Schileyko (1978: Figs. 33, 35) suggested a quite recent origin based on morphological characteristics, and that they could be Pleistocene derivatives of the Trochulinae subfamily. Diversification processes in Trissexodontidae (44.82 Ma) slightly predated that of the Helicodontidae (39.19 Ma) and Helicidae (37.59 Ma). Divergence times obtained for the Sphincterochilidae (represented only by *Sphincterochila candidissima*) and Elonidae (represented only by the two extant species) cannot be considered in terms of their diversification origin.

The role of the Iberian plate in the evolution of the western Palaearctic Helicoidea has not been considered previously. Nevertheless, the Iberian plate which became isolated from Laurasia at the beginning of the Cretaceous and remained isolated during large periods in the Cenozoic (see Smith et al., 1994) could explain the presence of many ancient taxa endemic to the Iberian Peninsula. Besides, the Thulean bridge directly connected North America with the British Isles and the Iberian Peninsula, suggesting that the latter could have played an important role in the colonization processes of Europe. Hence, some family level taxa of helicoideans, like Trissexodontidae, Elonidae, Plentuisinae, Ponentininae, Monserratininae and Leptaxinae share the Iberian peninsula as their unique distribution area (with Neogene colonization of Macaronesia by *Leptaxis* and related genera in Leptaxinae and of the Riffean region by several species of *Hatumia* and *Oestophora* in Trissexodontidae as the only exceptions).

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Supplementary material S1: Taxa used in this study: family, species, locality, voucher, and GenBank accession numbers for the 16S and 5.8S-ITS2-28S gene fragments. Superscript numbers refer to the voucher specimen institutions: 1 - MVHN, Museu Valencià d'Història Natural (Spain), 2 - National Museum Cardiff (UK), 3 - Zoology and Animal Cell Biology Department, University of the Basque Country (Spain). Superscript asterisks (*) mean that the GenBank accession number has been published in other studies.

Family	Species	Locality	Voucher	GenBank Accesion number 16S	GenBank Accesion number 5.8S-ITS2-28S
Bradybaenidae	*Acusta despecta* (Sowerby, 1839)	(16S): Amami Island, Japan; (5.8-ITS2-28S): Japan		AY137578*	AY841337*
	Aegista vulgivaga (Schmacker & Boettger, 1890)	Osaka City, Japan			AY014139*
	Ainohelix editha (A.Adams, 1868)	Shimamaki, Hokkaido, Japan			AY841338*
	Bradybaena similaris (Férussac, 1821)	(16S): Brisbaen, Queensland, Australia; (5.8-ITS2-28S): Sri Lanka		GQ851001*	AY014138*
	Chloraea intorta (Sowerby, 1841)	Bohol Island, Phillipines			AY841344*
	Euhadra amaliae (Kobelt, 1875)	(16S): Western and middle parts of Japan; (5.8-ITS2-28S): Osaka City, Japan		AF098712*	AY014140*
	Euhadra decorata Pilsbry & Hirase, 1903	(16S): Tamayama, Iwate, Japan; (5.8-ITS2-28S): Japan		AY445012*	AY251834*
	Euhadra sandai (Kobelt, 1878)	(16S): Imajyo, Fukui, Japan; (5.8-ITS2-28S): Osaka City, Japan		AY445021*	AY014141*
	Ezohelix gainesi (Pilsbry, 1900)	Sapporro, Hokkaido, Japan			AY841339*
	Fruticicola fruticum (O. F. Müller, 1774)	Anzù di Feltre (Feltre, Belluno), Italy		AY741450*	
	Nesiohelix bipyramidalis Kuroda & Emura, 1943	Ryukyu, Japan			AY841341*
	Paraegista takahidei Kuroda & Azuma, 1951	Hokkaido, Japan			AY841340*
	Trishoplita hachijoensis Pilsbry, 1902	Niijima Island, Izu Islands, Japan			AY841345*
Camaenidae	*Satsuma (Coniglobus) mercatorius* (Pfeiffer, 1845)	(16S): Ryukyu Islands, Japan; (5.8-ITS2-28S): Kikai Island, Ryukyu, Japan		AF098715*	AY841324*
	Satsuma (Coniglobus) nux Möllendorff, 1888	Unknown		EF204786*	EF204880*
	Satsuma (Satsuma) japonica (Pfeiffer, 1847)	(16S): Japan; (5.8-ITS2-28S): Osaka City, Japan		AF098716*	AY014122*
Cepolidae	*Cepolis streatori* (Pilsbry, 1889)	Grand Cayman			AY841346*
Cochlicellidae	*Cochlicella (Cochlicella) acuta* (Da Costa, 1778)	(16S):Lampedusa Island, Valle Imbriacole (Lampedusa e Linosa, Agrigento), Italy; (5.8-ITS2-28S): Porthcurnick, Cornwall, UK		AY741442*	AY014126*
	Cochlicella (Cochlicella) acuta (Da Costa, 1778)	Bakio, Bizkaia, Spain	[3]EHUMC-1003	KJ458503	KJ458599
	Cochlicella (Cochlicella) conoidea (Draparnaud, 1801)	Miramar, Portugal	[3]EHUMC-1004	KJ458504	KJ458600
	Cochlicella (Prietocella) barbara (Linnaeus, 1758)	Antequera, Málaga, Spain	[3]EHUMC-1005	KJ458550	
	Cochlicella (Prietocella) barbara (Linnaeus, 1758)	Gasteiz, Álava, Spain	[3]EHUMC-1006	KJ458551	KJ458633
Elonidae	*Elona quimperiana* (Férussac, 1821)	Arantzazu: Araotz, Gipuzkoa, Spain		FJ786408*	

	Elona quimperiana (Férussac, 1821)	Arantzazu: Araotz, Gipuzkoa, Spain		FJ786409*	JQ805023*
	Norelona pyrenaica (Draparnaud 1805)	Setcases, Girona, Spain	[3]EHUMC-1007	KJ458543	
	Norelona pyrenaica (Draparnaud 1805)	Queralbs: Daió, Girona, Spain	[3]EHUMC-1008	KJ458544	KJ458627
Helicidae	*Alabastrina (Alabastrina) alabastrites* (Michaud, 1833)	Honaine, Traras Massif, Algeria	[1]MVHN-2169	KJ458484	KJ458582
	Alabastrina (Atlasica) atlasica (Mousson, 1873)	Between Agadir and Essauira, Morocco	[1]MVHN-010211FR03	KJ458490	KJ458588
	Allognathus graellsianus (Pfeiffer, 1848)	Between Caiman and Sóller, Mallorca, Spain	[1]MVHN-1789	KJ458485	KJ458583
	Allognathus hispanicus minoricensis (Mittre, 1842)	Alaior, Menorca, Spain	[3]EHUMC-1009	KJ458531	KJ458618
	Arianta arbustorum (Linnaeus, 1758)	Stockholm, Sweden	[1]MVHN-2159	KJ458486	KJ458584
	Arianta xatartii (Farines, 1834)	Núria, Girona, Spain	[1]MVHN-2145	KJ458487	KJ458585
	Cantareus apertus (Born, 1778)	Djelfa, Algeria	[1]MVHN-2013	KJ458491	KJ458589
	Cepaea (Cepaea) hortensis (O. F. Müller, 1774)	Thurso, Highlands, Scotland, UK	[3]EHUMC-1010	KJ458497	KJ458594
	Cepaea (Cepaea) nemoralis (Linnaeus, 1758)	(16S): Pitarque, Teruel, Spain; (5.8-ITS2-28S): Marlborough Downs, Wiltshire, UK	[1]MVHN-2197	KJ458498	AY014130*
	Chilostoma (Cingulifera) cingulatum (Studer, 1820)	Tirol, Halltal, 9 km NE of Innsbruck, Austria		JF717812*	
	Chilostoma (Corneola) desmoulinsii atricha (Bofill, 1915)	Congost de Montrebu, Lleida, Spain	[1]MVHN-2164	KJ458499	KJ458595
	Chilostoma (Corneola) desmoulinsii bechi (Altimira, 1959)	La Riba, Tarragona, Spain	[1]MVHN-2165	KJ458500	KJ458596
	Chilostoma (Corneola) desmoulinsii desmoulinsii (Farines, 1834)	Portell, Rambla Celumbres, Castellón, Spain	[1]MVHN-2163	KJ458501	KJ458597
	Chilostoma (Corneola) squamatinum (Rossmässler, 1835)	Albanya, near Muga river, Girona, Spain	[1]MVHN-2166	KJ458502	KJ458598
	Codringtonia (Codringtonia) codringtonii Gray, 1834	Rodia 1.2 km before, Peloponnese, Greece		JQ240092*	
	Cornu aspersum (O. F. Müller, 1774)	(16S): Unknown; (5.8-ITS2-28S): Kettering, Northants, UK		AF434797*	AY014128*
	Eobania vermiculata (O. F. Müller, 1774)	Girona, Girona, Spain	[1]MVHN-080709DR04	KJ458509	
	Eobania vermiculata (O. F. Müller, 1774)	Murchante, Navarre, Spain	[3]EHUMC-1011	KJ458510	KJ458604
	Eobania vermiculata (O. F. Müller, 1774)	Calblanque Regional Park, Murcia, Spain	[3]EHUMC-1012	KJ458511	
	Erctella cephalaeditana (Giannuzzi-Savelli, Oliva & Sparacio, 2012)	Cefalú, La Rocca, Palermo, Sicily, Italy		GQ402397*	
	Erctella insolida (Monterosato, 1892)	San Vito lo Capo, Cala Mancina, Trapani, Sicily, Italy		GQ402423*	
	Eremina desertorum (Forsskål, 1775)	Unknown			AY841335*
	Helicigona lapicida andorrica (Bourguignat, 1876)	Serrat, Andorra	[3]EHUMC-1013	KJ458523	JQ805027*
	Helicigona lapicida lapicida (Linnaeus, 1758)	(16S): Luxembourg, La Roche-en-Ardenne, chateau, Belgium; (5.8-ITS2-28S): Deepdale, Derbyshire, UK		JF717817*	AY014137*
	Helix (*Helix*) *lucorum* Linnaeus 1758	Unknown		AF126144*	AY841334*
	Helix (*Helix*) *melanostoma* Draparnaud 1801	Llombai, Algeria	[1]MVHN-783	KJ458524	KJ458612

Family	Species	Locality	Voucher	GenBank Accesion number 16S	GenBank Accesion number 5.8S-ITS2-28S
Helicidae	*Helix (Helix) pomatia* Linnaeus, 1758	(16S): Unknown; (5.8-ITS2-28S): Pulpit Down, Buckinghamshire, UK		AF208297*	AY841333*
	Hemicycla (Hemicycla) bidentalis (Lamarck, 1822)	Anaya, Tenerife, Canary Islands, Spain	[1]MVHN-2160	KJ458528	KJ458615
	Hemicycla (Hemicycla) consobrina (Férussac, 1821)	Tenerife, Canary Islands, Spain		HM147230*	
	Hemicycla (Hemicycla) eurythyra O. Boettger, 1908	Tenerife, Canary Islands, Spain		HM147226*	
	Hemicycla (Hemicycla) fulgida Alonso & Ibáñez, 2007	Tenerife, Canary Islands, Spain		HM147200*	
	Iberus gualtieranus gualtieranus (Linnaeus, 1758)	Sierra Elvira, Granda, Spain	[3]EHUMC-1014	KJ458530	KJ458617
	Isognomostoma isognomostomos (Schröter, 1784)	Trento-Alto Adige, between Predazzo and Bellamonte, Italy		JF717821*	
	Levantina hierosolyma (Mousson, 1854)	Ksalon, Israel	[1]MVHN-050710FC01	KJ458534	KJ458620
	Marmorana (Ambigua) saxetana (Paulucci, 1886)	Giglio. Il Franco, Tuscany, Italy		GU391400*	
	Marmorana (Ambigua) signata (Férussac, 1821)	Monti Lepini, Latium, Italy		GU391405*	
	Marmorana (Marmorana) serpentina (Férussac, 1821)	Casa Cantoniera, Sardinia, Italy		GU391397*	
	Marmorana (Murella) muralis (O. F. Müller, 1774)	(16S): Castle, Fiumedinisi, Sicily; (5.8-ITS2-28S): Pompeya, Nápoles, Italy	[1]MVHN-1276	GU391399*	KJ458621
	Marmorana (Murella) muralis (O. F. Müller, 1774)	Caltabellotta, Chiesa di San Pellegrino, Sicily, Italy		EU189886*	
	Marmorana (Murella) scabriuscula (Deshayes, 1830)	Monte Nadore, top, Sicily, Italy		EU189888*	AY014132-AY014133*
	Maurohelix raymondi (Moquin-Tandon, 1848)	Bou-Saad, Algeria	[2]1984.306.14	KJ458535	KJ458622
	Otala (Dupotetia) sp	Detour to Honaine, between Orán and Tlemcén, Algeria	[1]MVHN-2171	KJ458507	KJ458603
	Otala (Dupotetia) sp	Ghar el Melh, Bizerte, Tunisia	[1]MVHN-260410DR01	KJ458508	
	Otala (Otala) lactea (O. F. Müller, 1774)	(16S): Nerja-Frigiliana: 1 Km, Málaga, Spain; (5.8-ITS2-28S): Unknown		AY937264*	AY841336*
	Otala (Otala) punctata (O. F. Müller, 1774)	Tlemcen, Algeria	[1]MVHN-2186	KJ458545	KJ458628
	Pseudotachea splendida Draparnaud, 1801	Sierra de Quibas, Murcia, Spain	[1]MVHN-2270	KJ458552	KJ458634
	Pseudotachea splendida Draparnaud, 1801	Náquera, Sierra Calderona, Valencia, Spain	[1]MVHN-080709DR01	KJ458553	
	Pseudotachea splendida Draparnaud, 1801	Sierra Espadán, Castellón, Spain	[1]MVHN-080709DR00	KJ458554	
	Rossmassleria olcesei (Pallary,1898)	Sefliane, Morocco	[2]1984.384.6	KJ458555	KJ458635
	Theba andalusica Gittenberger & Ripken, 1987	Tarifa, Cádiz, Spain	[1]MVHN-1383	KJ458558	
	Theba geminata (Mousson, 1857)	Teguise, Lanzarote, Las Palmas, Spain	[1]MVHN-241109AZ01	KJ458559	KJ458638
	Theba impugnata Mousson, 1857	Teguise, Lanzarote, Las Palmas, Spain	[1]MVHN-241109AZ02	KJ458560	KJ458639

	Theba pisana (O. F. Müller, 1774)	Almería, Almería, Spain	[1]MVHN-1283	KJ458561	
	Theba subdentata Férussac, 1821	El Alquian, Almería, Spain	[1]MVHN-1269	KJ458562	KJ458640
	Tingitana orientalis O. Boettger, 1884	Berkane, Morocco	[1]MVHN-080709DR03	KJ458563	KJ458641
	Tyrrheniberus ridens Von Martens, 1884	Caletta Fuili, Sardinia, Italy		GU391402*	
	Tyrrheniberus villicus (Paulucci, 1882)	Orosei, Sardinia, Italy		GU391410*	
Helicodontidae	*Atenia quadrasi* (Hidalgo, 1885)	Barranco de los Frailes, Pego, Alicante, Spain		FJ786403*	
	Atenia quadrasi (Hidalgo, 1885)	Celrá, Girona, Spain		FJ786404*	JQ805020*
	Helicodonta obvoluta (O. F. Müller, 1774)	Collsacabra, Girona, Spain		FJ786423*	JQ805021*
	Lindholmiola girva (Frivaldszky, 1835)	Igoumenitsa, Greece		AY741448*	
Humboldtianidae	*Humboldtiana fasciata* Burch & Thompson, 1957	El Chico, Hidalgo, Mexico		DQ324479*	DQ324510*
	Humboldtiana montezuma Pilsbry, 1940	Cumbre Infiernillo, Nuevo León, Mexico		DQ324467*	DQ324508*
	Humboldtiana nuevoleonis Pilsbry, 1927	Arteaga, Coahuila, Mexico		DQ324485*	DQ324524*
Hygromiidae	*Actinella (Actinella) lentiginosa* (Lowe, 1831)	Sao Vicente, Madeira, Portugal	[1]MVHN-2190	KJ458482	KJ458580
	Actinella (Plebecula) giramica (Lowe, 1852)	Pico Serrado, Corral das Freijas, Madeira, Portugal	[1]MVHN-2195	KJ458481	
	Actinella (Plebecula) nitidiuscula (Sowerby, I 1824)	Ponta Sao Lourenço, Madeira, Portugal	[1]MVHN-2193	KJ458483	KJ458581
	Ashfordia granulata (Alder, 1830)	Ordes, A Coruña, Spain	[3]EHUMC-1015	KJ458488	KJ458586
	Ashfordia granulata (Alder, 1830)	Gontan, Ourense, Spain	[3]EHUMC-1016	KJ458489	KJ458587
	Canariella (Canariella) hispidula (Lamarck, 1822)	Las Valladas, Tenerife, Canary Islands, Spain	[1]MVHN-2167	KJ458494	KJ458591
	Candidula corbellai Martínez-Ortí, 2011	Lleida, Lleida, Spain	[1]MVHN-2188	KJ458492	KJ458590
	Candidula gigaxii (L. Pfeiffer, 1847)	Igualeja, Málaga, Spain	[3]EHUMC-1017	KJ458493	
	Candidula intersecta (Poiret, 1801)	Mon Island, Tiornemarke, Denmark		AY741437*	
	Candidula najerensis (Ortiz de Zárate, 1950)	Miño de Medinaceli, Soria, Spain	[3]EHUMC-1018	KJ458495	KJ458592
	Candidula olisippensis (Servain, 1880)	Torro Lamario, Portugal		AY546346*	
	Candidula rugosiuscula (Michaud, 1831)	Carrières-sous-Poissy, France		AY546347*	
	Candidula spadae (Calcara, 1845)	Monte Cucco (Costacciaro, Perugia), Italy		AY741436*	
	Candidula unifasciata (Poiret, 1801)	Parco La Tebaide, Cetinale (Sovicille, Siena), Italy		AY741438*	
	Caseolus compactus (Lowe, 1832)	Ponta Sao Lourenço, Madeira, Portugal	[1]MVHN-2194	KJ458496	KJ45859
	Cernuella (Cernuella) cisalpina (Rossmässler, 1837)	Stazione di Castelnuovo Berardenga (Asciano, Siena), Italy		AY741423*	
	Cernuella (Cernuella) virgata (Da Costa, 1778)	(16S): Stazione di Castelnuovo Berardenga (Asciano, Siena), Italy; (5.8-ITS2-28S): Porthcumick, Cornwall, UK		AY741422*	AY014127*
	Cernuella (Xerocincta) neglecta (Draparnaud, 1805)	Torrente Arbia, Vallina (Castelnuovo Berardenga, Siena), Italy		AY741426*	
	Cernuella (Xerocincta) neglecta (Draparnaud, 1805)	Grasse, France	[3]EHUMC-1019	KJ458571	KJ458648
	Cernuellopsis ghisottii Manganelli & Giusti, 1987	Monte Pollino, Cozzo Vardo (Morano Calabro, Cosenza), Italy		AY741429*	
	Ciliella ciliata (Hartmann, 1821)	Ordesa valley, Torla, Huesca, Spain		FJ786407*	JQ805024*
	Cryptosaccus asturiensis Prieto & Puente, 1994	Somiedo, Asturias, Spain	[3]EHUMC-1020	KJ458505	KJ458601
	Discula (Discula) polymorpha (Lowe, 1831)	Sao Lourenço, Madeira, Portugal	[1]MVHN-2192	KJ458506	KJ458602

Family	Species	Locality	Voucher	GenBank Accesion number 16S	GenBank Accesion number 5.8S-ITS2-28S
Hygromiidae	*Euomphalia strigella* (Draparnaud, 1801)	Pitarque, Teruel, Spain	[1]MVHN-2198	KJ458512	
	Euomphalia strigella (Draparnaud, 1801)	Queralbs: Daió, Girona, Spain	[3]EHUMC-1021	KJ458513	KJ458605
	Ganula gadirana Muñoz, Almodóvar & Arrébola, 1999	Afueras de Algeciras, Cádiz, Spain	[1]MVHN-1382	KJ458514	
	Ganula gadirana Muñoz, Almodóvar & Arrébola, 1999	Valdevaqueros-Punta Paloma, Cádiz, Spain	[3]EHUMC-1022	KJ458515	KJ458606
	Ganula gadirana Muñoz, Almodóvar & Arrébola, 1999	Valdevaqueros-Punta Paloma, Cádiz, Spain	[3]EHUMC-1023	KJ458516	
	Ganula lanuginosa (Boissy, 1835)	Andratx-Sant Elm, Mallorca, Spain	[3]EHUMC-1024	KJ458517	KJ458607
	Ganula lanuginosa (Boissy, 1835)	Coll de Sóller, Mallorca, Spain	[3]EHUMC-1025	KJ458518	
	Ganula sp	Honaine, Traras Massif, Algeria	[1]MVHN-2179	KJ458519	KJ458608
	Helicella itala (Linnaeus, 1758)	Enol, Asturias, Spain	[1]MVHN-2140	KJ458522	KJ458611
	Helicella orzai Gittenberger & Manga, 1981	Aralar, Navarra, Spain	[3]EHUMC-1026	KJ458525	KJ458613
	Helicella stiparum (Rossmässler, 1854)	Los Alcores, Almería, Spain	[1]MVHN-1285	KJ458526	
	Helicopsis striata (Müller, 1774)	Kyffheauser, Germany		AY546362*	
	Helicopsis turcica (Holten, 1802)	Between Essauira and Agadir, Morocco	[1]MVHN-010211FR04	KJ458527	KJ458614
	Helicotricha carusoi Giusti, Manganelli & Crisci, 1992	Linosa Island, Monte Calcarella (Lampedusa e Linosa, Agrigento), Italy		AY741434*	
	Hygromia (Hygromia) cinctella (Draparnaud, 1801)	Pian di Giuncheto (Cetona, Siena), Italy		AY741421*	
	Hygromia (Riedelia) limbata (Draparnaud, 1805)	Queralbs: Daió, Girona, Spain	[3]EHUMC-1027	KJ458529	KJ458616
	Ichnusomunda sacchii Giusti & Manganelli, 1998	Is Arenas, Cuccuru Pranu (Arbus, Oristano), Italy		AY741424*	
	Leptaxis (Cryptaxis) groviana (A. Ferussac, 1832)	Ponta Sao Lourenço, Madeira, Portugal	[1]MVHN-2189	KJ458533	KJ458619
	Leptaxis (Leptaxis) drouetiana (Morelet, 1860)	Faial island, Azores islands, Portugal		AY748301*	
	Leptaxis (Leptaxis) simia (A. Ferussac, 1832)	(16S-ITS2): Sao Vicente, Madeira, Portugal; (28S): Portela and Santa‡, Madeira, Portugal	[1]MVHN-2191	KJ458532	KJ458653-AJ550969*
	Mengoana jeschaui (Kobelt, 1878)	Pola de Somiedo, Asturias, Spain	[3]EHUMC-1028	KJ458536	KJ458623
	Mengoana jeschaui (Kobelt, 1878)	Between Cangas and Llanes, Asturias, Spain	[3]EHUMC-1029	KJ458537	
	Monacha (Monacha) cantiana (Montagu, 1803)	(16S): Sopelana, Bizkaia, Spain; (5.8-ITS2-28S): Pulpit Down, Buckinghamshire, UK	[3]EHUMC-1030	KJ458539	AY841332*
	Monacha (Monacha) cartusiana (O. F. Müller, 1774)	Cañón del río Dulce, Guadalajara, Spain	[3]EHUMC-1031	KJ458540	KJ458625
	Monacha (Monacha) martensiana (Tiberi, 1869)	Piana di Colfiorito (Foligno, Perugia), Italy		AY741420*	
	Monacha (Monacha) parumcincta(Menke, 1828)	Medane (Asciano, Siena), Italy		AY741418*	
	Monachoides incarnatus (O. F. Müller, 1774)	Enney, Switzerland		AY546371*	
	Montserratina bofilliana (Fagot, 1884)	Bagés, Barcelona, Spain	[1]MVHN-2139	KJ458538	KJ458624
	Montserratina martorelli (Bourguignat, 1870)	Les Planes, Barcelona, Spain	[1]MVHN-2137	KJ458541	KJ458626
	Montserratina martorelli (Bourguignat, 1870)	Collserola, Barcelona, Spain	[1]MVHN-2138	KJ458542	
	Plentuisa vendia Puente & Prieto, 1992	Tielve, Asturias, Spain	[3]EHUMC-1032	KJ458546	KJ458629

Ponentina revelata (Michaud, 1831)	Valdenoceda, Burgos, Spain	[3]EHUMC-1033	KJ458547	KJ458630
Ponentina revelata (Michaud, 1831)	Ordes, A Coruña, Spain	[3]EHUMC-1034	KJ458548	KJ458631
Portugala inchoata (Morelet, 1845)	Conimbriga, Coimbra, Portugal	[3]EHUMC-1035	KJ458549	KJ458632
Pyrenaearia cantabrica (Hidalgo, 1873)	Los Beyos defile, Asturias, Spain		EU310145*	JQ805025*
Trochoidea (Trochoidea) elegans (Gmelin, 1791)	L'Alcudia, Valencia, Spain	[1]MVHN-1310	KJ458564	KJ458642
Trochoidea (Trochoidea) pyramidata (Draparnaud, 1805)	Cala de la Mosca, Orihuela, Alicante, Spain	[1]MVHN-120110XT04	KJ458565	KJ458643
Trochoidea (Trochoidea) trochoides (Poiret, 1789)	Populonia, Italy		AY546379*	
Trochulus (Trochulus) alpicolus (Eder, 1921)	Bannalppass, Nidwalden, Switzerland		DQ217812*	
Trochulus (Trochulus) biconicus Eder, 1917	Bannalppass, Nidwalden, Switzerland		DQ217811*	
Trochulus (Trochulus) caelatus Studer, 1820	Birseschlucht, Bern, Switzerland		DQ217803*	
Trochulus (Trochulus) hispidus (Linnaeus, 1758)	(16S): Between Lastras and Valle, Cantabria, Spain; (5.8-ITS2-28S): Deepdale, Derbyshire, UK		FJ786447*	AY014125*
Trochulus (Trochulus) piccardi Pfenninger & Pfenninger, 2005	Chateau d'Oex, Vaud, Switzerland		AY738397*	
Trochulus (Trochulus) sericeus (Draparnaud, 1801)	Enney, Switzerland		AY546374*	
Trochulus (Trochulus) striolata (Pfeiffer, 1828)	Deepdale, Derbyshire, UK			AY014124*
Xerocrassa (Xerocrassa) grabusana Hausdorf & Sauer, 2009	Crete, Greece		JN701847*	
Xerocrassa (Xerocrassa) mesostena (Westerlund, 1879)	Crete, Greece		JN701876*	
Xerocrassa barceloi (Hidalgo, 1878)	Calpe, Alicante, Spain	[1]MVHN-1304	KJ458570	
Xerocrassa grata (F. Haas, 1924)	Benifallet, Tarragona, Spain	[1]MVHN-2196	KJ458575	
Xerolenta obvia (Menke, 1828)	Fiume Tagliamento, Lago di Cornino (Folgaria nel Friuli, Udine), Italy		AY741431*	
Xeromunda durieui (L. Pfeiffer, 1848)	Marina di Pescoluse (Salve, Lecce), Italy		AY741432*	
Xerosecta (Polloneriella) contermina(L. Pfeiffer, 1848)	Lago di Burano (Capalbio, Grosseto), Italy		AY741425*	
Xerosecta (Xeromagna) adolfi (L. Pfeiffer, 1854)	Castala, Almería, Spain	[1]MVHN-1298	KJ458566	KJ458644
Xerosecta (Xeromagna) adolfi (L. Pfeiffer, 1854)	Nijar, Almería, Spain	[3]EHUMC-1036	KJ458567	KJ458645
Xerosecta (Xeromagna) arigonis (A. Schmidt, 1853)	Pitarque, Teruel, Spain	[1]MVHN-2199	KJ458569	KJ458647
Xerosecta (Xeromagna) promissa (Westerlund, 1893)	Sierra de Benaoján, Málaga, Spain	[1]MVHN-1375	KJ458576	KJ458651
Xerosecta (Xeromagna) reboudiana (Bourguignat, 1863)	Antequera, Málaga, Spain	[3]EHUMC-1037	KJ458577	KJ458652
Xerosecta (Xerosecta) cespitum (Draparnaud 1801)	La Garde, France		AY546351*	
Xerosecta (Xerosecta) explanata (O. F. Müller, 1774)	Playa de Daimuz, Valencia, Spain	[1]MVHN-2168	KJ458573	KJ458650
Xerosecta (Xerosecta) explanata (O. F. Müller, 1774)	Cap d'Agde - Sete, Hérault, France	[3]EHUMC-1038	KJ458574	
Xerotricha apicina (Lamarck, 1822)	Málaga, Málaga, Spain	[1]MVHN-2157	KJ458520	KJ458609
Xerotricha apicina (Lamarck, 1822)	Between Portimao and Algarve, Portugal	[1]MVHN-2155	KJ458568	KJ458646
Xerotricha conspurcata (Draparnaud, 1801)	Jadraque, Guadalajara, Spain	[3]EHUMC-1039	KJ458572	KJ458649
Xerotricha gonzalezi (Azpeitia, 1925)	Pancorbo, Burgos, Spain	[3]EHUMC-1040	KJ458521	KJ458610
Xerotricha vatonniana (Bourguignat, 1867)	Between Matagañanes and El Cmpillo, Córdoba, Spain	[3]EHUMC-1041	KJ458578	
Xerotricha vatonniana (Bourguignat, 1867)	Between Matagañanes and El Cmpillo, Córdoba, Spain	[3]EHUMC-1042	KJ458579	

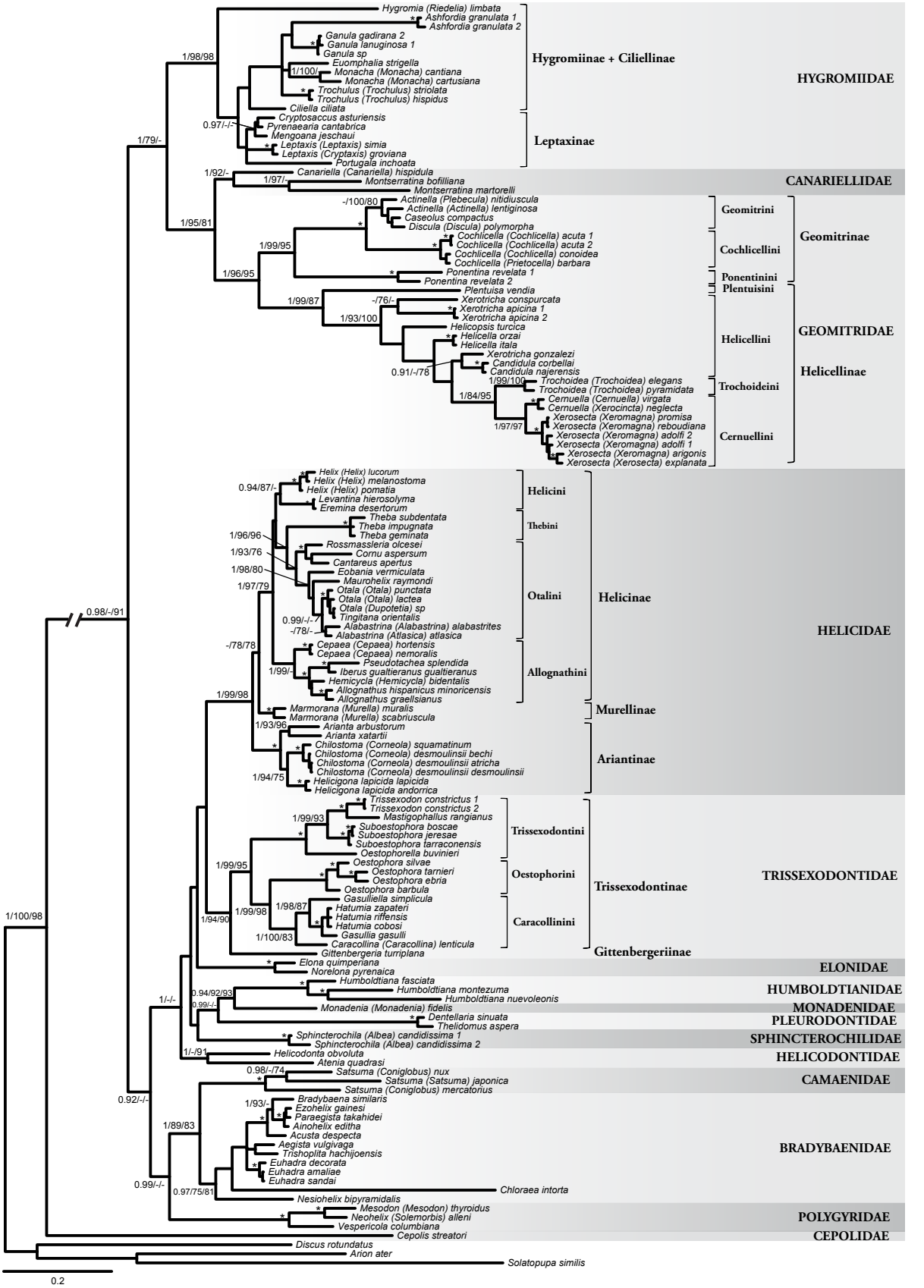
Family	Species	Locality	Voucher	GenBank Accesion number 16S	GenBank Accesion number 5.8S-ITS2-28S
Monadeniidae	*Monadenia (Monadenia) fidelis* (J.E. Gray, 1834)	Oregon, USA			AY014142*
Pleurodontidae	*Dentellaria sinuata* (O. F. Müller, 1774)	Green Grot Cave, Jamaic			AY841322*
	Thelidomus aspera (Férussac, 1821)	Windsor, Jamaic			AY841321*
Polygyridae	*Mesodon (Mesodon) thyroides* (Say, 1816)	York Co. Pennsylvania, USA			AY841315*
	Neohelix (Solemorbis) alleni (Wetherby, 1881)	Williams Creek, Iowa, USA			AY841316*
	Vespericola columbiana Henderson, 1928	Eugene, Oregon, USA			AY014120*
Sphincterochilidae	*Sphincterochila (Albea) candidissima* (Draparnaud, 1801)	Bardenas Reales, Navarra, Spain	[3]EHUMC-1043	KJ458556	KJ458636
	Sphincterochila (Albea) candidissima (Draparnaud, 1801)	Cervera del Maestre, Castellón, Spain	[1]MVHN-280610ZB14	KJ458557	KJ458637
Trissexodontidae	*Caracollina (Caracollina) lenticula* (Michaud, 1831)	(16S): Tarifa, Cádiz, Spain; (5.8-ITS2-28S): Valverde del Camino, Huelva, Spain		FJ786406*	JQ805002*
	Gasullia gasulli (Ortiz de Zárate & Ortiz de Zárate, 1961)	Nerva, Huelva, Spain		FJ786411*	JQ805000*
	Gasulliella simplicula (Morelet, 1845)	Tharsis, Huelva, Spain		FJ786413*	JQ805001*
	Gittenbergeria turriplana (Morelet, 1845)	(16S): Tavira, Portugal; (5.8-ITS2-28S): Silves, Portugal		FJ786416*	JQ805026*
	Hatumia cobosi (Ortiz de Zárate, 1962)	Enix, Almería, Spain		FJ786420*	JQ804999*
	Hatumia riffensis (Ortiz de Zárate, 1962)	Monte Gurugú, Melilla		FJ786422*	JQ804998*
	Hatumia zapateri (Hidalgo, 1870)	Cerro del Olivo, Córdoba, Spain		FJ786421*	JQ804997*
	Mastigophallus rangianus (Michaud, 1831)	Port de la Selva, Girona, Spain		FJ786426*	JQ805019*
	Oestophora barbula (Rossmässler, 1838)	Vilches, Jaén, Spain		FJ786437*	JQ805013*
	Oestophora ebria (Corbellá, 2004)	Sierra de las Nieves Natural Park, Málaga, Spain		FJ786430*	JQ805010*
	Oestophora silvae (Ortiz de Zárate, 1962)	Arredondo, Cantabria, Spain		FJ786435*	JQ805011*
	Oestophora tarnieri (Morelet, 1854)	Bobadilla, Los Alcornocales Natural Park, Málaga, Spain		FJ786433*	JQ805009*
	Oestophorella buvinieri (Michaud, 1841)	Matienzo, Cantabria, Spain		FJ786440*	JQ805007*
	Suboestophora boscae (Hidalgo, 1869)	Bobadilla, Los Alcornocales Natural Park, Málaga, Spain		FJ786445*	JQ805016*
	Suboestophora jeresae (Ortiz de Zárate, 1962)	Valencia, Spain		FJ786444*	JQ805014*
	Suboestophora tarraconensis (Aguilar-Amat, 1935)	Cunit, Tarragona, Spain		FJ786443*	JQ805015*
	Trissexodon constrictus (Boubée, 1836)	(16S): Kakouetta, Zuberoa, France; (5.8-ITS2-28S): Pagasarri, Bizkaia, Spain		FJ786448*	JQ805017*
	Trissexodon constrictus (Boubée, 1836)	Pagasarri, Bizkaia, Spain		FJ786450*	JQ805018*
Outgroups	*Arion ater (Linnaeus, 1758)*	(16S): France; (5.8-ITS2-28S): Kirk Ireton, Derbyshire, UK		HQ659926*	AY014144*
	Discus rotundatus (O. F. Müller, 1774)	(16S): Frankfurt, Germany; (5.8-ITS2-28S): Kirkdale, Derbyshire, UK		FJ917265*	AY014097*
	Solatopupa similis (Bruguiere 1792)	(16S): Montpellier, France; (5.8-ITS2-28S): Verdon Gorge, Fr		DQ305057*	AY014033*

Supplementary material S2: Results of the phylogenetic signal analyses measuring substitution saturation. Steel's test results are given as the range of the mean Phi. Results of Xia's test are based on simulations with 10.000 replicates and 32 OTUs (for families containing more than 32 taxa). Iss = Index of substitution saturation; Iss.cSym and Iss.cAsym = critical substitution saturation index if the true tree is symmetrical or asymmetrical, respectively. Significant substitution saturation exists in the dataset if Iss > Iss.c.

DNA fragment	Family	Steel´s test (mean Phi)	Xia´s test				
			Iss	Iss.cSym	P	Iss.cAsym	P
16S rRNA	Bradybaenidae	0.3993-03659	0.2954	0.7478	0	0.6788	0
	Cochlicellidae	0.9962	0.153	0.7863	0	0.7561	0
	Elonidae	0.9979	0.128	0.7879	0	0.7566	0
	Helicidae	0.0514-0.0223	0.305	0.692	0	0.379	0.0995
	Helicodontidae	0.5248	0.2679	0.7837	0	0.7555	0
	Hygromiidae	0.0348-0.0135	0.306	0.732	0	0.456	0.0081
	Trissexodontidae	0.1725-0.1116	0.2509	0.6792	0	0.4574	0
5.8S-ITS2	Bradybaenidae	0.2687-0.2189	0.1127	0.7008	0	0.5348	0
	Cochlicellidae	0.9997	0.0275	0.7964	0	0.7626	0
	Helicidae	0.0943-0.0632	0.116	0.683	0	0.354	0
	Hygromiidae	0.0764-0.0421	0.195	0.689	0	0.372	0
	Trissexodontidae	0.2256-0.1556	0.1437	0.6953	0	0.4799	0
28S	Bradybaenidae	0.3522-0.2582	0.0158	0.7781	0	0.6704	0
	Helicidae	0.1068-0.0691	0.022	0.731	0	0.413	0
	Hygromiidae	0.1096-0.0554	0.022	0.722	0	0.398	0
	Trissexodontidae	0.2129-0.1808	0.0217	0.7604	0	0.5562	0

Supplementary material S3: Phylogenetic tree of the Helicoidea based on Bayesian inference (BI), maximum likelihood (ML) and neighbor joining (NJ) analysis of the 16S rRNA gene. Numbers correspond to BI posterior probabilities, ML bootstrap values and NJ bootstrap, respectively. Asterisks (*) indicate full support of nodes: BI posterior probabilities = 1.00 ML bootstrap values = 100% and NJ bootstrap = 100%.

Supplementary material S4: Phylogenetic tree of the Helicoidea based on Bayesian inference (BI), maximum likelihood (ML) and neighbor joining (NJ) analysis of the nuclear 5.8S, ITS2 and 28S sequence data set. Numbers correspond to BI posterior probabilities, ML bootstrap values and NJ bootstrap, respectively. Asterisks (*) indicate full support of nodes: BI posterior probabilities = 1.00 ML bootstrap values = 100% and NJ bootstrap = 100%.



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4

CHAPTER 4

Pyramidula

PAPER II

Species delimitation for cryptic species complexes: case study of *Pyramidula* (Gastropoda, Stylommatophora)

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Cladistics (under review)

Abstract

Species discovery and validation methods were used to delimit the species of a cryptic species complex. Species boundaries were inferred from multiple lines of evidence arising from mitochondrial and nuclear DNA sequencing and ecological niche modeling. Phylogenetic relationships among species were assessed by Bayesian inference, maximum likelihood and maximum parsimony procedures. The approach was applied to the terrestrial gastropod genus *Pyramidula.* By examining 211 specimens collected from the western Palaearctic region, we here identified 9 putative species for this region. So far, descriptions of the morphospecies of this genus had been based exclusively on shell characters. Hence, we also studied the variation of shell characters to assess their utility to differentiate the species defined, and to examine whether shell shape in *Pyramidula* is influenced by environmental factors. Our findings indicate that although shell characters serve to discriminate between some putative species, they are not sufficient as unique taxonomic characters and other lines of evidence are needed. According to our ecological niche modeling results, shell shape may be influenced by environmental factors.

Keywords

Pyramidula, species delimitation, ecological niche modeling, molecular phylogeny

1. Introduction

The essential goals of systematics are to discern species and to examine their phylogenetic relationships as a means to devise a natural classification system and describe the diversity of life (Simpson, 1951; Sites and Marshall, 2003; Wiens, 2007). Defining species boundaries is another important goal with significant impacts in fields such as evolution, ecology and conservation (Sites and Marshall, 2003; Agapow et al., 2004; Isaac et al., 2004; Padial and de la Riva, 2006).

For centuries, species descriptions have been based on morphology. However, morphology-based taxonomic inferences can be ambiguous due to high phenotypic plasticity in some species or the existence of cryptic species (Mayden, 1997; Agapow et al., 2004). Advances in DNA sequencing technologies have led to the detection of many morphologically cryptic lineages (Bickford et al., 2007) in which species delimitation is particularly difficult. Morphology alone or any other single data set may not serve to establish species boundaries and relationships accurately, and hence the usage of different lines of evidence is needed (Padial et al., 2010; Derkarabetian and Hedin, 2014; Melville et al., 2014).

The expansion of molecular techniques, especially DNA sequencing, has led to the use of tree-based methods to search for monophyletic groups representing candidate species, despite there being no objective way to determine which supported clades reflect real species (Padial et al., 2010). In the past few years, several methods for molecular species delimitation have been developed (Wiens, 2007; Camargo and Sites, 2013), many of which are based on the coalescent theory (Pons et al., 2006; Yang and Rannala, 2010; Ence and Carstens, 2011; Fujita et al., 2012).

Ecological niche modeling (ENM) is a powerful tool to predict the habitat suitability of a species by combining information of environmental variables and species occurrence sites (Graham and Hijmans, 2006; Warren et al., 2008). Wiens and Graham (2005) proposed that niche modeling could be useful for species delimitation, as geographic isolation between populations would preclude gene flow between them, and hence support the hypothesis of being distinct species. Warren et al. (2008) developed comparative similarity measures and statistical tests that enable the quantitative comparison of ecological niche models (ENMs) between two species. There has been some discussion about what ENMs actually model (Kearney, 2006) and many researchers have replaced the term "environmental (or ecological) niche modeling" (ENM) with "species distribution modeling" (SDM) (Phillips and Dudík, 2008; Wisz et al., 2008; Marmion et al., 2009; Molloy et al., 2014). Warren (2012) suggested the term "niche modeling" for evolutionary studies such as species delimitation, since models usually consider some elements of the niche. Therefore, in this paper, we opted for the term "ecological niche modeling" (ENM).

By combining genetic information from multiple loci and ENM, species delimitation hypotheses gain in objectivity and strength. In effect, integration of genetic and ecological approaches is becoming ever more common (Raxworthy et al., 2007; Rissler and Apodaca,

2007; Leaché et al., 2009; Hawlitschek et al., 2011; Hidalgo-Galiana et al., 2014).

The land snail genus *Pyramidula* is distributed across almost all of Europe, the Mediterranean area, Central Asia and Japan (Gómez-Moliner, 1988; Welter-Schultes, 2011). Its species inhabit limestone rocks (Gittenberger and Bank, 1996; Kerney, 1999; Martínez-Ortí et al., 2007) and can occupy areas from sea level to altitudes of 3,800 m (Schileyko and Balashov, 2012). They feed on algae and lichens and are often passively dispersed, primarily by birds (Kerney, 1999). Species are hermaphroditic and ovoviviparous, and in at least four species, the morphology of the reproductive system is simple, hindering anatomy-based species characterization (Martínez-Ortí et al., 2007). Several species show a sympatric distribution and up to four different morphospecies have been identified within the same locality in Greece (Gittenberger and Bank, 1996). Shells are small, not exceeding 3 mm in diameter, and trochoid in shape, from high to low conical, with a broad umbilicus. Shell colour is yellowish grey to dark brownish (Gittenberger and Bank, 1996).

During most of the twentieth century, the general trend was to consider a single species within the genus, *P. rupestris*. The presence of six species in Europe based exclusively on shell characters was subsequently suggested in a review by Gittenberg and Bank (1996), and this is the taxonomy today proposed in Fauna Europaea (Bank, 2014): (1) *Pyramidula pusilla* (Vallot, 1801) is the most widespread and common in Europe, occupying the entire Mediterranean region and Central and Western Europe. It is characterized by a moderately low conical shell, broader than high; (2) *P. rupestris* (Draparnaud, 1801) occurs in the entire Mediterranean region and has a conical shell with practically straight sides; (3) *P. umbilicata* (Montagu, 1803) shows a Lusitanian-Atlantic distribution, and is described as having the lowest shell shape, clearly broader than high; (4) *P. jaenensis* (Clessin, 1882) inhabits in the southern Iberian Peninsula and shows a high conical shell that is much higher than broad; (5) *P. chorismenostoma* (Westerlund and Blanc, 1879) with disjunct coiling shell (body whorl separated from the rest of the shell) is found in Greece, Crete, Aegean islands and western Turkey; and (6) *P. cephalonica* (Westerlund, 1898) with a low conical shell occurs in Croatia, Greece and Turkey.

So far, species identification has been exclusively based on the shell characters: maximum diameter, umbilicus diameter, height and coloration (Gittenberger and Bank, 1996; Martínez-Ortí et al., 2007). Supplementary material 1 provides some of the shell characters used to define the six European species according to Gittenberger and Bank (1996) and Martínez-Ortí et al. (2007). These characters are highly correlated between each other and are sometimes influenced by environmental factors such that they are likely insufficient to fully resolve the taxonomy of the genus. The need of complementary characters becomes evident for this genus.

The present study was designed to: 1) reconstruct the phylogeny of the genus *Pyramidula* in the western Palaearctic using molecular markers; 2) delimit its species by integrating genetic and ecological data; 3) explore the utility of shell characters to differentiate the species

delimited; 4) explore whether shell shape in *Pyramidula* is influenced by environmental factors; and (5) review the taxonomy of the genus (when possible topotypes of western Palaearctic *Pyramidula* species were included in the analyses). To the best of our knowledge the present study is the first work applied to land snails, combining genetic, niche modeling and morphological data.

2. Methodology

2.1. Specimens

The collection sites and codes assigned to the 211 specimens of the genus *Pyramidula* examined here are detailed in Supplementary material 2. The snails were collected throughout their European distribution range and adjacent Mediterranean areas. Some of the samples were collected specifically for this study, whereas other specimens were kindly provided by museums. All specimens were photographed and preserved in 96% ethanol before DNA extraction.

2.2. DNA extraction, PCR and sequencing

Total genomic DNA was extracted from the entire specimen using the DNAeasy Tissue kit (Qiagen, Valencia, CA, USA). Five gene fragments were selected for multi-locus analyses: two mitochondrial markers [621 bp of the *cytochrome c oxidase subunit I* (*COI*) and around 335 bp of the *16S ribosomal RNA* gene] and three nuclear fragments [around 1,420 bp of the rRNA gene cluster, including the 3' end of the *5.8S* gene (-50 bp), the complete *ITS2* region (-530 bp) and the 5' end (-840 bp) of the large subunit (LSU; *28S*) gene]. Being only partial and short (-50 bp), we considered the *5.8S* fragment together with the *ITS2* region as a single gene/partition (*5.8S-ITS2*) for posterior analyses. The general PCR cycling conditions used for DNA amplification were: (1) 1 min at 96ºC, [30 s at 94ºC, 30 s at 50ºC, 1 min at 72ºC] (repeated for 35 cycles) and 10 min at 72ºC for *COI* and nuclear fragments, and (2) 20 s at 94ºC, [20 s at 94ºC, 30 s at 55ºC, 30 s at 72ºC] (repeated for 40 cycles) and 30 s at 72ºC for *16S rRNA*. The primers used are listed in Supplementary material 3. PCR products were purified and sequenced at Macrogen in Korea or Amsterdam using an ABI3730XL or ABI3700 sequencer. Genbank accession numbers (KP726936-KP727570) are provided in Supplementary material 2.

2.3. Phylogenetic analysis

Sequences were aligned with Mafft v7 online version (Katoh, 2002; Katoh and Standley, 2013) as this method has been described to perform better than other pairwise alignment methods (Golubchik et al., 2007). We used the Q-INS-i algorithm for rRNA, which considers the secondary structure of RNA (Katoh and Toh, 2008), and the Auto algorithm for *COI*. Default values were assigned to the remaining parameters.

COI protein coding sequences were translated into amino acids using DnaSP v5.10.1 (Librado and Rozas, 2009) to check for stop codons. Substitution saturation for each gene

and for each codon position in *COI* was assessed using the entropy-based information method of Xia (Xia et al., 2003) implemented in DAMBE v5.2.38 (Xia, 2013).

For phylogenetic reconstruction, Bayesian inference (BI) maximum likelihood (ML) and maximum parsimony (MP) methods were used on three data sets: mitochondrial, nuclear and the combined data set. For BI and ML analyses, each data set was partitioned by genes, allowing each one to evolve at different rates. For *COI*, the data set was divided into three partitions according to codon positions. Evolutionary models were estimated independently for each of the gene partitions using jModelTest v2.1.1 (Darriba et al., 2012) applying Akaike weights as the selection criterion. We used MrBayes v3.2.2 (Ronquist et al., 2012) to estimate the topologies provided. These were run for $20x10^6$ generations saving trees each 100 generations with a burn-in value of 25%. ML phylogenies were inferred with RAxML v8.0.24 (Stamatakis, 2014) implemented at CIPRES Science Gateway (Miller et al., 2010) using a GTRGAMMA model of evolution and 1,000 bootstrapping replicates. MP analyses were conducted in TNT v1.1 (Goloboff et al., 2008). Tree searches were conducted under the tree bisection reconnection (TBR) algorithm with 100 replicates and 10 trees held per replicate. Nodes supports were estimated using 1000 bootstrapping replicates. For the different topologies obtained, we interpreted as significant support values above 75% for bootstrapping procedures in ML and MP and 0.95 for posterior probabilities obtained by BI. Three species were used as outgroup (*Chondrina avenacea*, *Arianta arbustorum* and *Cochlicella acuta*).

2.4. Species delimitation

Two approaches were used on the different data sets, following the terminology of Carstens et al. (2013). Firstly, *species discovery* methods were used on the molecular data without assigning a priori information on species memberships, and then s*pecies validation* tests were used on the molecular and ecological data, assigning samples to the recovered candidate species.

2.4.1. Molecular species discovery

Two species discovery approaches were used on the mitochondrial sequence data:

(1) Generalized Mixed Yule Coalescent method (GMYC) (Pons et al., 2006). This procedure uses a maximum-likelihood framework to delimit species based on single-locus ultrametric trees. The method estimates the transition point on a tree, before which all nodes reflect species diversification events (Yule model) and after which all nodes represent a population coalescent process (Pons et al., 2006). A new mitochondrial tree (*COI-16S rRNA*) was recovered by BI after removing identical sequences from the data set because zero length terminal branches hinder likelihood estimates (Fujisawa and Barraclough, 2013). The mitochondrial tree was converted to ultrametric form using Mesquite v2.75 (Maddison and Maddison, 2011). GMYC tests were run using the web server (http://species.h-its.org/gmyc/) under the single-threshold model; this model performs better than the multiple-

threshold model (Fujisawa and Barraclough, 2013).

(2) Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012). This procedure groups the recovered sequences into candidate species based on the "barcode gap". This gap can be observed in the distribution of pairwise differences of a barcode data set, between intraspecific and interspecific divergence (Puillandre et al., 2012). The method finds the distance where the barcode gap is detected and subsequently, the data is recursively partitioned in groups so that the distance between two samples from different groups is greater than the given distance. *COI* sequence alignments were uploaded at the ABGD web server (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) and the method was run with the following settings: Pmin: 0.001; Pmax: 0.1; Steps: 10; X (relative gap width): 0.5; Nb bins: 20 and Jukes-Cantor (J69): 2.0 distances. Since different partitions are proposed by the method, Puillandre et al. (2012) suggest using independent data to choose among different partitions.

Candidate groups containing only one or two specimens were not considered valid and were either grouped with other candidate species or removed from further analyses after considering the phylogenetic and geographical data. Consensus delimitations for species discovery were obtained by comparing the GMYC and ABGD results, and considering the phylogenetic inferences and geographical information for each sample.

2.4.2. Molecular species validation

Two multilocus methods were used on the mitochondrial and nuclear sequence data to validate the candidate species discovered. Although both methods rely on the coalescent theory, they are complementary because they simplify the parameter space via different strategies (Carstens et al., 2013):

(1) The Bayesian species-delimitation method was performed using BPP software (Yang and Rannala, 2010). The method calculates posterior probabilities of potential species delimitations after defining plausible species using a guide phylogeny. They suggest using species tree methods to construct the guide tree. Thus, we estimated the topology of the species tree in a Bayesian framework using *BEAST implemented in BEAST v1.8 (Drummond and Rambaut, 2007) for the concatenated data set of the four genes (after removing identical sequences). The species tree was tested in BPP. The rjMCMC was run for 500.000 generations (sampling every five generations) with a burn-in of 50,000. Based on Leaché and Fujita (2010), we used algorithm 0 (ε= 15.0). Three different combinations of priors were modelled because the prior distributions on the ancestral population size (θ) and root age (τ0) can affect the models' posterior probabilities: G (1, 10) for θ and τ0; G (2, 2000) for θ and τ0; and G (1, 10) for θ and G (2, 2000) for τ0. Each analysis was repeated twice using different seed numbers.

An inaccurate guide topology could result in the delimitation of all candidate species due to overestimated genetic divergence between sister lineages (Leaché and Fujita, 2010). Since

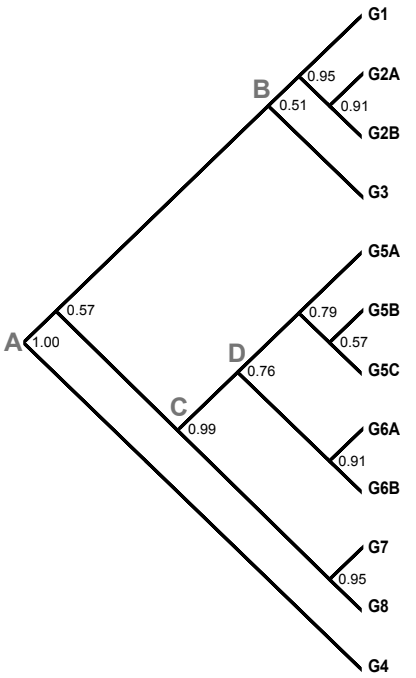


Figure 1: Species tree generated by *BEAST. Numbers indicate the posterior probability of each node. Grey letters indicate the four clades that were separately examined by BPP.

we obtained no supported nodes in the species tree (Figure 1), we analysed 4 partitions of the tree to minimize errors arising from an inaccurate guide tree. Clades A, B, C and D in Figure 1 represent the clades that were analysed separately. The three combinations of priors described above were applied to the 4 partitions such that we ran 12 BPP procedures.

(2) SpedeSTEM (Ence and Carstens, 2011). This software package calculates the maximum likelihood species tree taking into account previously estimated gene trees. The procedure assumes that the only discordance between the topology of gene trees and the species phylogeny occurs via the coalescent process. Accordingly, the accuracy of the method relies on the quality of the gene tree estimates (Ence and Carstens, 2011).

To recover accurate gene trees, the data set was reduced to 10 specimens per candidate species, selecting a representative portion of the geographical distribution and genetic diversity of each group. Each gene tree (*COI, 16S rRNA* and nuclear genes fragment) was estimated using BI and converted to ultrametric form using Mesquite v2.75 (Maddison and Maddison, 2011). Together with the gene trees, the web server (https://spedestem.osu.edu/) was provided with a setting file compiling the group membership information (candidate species), scaling factors for each gene, and BT values. Scaling factors and BT values were obtained using DnaSP v5 (Librado and Rozas, 2009).

2.4.3. Ecological species validation

To validate candidate species using ecological data, we used the ENM procedure. Maxent v3.3.3 (Phillips et al., 2006) software was used to create ENMs of each candidate species as it performs better than other methods (Phillips et al., 2006; Wisz et al., 2008). Maxent is a

maximum entropy method that combines presence only data with environmental layers to create potential geographical distributions of target species. We ran the method applying a random test percentage of 25%, choosing the logistic output format and creating response curves. The area under the ROC curve (AUC) of the tested data was used as a measure of the model's predictive power. AUC is the probability that a randomly chosen presence site will be ranked above a random background site (Phillips et al., 2006). It ranges from 0.5 (random discrimination ability) to 1.0 (perfect discrimination ability); values above 0.75 are considered as potentially useful (Elith, 2000).

Environmental layers (altitude and 19 bioclimatic variables) were downloaded from the WorldClim database (Hijmans et al., 2005; http://www.worldclim.org/) at a 30 arc-seconds resolution. So as not to overparameterize niche models, we conducted correlation tests for the 19 bioclimatic data and then removed highly correlated variables. First, we ran Maxent for each bioclimatic layer separately using the collection localities of all the samples to obtain an AUC value for each variable. Next, setting a Pearson correlation coefficient cutoff of 0.75, we identified highly correlated variable pairs and kept the variable of a pair with the highest AUC value. The final stage of selection was based on altitude and the 10 climatic variables: isothermality (bio3), temperature seasonality (bio4), maximum temperature warmest month (bio5), mean temperature wettest quarter (bio8), annual precipitation (bio12), precipitation wettest quarter (bio13), precipitation driest month (bio14), precipitation wettest quarter (bio16), precipitation warmest quarter (bio18) and precipitation coldest quarter (bio19).

After running Maxent for each candidate species, we used ENMTools software (Warren et al., 2010) to calculate similarity measures and compare the generated ENMs. First, we determined *niche overlap* between predicted ENMs of candidate species using two different statistics, Schoener's *D* (Schoener, 1968) and Warren's *I* statistic (Warren et al., 2008). These metrics ranged from 0 (species with completely discrepant ENMs) to 1 (species with identical ENMs) (Warren et al., 2010). Secondly, we conducted *niche identity* tests, also implemented in ENMTools, using 100 replicates. These tests determine whether ENMs produced from two species are statistically identical or not by generating a distribution of overlap scores between species. This null distribution is compared to the actual observed *niche overlap* measure, and niche identity will be rejected when the observed values for *I* and *D* are significantly lower than the values expected from the null distribution.

Three candidate species were not included in these tests since they represented only 4 sampled sites.

2.4.4. Consensus species validation

By comparing the molecular and ecological species validation results, we obtained a consensus delimitation of putative species to be tested in subsequent analyses.

2.5. Morphological inferences

The shells of the specimens used for the genetic tests were photographed to obtain "height/maximum diameter" (H/D) and "umbilicus diameter/maximum diameter" (UD/D) measures for each specimen. When combined, these ratios provide a general description of shell shape and are the main measurable characters that have been used to define or describe the morphospecies of the genus *Pyramidula* (Gittenberger and Bank, 1996; Martínez-Ortí et al., 2007; Balashov and Gural-Sverlova, 2011; Schileyko and Balashov, 2012). Based on the data provided by Gittenberger and Bank (1996) and Martínez-Ortí et al. (2007) (Supplementary material 1), we defined different ranges for H/D and UD/D accompanied by their corresponding descriptions (Table 1). This served to objectively describe the morphology of the phylogenetic clades and putative species using the measures obtained for each specimen. To summarize the descriptive statistics, boxplots were created for both characters and for each phylogroup using R statistical software v3.0.2 (R Core Team 2013). Another character used for classification is the disjunct coiling shell (Table 1), only present in the morphospecies *P. chorismenostoma*. Given this character is not a continuous or quantitative character it was not treated in the same way as the two measures described above. Instead, it was used to assess its utility in delimiting the putative species yielded.

Three of the major clades were not included in this analysis as they comprised fewer than 5 specimens with available measurements.

According to H/D and UD/D, and information in Supplementary material 1, each specimen was assigned to the corresponding morphospecies. In Supplementary material 2, we provide H/D and UD/D measures for each specimen along with the corresponding morphospecies.

2.5.1. Utility of shell dimensions for species delimitation

H/D and UD/D were used to assess the utility of shell dimensions to differentiate the

Table 1: Ranges and descriptions defined for three shell characters based on the information of Supplementary data 1. "H/D": height/maximum diameter; "UD/D": umbilicus diameter/maximum diameter. Ranges were defined to objectively describe the morphology of the different phylogroups.

Shell character	Range	Description
H/D	<0.70	low conical
	0.7-0.9	moderately low conical
	0.9-1.1	conical
	>1.1	high conical
UD/D	<0.25	narrow umbilicus
	0.25-0.33	medium-width umbilicus
	>0.33	wide umbilicus
Disjunct coiling shell	Yes/No	Apical whorls are coiled and contact each other, while the last ones are increasingly wide apart

species defined according to genetic and ecological data. Putative species were compared by examining differences in variable means by MANOVA and means that significantly varied between species were identified by the multiple comparisons Tukey test using R statistical software v3.0.2.

2.5.2. Influence of environmental factors on shell dimensions

To determine whether the shell shape of this genus could be influenced by environmental factors, we combined morphological and ecological (ENMs) data. The specimens were first divided into two groups of opposite shell shapes based on the H/D data given in Table 1: ENM1, comprising specimens with low or moderately low shells (H/D = < 0.9); and ENM2, comprising specimens with conical or high conical shells (H/D = ≥ 0.9). Then to explore whether differences existed between the habitats of groups ENM1 and ENM2, we used Maxent to model the ranges for each group and the statistical tests implemented in ENMTools to compare the groups, as described in section 2.4.3.

3. Results

3.1. Gene sequences

No stop codons were detected in the *COI* sequences. No genes or codon positions showed signs of saturation, indicated by an Iss (index of substitution saturation) significantly lower than the Iss.c (critical substitution saturation index) (Supplementary material 4).

The data set used for the phylogenetic tests corresponded to 211 representatives of the genus, with 2,493 aligned characters. More information about the sequences of each gene and the complete data set (sequence lengths, number of polymorphic sites, selected substitution models and their parameters) are shown in Supplementary material 5. Alignment of the combined data set, outgroups included, is available in Supplementary material 6.

3.2. Phylogenetic inference

The phylogeny obtained by concatenating the mitochondrial and nuclear genes is shown in Figure 2. The figure also indicates the distributions of each clade. The topology of the phylogeny is based on BI but all BI posterior probabilities and ML and MP bootstrap values are indicated at the nodes of main clades when BI ≥ 0.90, and ML and MP ≥ 70%. Using the three procedures, all taxa were grouped into eight major clades (Groups 1-8), with the exception of five individual specimens (indicated with a red cross). Descriptive statistics for the morphological variables of each clade are shown in the box plots in Figure 3. These results are mentioned below to describe the shell morphology of each clade.

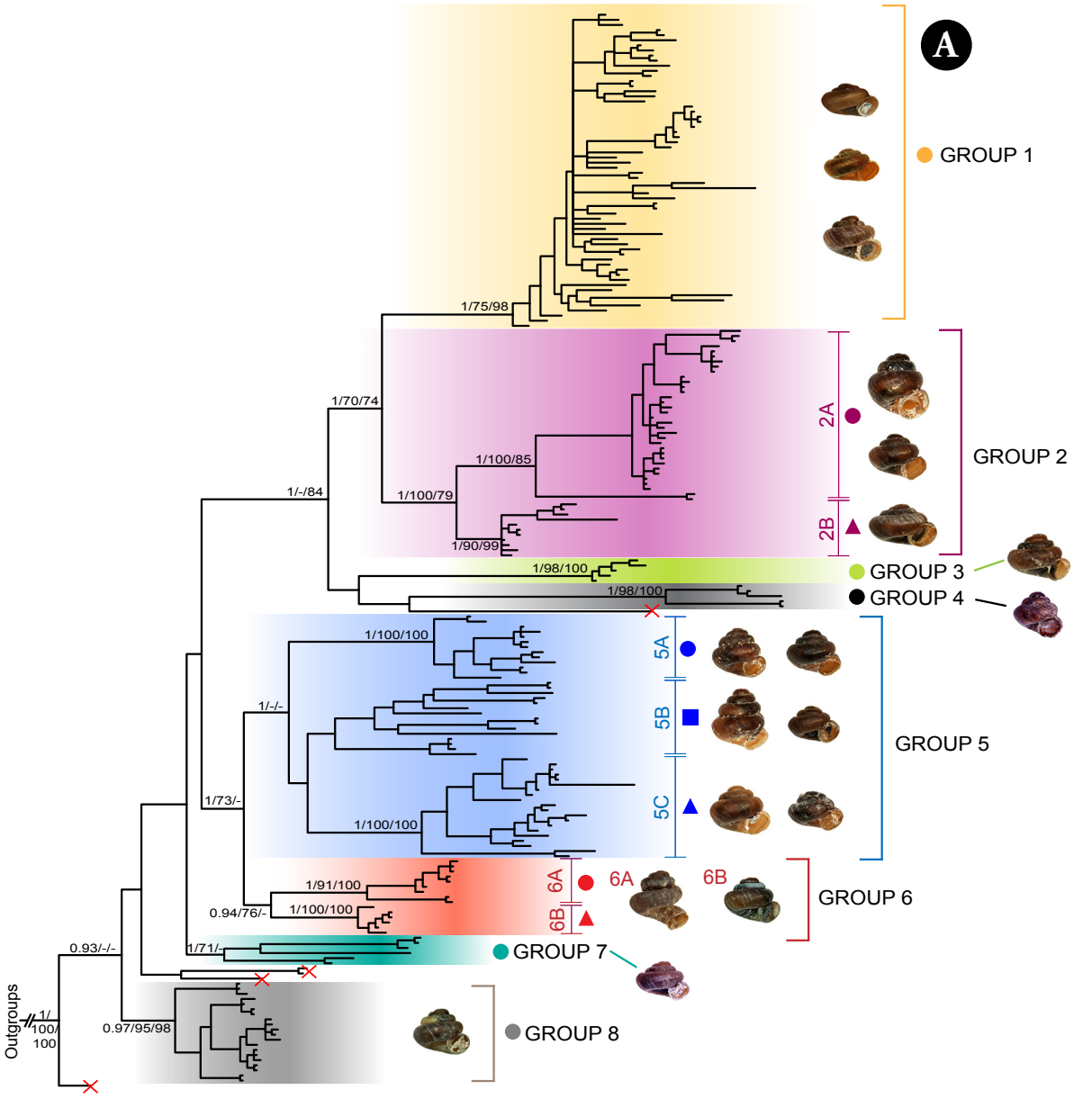
The monophyly of Group 1 was recovered by all analyses (BI=1; ML=75; MP=98). Specimens grouped in this clade inhabit the Iberian Peninsula, British Isles, France, Italian Peninsula, the Alps, the Carpathians, the Dinaric Alps, the Balkan Peninsula, Anatolia and the Crimean Peninsula. Shell shape varied from low to moderately low conical and umbilicus size from medium-width to wide.

Figure 2:

A. Phylogenetic tree of *Pyramidula* based on Bayesian inference (BI) analysis of the concatenated data set including *COI, 16S rRNA* and nuclear *5.8S, ITS2* and *28S* sequences. Numbers correspond to BI posterior probabilities, and ML and MP bootstrap values, respectively. The tree is coloured to distinguish eight major supported clades and the internal clades of some groups. Photographs of some of the specimens included in the phylogeny are shown. The reader is referred to this tree as a guide for the phylogeny descriptions.

B. Localization of the samples assigned to groups 1-4. Samples in each group appear in the same colours as used in the phylogenetic tree (A).

C. Localization of the samples assigned to groups 5-8. Samples in each group appear in the same colours as used in the phylogenetic tree (A).



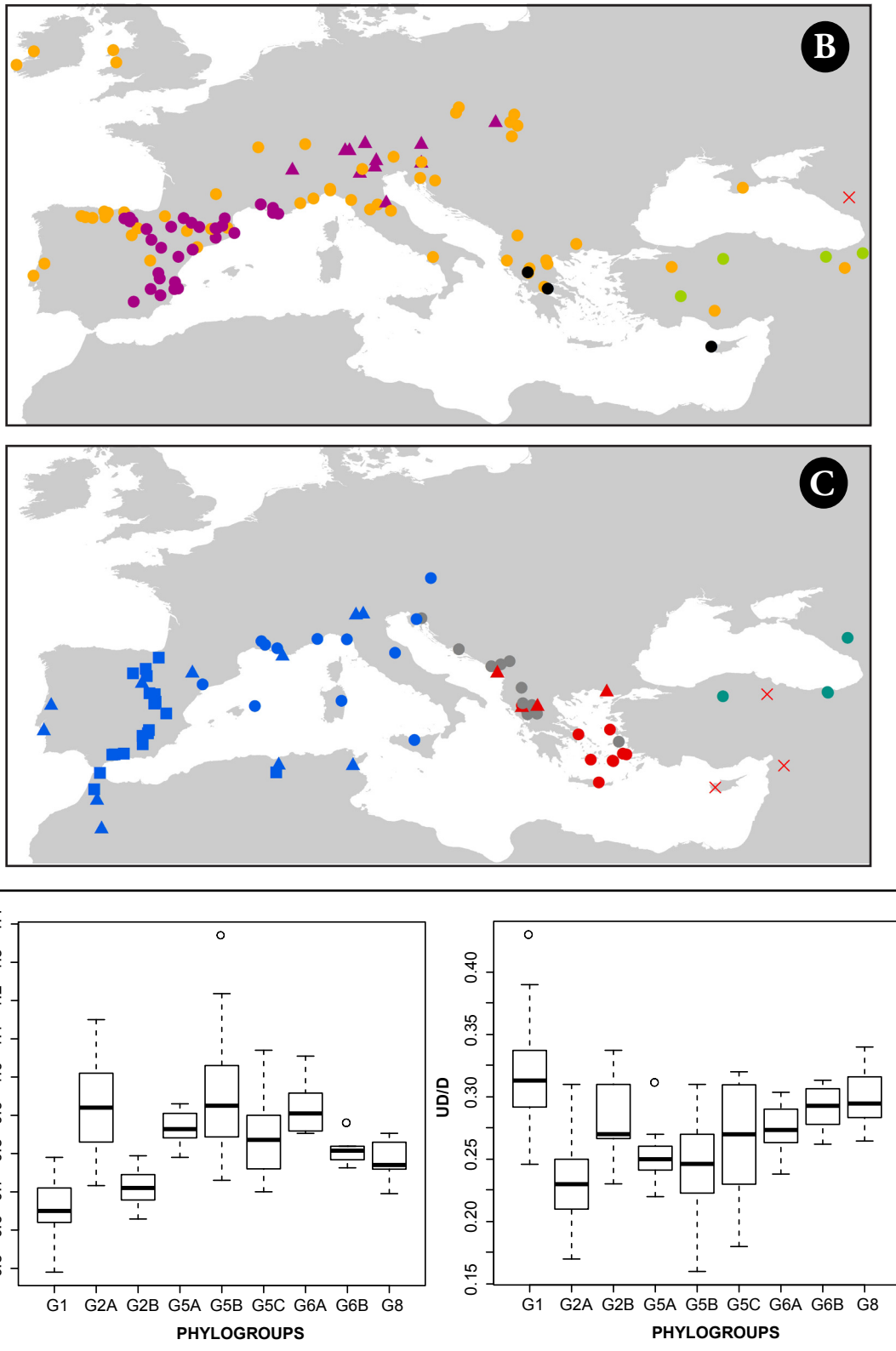


Figure 3: Box plots showing the variation produced in the two shell morphological characters in each phylogroup. "H/D": height/maximum diameter; "UD/D": umbilicus diameter/maximum diameter.

Group 2 was supported (BI=1; ML=100; MP=79) and divided in two supported sister clades (2A: BI=1; ML=100; MP=85; and 2B: BI=1; ML=90; MP=99). Clade 2A grouped together specimens from the Iberian Peninsula and Southern France, with shell shapes ranging from moderately low to high conical, and umbilicus size from narrow to medium-width. Clade 2B grouped specimens from the Alps and the Carpathians. Shell shape varied from low to moderately low conical, and umbilicus size from narrow to wide.

Groups 3 and 4 were recovered with strong support (BI=1; ML=98; MP=100) but were represented only by five samples each. The specimens in Group 3 were from Turkey, and specimens in Group 4 from Greece and Cyprus.

Group 5, recovered by all analyses, was supported only by bayesian inference (BI=1). Specimens grouped in this clade inhabit the Iberian Peninsula, North Africa, Menorca, Sicily, Sardinia, Southern France, Italy, Croatia and Austria. This group included 3 clades: 5A (BI=1; ML=100; MP=100); 5B (not supported) and 5C (BI=1; ML=100; MP=100). Shell shape in group 5 varied from moderately low to high conical and umbilicus size from narrow to medium-width.

The monophyly of group 6 was moderately supported (BI=0.94; ML=76; MP=-) and the group was divided into two supported sister clades (6A: BI=1; ML=91; MP=100 and 6B: BI=1; ML=100; MP=100). Clade 6A included specimens from Aegean islands and continental coast, all of which had disjunct coiling shells. Clade 6B included specimens from the continental part of Greece and Montenegro, and had moderately low conical shells and a medium-width umbilicus.

Group 7 (BI=1; ML=71; MP=-) included six specimens, inhabiting Turkey, Georgia and Russia.

Group 8 was well supported by all analyses (BI=0.97; ML=95; MP=98). Specimens of this group were present in Greece, Croatia, Montenegro and Albania. They showed moderately low conical shells and a medium-width umbilicus. Their geographical distribution and morphology was similar to those of the clade 6B specimens.

The phylogenies obtained for both mitochondrial (a) and nuclear (b) genes separately are shown in Supplementary material 7. Both trees recovered the monophyly of Group 2 (a: BI=1; ML=90; MP=83; b: BI=1; ML=92; MP=<70), Group 3 (a: BI=1; ML=100; MP=100; b: BI=1; ML=98; MP=97) and Group 4 (a: BI=1; ML=84; MP=100; b: BI=1; ML=97; MP=100). Group 1 was supported for both phylogenies by BI, but not by ML and MP (a: BI=0.91; ML= <70; MP=82; b: BI=0.97; ML= <70; MP=74). Groups 5 and 6 emerged as monophyletic clades only in the mitochondrial tree (Group 5: BI=1; ML=77; MP=<70; Group 6: BI=0.94; ML=82; MP=<70), while Group 7 was only supported by the nuclear data set (BI=1; ML=89; MP=80). The monophyly of Group 8 was supported by both analyses in the mitochondrial tree (BI=0.98; ML=92; MP=84) and only by ML and MP in the nuclear tree (BI=<90; ML=83; MP=91), but included some clade 6B specimens.

3.3. Species delimitation

3.3.1. Molecular species discovery

GMYC The maximum likelihood obtained in the GMYC model was significantly higher than the likelihood observed in the null model: GYMC model=881.36; null model=875.54; likelihood ratio=11.64 and LR test=0.0092. This test generated 22 entities, 8 of which included only one or two specimens (Figure 4).

ABGD Different numbers of groups were obtained for the recursive partition with differing prior maximal distances (p): 86 (p=0.0010), 80 (p=0.0017), 80 (p=0.0028), 78 (p=0.0046), 29 (p=0.0077), 23 (p=0.0129) and 1 (p=0.0215). According to the suggested by Puillandre et al. (2012) of using independent data to choose among different partitions, we compared these results with the GMYC data. Taking into account this comparison and the fact that initial and recursive partitions were stable for p=0.0129, we consider the results of 23 groups (p=0.0129) as accurate. 11 of these 23 groups only included one or two samples (Figure 4).

Consensus delimitation for species discovery was obtained after grouping or removing candidate groups/entities containing only one or two specimens. As shown in Figure 4, 12 candidate species were considered for subsequent validation analyses and named according to the phylogenetic tree.

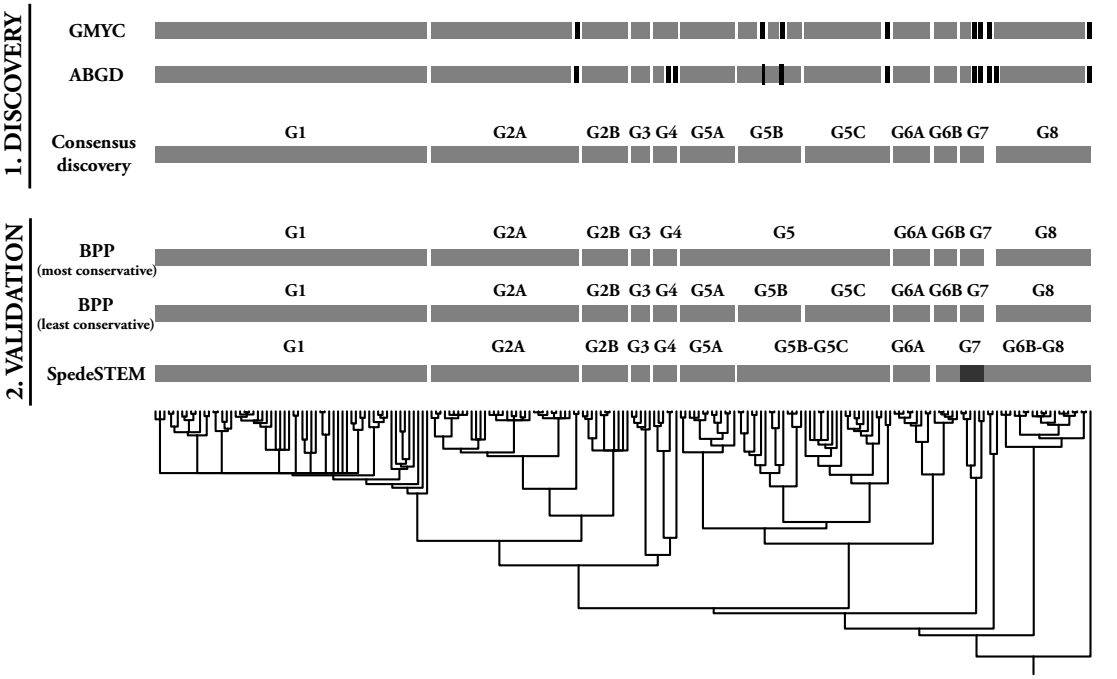


Figure 4: Summarized results of all the molecular delimitation analyses (including both discovery and validation approaches) represented on a phylogenetic tree of the concatenated data set. Grey boxes represent resultant candidate species (identified in the discovery analyses) or putative species (identified in the validation analyses). The black lines along GMYC and ABGD indicate that only one or two individuals were included in these candidate groups.

3.3.2. Molecular species validation

BPP In 11 of the 12 tests run, all candidate species of each clade were delimited with a posterior probability of 1 at all nodes. Only the analysis of clade D of the species tree (Figure 1), with prior combinations of relatively small ancestral population sizes and shallow divergences among species, yielded a different result; grouping G5A, G5B and G5C into one species with a posterior probability of 1. Figure 4 shows the two different delimitations offered by BPP; the least conservative one delimited all the candidate species and the most conservative one delimited all the candidate species but G5A, G5B and G5C, which were clustered into one (G5).

SpedeSTEM These results are provided in Supplementary material 8. The model showing the highest probability (ωi = 0.585) collapsed G5B and G5C, and G6B and G8 into single species, while remaining candidate species were delimited as different.

3.3.3. Ecological species validation

Using Maxent we obtained predicted distribution maps for each candidate species. All models returned high AUC values (> 0.75) indicating good model performance except for the prediction of the candidate species G5C (AUC = 0.640). Habitat suitability maps obtained for each candidate species together with their AUC values are shown in Supplementary material 9. In these maps, darker colours indicate areas showing the best predicted conditions.

Results of niche model comparisons between candidate species are shown in Table 2. ENMTools detected no significant differences between any of the pairwise comparisons of G5A, G5B and G5C. Another Maxent analysis was therefore run for all the samples included in phylogroup G5 (clustering G5A, G5B and G5C) and the resulting niche model was used to conduct subsequent *niche overlap* and *niche identity* comparisons with the remaining candidate species. No significant differences were observed between the ENMs of candidate species G1 and G2B, and G6B and G8. For all other comparisons, ENMTools did detect significant differences between the ENMs of the candidate species.

3.3.4. Consensus species validation

Although no significant differences were found between the ENMs of candidate species G1 and G2B, both species were delimited by the two molecular validation approaches (BPP and SpedeSTEM). Moreover, in Figure 2 it may be observed that the distribution range of G2B overlaps part of the distribution range of G1 (Alps and surroundings), but G2A emerges as the sister group of G2B rather than of G1 in all phylogenetic tests on mitochondrial, nuclear or concatenated data sets (Figure 2 and Supplementary material 7). Accordingly, we considered G1 and G2B as putative species.

G2A and G6A were delimited as distinct species by all the molecular and ecological validation approaches and thus also considered as putative species.

Table 2: ENMTools results. Results are provided as both Schoener's *D* and Warren's *I* statistics (D/I). The *niche overlap* results for all pairwise comparison between candidate species are given as values between 0 (species with completely discordant ENMs) and 1 (species with identical ENMs). The *niche identity* test results are also given using asterisks, to indicate the significance of niche dissimilarity. Significance codes: "**": null hypothesis of niche similarity was rejected (α=0.01); "*": null hypothesis of niche similarity was rejected (α=0.05); and "n.s.": null hypothesis of niche similarity was not rejected, and therefore, no significant differences were found between ENMs.

Candidate species	**G5A**					
G5B	0,458$^{n.s.}$ / 0,645$^{n.s.}$	**G5B**				
G5C	0,664$^{n.s.}$ / 0,778$^{n.s.}$	0,683$^{n.s.}$ / 0,784$^{n.s.}$				
Candidate species	**G1**					
G2A	0,473** / 0,657**	**G2A**				
G2B	0,608$^{n.s.}$ / 0,752$^{n.s.}$	0,298** / 0,546**	**G2B**			
G5	0,569** / 0,717**	0,584** / 0,724**	0,350** /0,585**	**G5**		
G6A	0,076** / 0,375**	0,098** / 0,376**	0,056** / 0,357**	0,156** / 0,436**	**G6A**	
G6B	0,337** / 0,548**	0,196** / 0,452**	0,222** / 0,495**	0,297** / 0,535**	0,214* / 0,457*	**G6B**
G8	0,542** / 0,688**	0,263** / 0,507**	0,314** / 0,561**	0,440** / 0,639**	0,157** / 0,428**	0,623$^{n.s.}$ / 0,726$^{n.s.}$

G5A, G5B and G5C clustered as a single species in one of the BPP tests, and G5B and G5C were joined together by SpedeSTEM. ENMTools detected no significant differences in pairwise comparisons of G5A, G5B and G5C. In addition, in the phylogenetic inference (Figure 2), rather than a restricted geographical zone for each candidate species, the similar distributions of the three candidate groups show considerable overlap. We therefore consider that G5A, G5B and G5C should be clustered into one putative species (G5).

Candidate species G6B and G8 were defined as distinct species by all the BPP analyses. However, using spedeSTEM, the two candidate species clustered together as a single species. Ecological species validation also detected no significant differences between the ENMs of both candidate species, and their distribution range is also common to both (Figure 2). The phylogenetic relationships between G6B and G8 recovered by the mitochondrial and nuclear data (Supplementary material 7) were incongruent. In the mitochondrial phylogeny (Supplementary material 7-A), candidate species G6B comprises a fully supported clade (BI=1; ML=100; MP=91) closely related to species G6A (BI=0.94; ML=82) while the nuclear phylogeny (Supplementary material 7-B) failed to recover the monophyly of G6B with samples grouping together with the G8 samples, though without support for BI (ML=82; MP=91). In this case, although we had molecular and ecological evidence to consider G6B and G8 as the same species, we decided to keep them as putative species for subsequent morphological analyses.

Candidate species G3, G4 and G7 were only validated by the molecular tests, because ENM data were unavailable. Nevertheless, the three species were delimited as separate species in all the analyses.

In summary, according to the molecular and ecological validation approaches, 10 groups were identified as putative species for subsequent analyses: G1, G2A, G2B, G3, G4, G5, G6A, G6B, G7 and G8.

3.5. Morphological inferences

3.5.1. Utility of shell dimensions for species delimitation

Candidates G3, G4 and G7 were not included in these analyses as they comprised less than five specimens with available shell measurements. The results presented below refer to the remaining seven putative species. MANOVA revealed differences in means between the seven putative species for the two morphological variables (H/D and UD/D) (Pillai trace F = 13.512, $P < 0.001$). In contrast, not all pairwise comparisons returned significant differences in the multiple comparisons Tukey test (Table 3). Significant differences in both shell dimensions were identified for the following pairs of species: G1 vs G2A, G1 vs G5, G1 vs G6A, G2A vs G2B, G2A vs G8 and G5 vs G8. In contrast, no differences in either variable were recorded for G2A vs G5, G2B vs G6B, G2B vs G8, G5 vs G6A, G5 vs G6B, G6A vs G6B or G6B vs G8.

The character of disjunct coiling shell differentiated G6A from the rest of the candidate species as it is the only one showing this peculiarity.

3.5.2. Influence of environmental factors on shell dimensions

The AUC values obtained in Maxent were 0.868 for group ENM1 (specimens with low and moderately low shells (H/D = < 0.9)) and 0.927 for group ENM2 (specimens with conical and high conical shells (H/D = ≥ 0.9). The ENMs of the two differentiated morphological groups are displayed in Figure 5 (A and B), in which darker colours indicate areas with better predicted conditions.

The measured niche overlap values between groups ENM1 and ENM2 were $I = 0.711$ and $D = 0.572$, significantly lower than the null distribution obtained by ENMTools. This

Table 3: Multiple comparisons Tukey test for measures of two shell morphological characters ("H/D"/"UD/D"). "H/D": height/maximum diameter; "UD/D": umbilicus diameter/maximum diameter. Significance codes: "**": α=0.01; "*": α=0.05; and "n.s.": not significant.

Putative species	G1					
G2A	** / **	G2A				
G2B	n.s. / *	** / **	G2B			
G5	** / **	n.s. / n.s.	** / n.s.	G5		
G6A	** / *	n.s. / **	** / n.s.	n.s. / n.s.	G6A	
G6B	** / n.s.	n.s. / **	n.s. / n.s.	n.s. / n.s.	n.s. / n.s.	G6B
G8	** / n.s.	** / **	n.s. / n.s.	** / **	** / n.s.	n.s. / n.s.

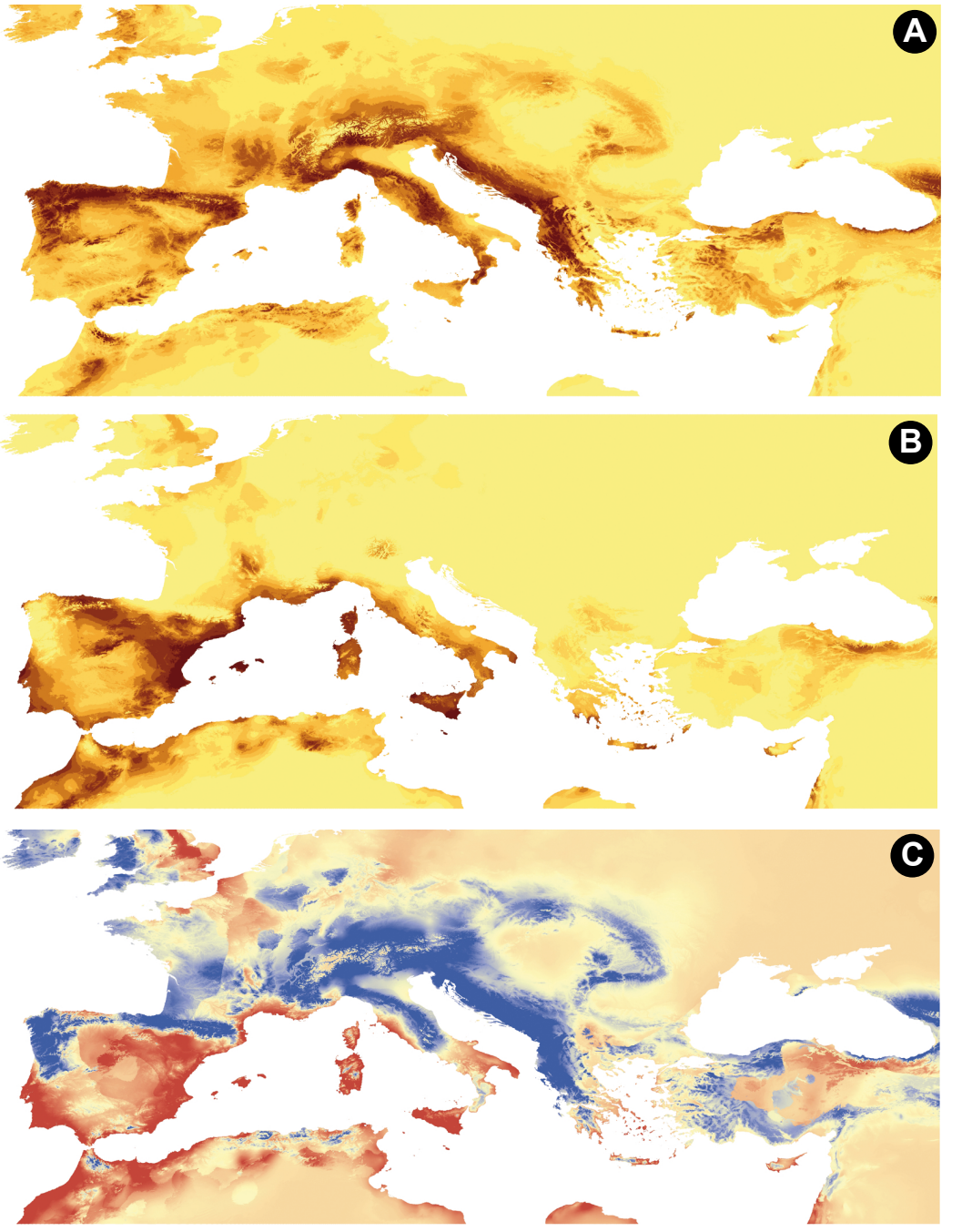


Figure 5:

A. Habitat suitability map provided by the Maxent model for Group ENM1 (specimens with low or moderately low shells (H/D = < 0.9)). Darker colours indicate areas with better predicted conditions.

B. Habitat suitability map provided by the Maxent model for Group ENM2 (specimens with conical or high conical shells (H/D = ≥ 0.9)). Darker colours indicate areas with better predicted conditions.

C. Subtraction map "Maxent model ENM2 – Maxent model ENM1". Bluish colours indicate areas with better predicted conditions for group ENM1. Reddish colours indicate areas with better predicted conditions for group ENM2.

indicates that the identity test rejected the hypothesis of niche identity assuming that both ENMs generated by Maxent were significantly different.

Maximum probabilities of the predicted ecological suitability of group ENM1 were mainly assigned to the northern and west-northern Iberian Peninsula, Central Europe, British Isles, major mountain ranges of Europe (Pyrenees, Alps, Apennines, Carpathians, Dinaric Alps, Pindus mountains), western Anatolia, the Caucasus, the Rif and the eastern Tell Atlas (Figure 5-A, 5-C). In contrast, maximum probabilities for ENM2 corresponded to areas where the Mediterranean climate is more pronounced: central, south-western and eastern Iberian Peninsula, south coast of France, Balearic islands, Sicily, Sardinia, Corsica, some areas of Italy, Aegean islands, Crete, Cyprus, northern Anatolia and northern Africa (Figure 5-B, 5-C).

Table 4 summarizes the phylogenetic supports, species validation, and morphological ENM groups involved for each putative species.

4. Discussion

4.1. Species delimitation

In biology, species are considered fundamental units (Mayr, 1982) and their delimitation is pivotal for systematic biology with implications for biogeography, ecology, and evolution and conservation biology (Avise, 2000; Goldstein et al., 2000; Hey et al., 2003). Thus,

Table 4: Summary of the phylogenetic supports, species validation, and morphological ENM groups involved for each putative species. "YES" = ecological niche model differentiated from that of the remaining putative species; "NO" = ecological niche model not differentiated from that of the all remaining species; "ENM1" = specimens with low or moderately low shells; "ENM2" = specimens with conical or high conical shells.

Putative species	Phylogenetic support (concatenated / mitochondrial / nuclear data)	Molecular species validation	Ecological species validation	Mophological ENM groups involved
G1	BI=1/0.91/0.97, ML=75/-/-, MP=98/82/74	SpedeSTEM & BPP	NO (G1=G2B)	ENM1
G2A	BI=1/1/1, ML=100/79/97, MP=85/-/80	SpedeSTEM & BPP	YES	ENM1, ENM2
G2B	BI=1/1/-, ML=90/92/73, MP=99/90/95	SpedeSTEM & BPP	NO (G1=G2B)	ENM1
G3	BI=1/1/1, ML=98/100/98, MP=100/100/97	SpedeSTEM & BPP	NA	ENM1, ENM2
G4	BI=1/1/1, ML=98/84/97, MP=100/100/100	SpedeSTEM & BPP	NA	ENM1, ENM2
G5 (G5A+G5B+G5C)	BI=1/1/-, ML=-/77/-, MP=-/-/-	BPP	YES	ENM1, ENM2
G6A	BI=1/1/-, ML=91/100/-, MP=100/99/85	SpedeSTEM & BPP	YES	ENM1, ENM2
G6B + G8	BI=-/-/-, ML=-/-/83, MP=-/-/91	SpedeSTEM	YES	ENM1
G7	BI=1/1/1, ML=71/96/89, MP=-/100/80	SpedeSTEM & BPP	NA	ENM1

accurate species delimitation is the key to defining species boundaries and understanding evolutionary processes and mechanisms (Sites and Marshall, 2003).

Most snail species descriptions to date have been based on morphological characters. However, cryptic taxa (like the species comprising *Pyramidula*) are morphologically indistinguishable and therefore lack diagnostic characters (Bickford et al., 2007). In the present study, multiple lines of evidence were obtained through molecular tests, examining mitochondrial and nuclear loci, and ecological niche modeling. We defined species by intersecting evidence from two or more independent taxonomic characters and assumed that congruent patterns of evidence exist among different characters, in this case, among molecular and ecological data. These two lines of evidence were applied in an objective species delimitation framework.

We detected absolute congruence between molecular and ecological validation approaches defining the candidate species G2A and G6A as delimited species.

Candidate species G1 and G2B were validated by all the molecular validation tests. However, the ecological validation tools used detected no significant differences between the ENMs of G1 and G2B, though distribution maps predicted by Maxent were quite different (Supplementary material 9). Thus, while candidate species G1 showed a wide distribution range, candidate species G2B was restricted to the Alps and Carpathians. Indeed, specimens clustered in G2B were collected from the Alps (one sample from the Italian Peninsula and the other from the Carpathians). Climate changes during glaciations had a significant influence on the distributions of various organisms. The general opinion until the end of the past century was that refuges during glacial periods occurred in southern regions, mainly in the three southern peninsulas (Balkan, Italian and Iberian) (Hewitt, 1996, 2000; Taberlet et al., 1998). However, recent studies suggest that the Central European mountain regions served as suitable glacial refugia. In effect, Tribsch and Schönswetter (2003) found palaeoenvironmental and geological evidence of Pleistocene refugia in the eastern European Alps for mountain vascular plants. Based on macrofossil data, Willis and Vanandel (2004) indicated that during the last glacial maximum, coniferous and some deciduous trees grew further north and eastwards than previously considered. Molecular genetic studies have offered new evidence of peripheral Alpine refuges for several terrestrial gastropods: *Arianta arbustorum* (Gittenberger et al., 2004; Haase et al., 2013), *Arion fuscus* (Pinceel et al., 2005), *Trochulus villosus* (Dépraz et al., 2008), *Trochulus caelatus* (Ursenbacher et al., 2010), *Carychium minimum* and *C. tridentatum* (Weigand et al., 2012), *Orcula dolium* (Harl et al., 2014). In addition, a study by Juřičková et al. (2014) of fossil shells of land snail species indicates that molluscs survived the last central European glacial period in isolated patches of broadleaf forests. Consequently, we consider that G2B may have been isolated in Alpine refuges during glacial periods, and is thus a delimited species, although its current ecological niche is similar to that of the candidate species G1. Although the putative species G2A and G2B form a monophyletic group, their respective distributions indicate that each species may have come from different glacial refugia: G2A from the

Iberian refugium and G2B from the Alpine refugium, as mentioned above.

Candidate species G5A, G5B and G5C clustered as a single putative species in our ecological validation study and in one of the molecular validation tests performed (BPP). The highest probability model indicated by SpedeSTEM grouped G5B and G5C into a single species, while it delimited G5A as a different taxon. Carstens et al. (2013) suggest the use of different species delimitation analyses and choosing delimitations that are concordant between them. These authors argue that spedeSTEM and BPP are particularly useful for this purpose as they are based on complementary strategies, and incongruence across results indicates that assumptions of some of the methods were not fulfilled. Leaché and Fujita (2010) showed that BPP relies on a correct topology of the species tree to accurately delimit species because on the contrary, BPP tends to delimit all candidate species. In our species tree generated by *BEAST, some nodes were not strongly supported, especially those reflecting relationships among G5A, G5B and G5C (Figure 1). An inaccurate guide topology could explain the delimitation of these three candidate species in many of the BPP analyses. Notwithstanding, congruence between the two molecular validation methods suggests G5A, G5B and G5C belong to the same species. Moreover, our ecological species validation tool detected no significant differences between the ENMs of the three candidates (Table 2).

The clustering of G6B and G8 into a single putative species was validated by ecological niche modeling and by the molecular species validation method SpedeSTEM, but not by BPP. Besides, no significant morphological differences were detected between them. G6B and G8 formed two different mtDNA haplogroups, but only one nuclear DNA haplogroup (Supplementary material 7). The G6B mtDNA haplogroup clustered with G6A instead of G8 (Supplementary material 7). These discrepancies between mitochondrial and nuclear phylogenies could be the result of introgressive hybridization events or incomplete lineage sorting of ancestral polymorphisms. Phylogenetic approaches based on the inspection of independent loci help to elucidate cases of discordant evolution (Zhong, 2000; Ballard and Whitlock, 2004; Peters et al., 2007). However, it is difficult to distinguish the patterns caused by the two processes mentioned (Nielsen and Wakeley, 2001). Introgression of mtDNA from one species into another may be more likely than nDNA (Ballard and Whitlock, 2004) and indeed, several examples exist where mitochondrial introgression occurs in the absence of nuclear introgression (Ferris et al., 1983; Powell, 1983; Shimizu and Ueshima, 2000; Melo-Ferreira et al., 2012). In most of these cases, introgression levels are higher in areas where taxa inhabit in sympatry or parapatry than in allopatry (Ballard and Whitlock, 2004). However, here we could observe that the samples examined from phylogroups G6A and G6B were not sympatric and hence, the hypothesis of recent gene flow is less plausible. The alternative explanation is that an ancestral mtDNA polymorphism was retained after a recent and rapid speciation process (Avise, 2000). Incomplete lineage sorting has been effectively documented in molluscs (Wilding et al., 2000; Wilke et al., 2005). Besides, as a result of incomplete lineage sorting, heterotypic lineages (G6B) should be divergent from homotypic lineages (G6A) due to the long length of time that ancestor populations

were divided (Avise, 2000), as occurred in the present study, where each one comprises a monophyletic group. Further work, however, involving more molecular markers would be needed to ascertain which one of these two processes is the cause of the discordance between the different gene trees.

Ecological data were not available to validate candidate species G3, G4 and G7, but the two molecular validation procedures delimited them as distinct species. More specimens and ecological data are needed to accurately delimit and define these species.

4.2. Shell characters in *Pyramidula*

Our morphological inferences indicate that no single shell dimension (H/D or UD/D) was able to discriminate between all the delimited species. Any discrimination is further aggravated by a high level of polymorphism existing within species. Although the two shell dimensions mentioned served to discriminate between some of the putative species, they did not emerge as valid unique taxonomic characters for the genus *Pyramidula*, such that other lines of evidences are needed. Disjunct coiling shell shape distinguished putative species G6A from the rest; we therefore consider it a valid shell character.

Several authors have warned of the limited utility of morphological characters to decipher the taxonomy of terrestrial snails (Giusti and Manganelli, 1992; Uit de Weerd et al., 2004; Holland and Hadfield, 2007). In parallel with the growing number of phylogenetic studies, examples of incongruences between morphology and phylogeny have also grown in number (Chiba, 1999; Parmakelis et al., 2003; Pinceel and Jordaens, 2004; Uit De Weerd et al., 2004; Alonso et al., 2006; Pfenninger et al., 2006; Elejalde et al., 2008). Many studies have shown that shell morphology may be correlated with environmental factors (Cain, 1977, 1978a, 1978b; Cook and Jaffar, 1984; Goodfriend, 1986; Harley et al., 2009; Stankowski, 2011, 2013).

Assuming that the shell dimensions traditionally used to diagnose the species of *Pyramidula* (Gittenberger and Bank, 1996; Martínez-Ortí et al., 2007) cannot differentiate all the species delimited in the present study, we assessed the existence of plasticity in the shells of this complex. Moreover, given that *Pyramidula* species inhabit the surfaces of limestone rocks (Gómez-Moliner, 1988; Gittenberger and Bank, 1996; Martínez-Ortí et al., 2007; Welter-Schultes, 2011), we could say they are very exposed to the climate conditions. Our ENM results revealed significant differences between the predicted habitats of two morphologically distinct groups (low *versus* high conical shell shape). The group comprising shells of lower relative height (low conical shells) showed maximum predicted habitat probabilities mainly in the northern Iberian Peninsula and main mountain ranges of Europe. In contrast, the group clustering shells of greater relative height (high conical shells) showed maximum predicted habitat probabilities in areas with a Mediterranean climate. We thus observed that the lower shells appeared in areas of higher altitude, lower temperatures and more precipitation while the higher shells inhabited areas of lower altitude, higher temperatures and less precipitation. Cain (1977, 1978a, 1978b) described

that the relative heights (H/D) of the shells of land snails in many regions can vary from flattened to elongated, the rarest being the intermediate forms. Cain (1977) proposed the mechanism of balancing as a possible cause of the existence of the two extreme shapes (flattened and elongated), considering the energy that land snails employ to carry their shells during locomotion. Cook and Jaffar (1984) found consistent correlation between the relative height of a snail and the surface (vertical or horizontal) it chose to inhabit. Goodfriend (1986) also mentioned several examples of correlation between relative shell height and environmental variation (e.g. rainfall) within species. Several studies examining within-species variation have suggested negative correlation between relative height and rainfall in *Pleurodonte lucerna* (Goodfriend, 1983), *Palaina* spp. (Tillier, 1981), and *Achatinella mustelina* (Welch, 1938). Goodfriend (1986) proposed that drier conditions may select relatively higher shells, as they tend to have more numerous and narrower whorls. Elejalde et al. (2008) found different habitat preferences between morphologically distinct *Iberus* specimens including keeled-flat shelled snails living in arid environments on karstic mountains. Additionally, some species of intertidal limpets show a shell morphology seemingly adapted to deal with heat stress. Harley et al. (2009) examined the effects of shell morphology on predicted body temperature in three limpet species. These authors found that body heat was lost more readily by higher shells, independently of the species, suggesting that shell morphology variation could be a phenotypic response to variations in environmental temperature.

4.3. Taxonomic re-interpretation

Through the delimitation of species within *Pyramidula*, we were able to review the taxonomy of this complex in the western portion of its distribution range. Below we describe the species identified, the methods used to validate their identity, their distribution ranges, and their morphology. Table 5 shows the correlation between the group numbers of the putative species and the assigned available names.

G1. Validated by molecular and ecological methods. This group included almost all

Table 5: Correlation between the group numbers of the putative species and the assigned available names.

Group	Available name
G1	*Pyramidula pusilla* (Vallot, 1801) [= *P. umbilicata* (Montagu, 1803)]
G2A	*Pyramidula rupestris* (Draparnaud, 1801)
G2B	*Pyramidula saxatilis* (Hartman, 1842)
G3	*Pyramidula cf. hierosolymitana* (Bourguignat, 1852)
G4	*Pyramidula* sp1*
G5 (G5A+G5B+G5C)	*Pyramidula jaenensis* (Clessin, 1882)
G6A	*Pyramidula chorismenostoma* (Westerlund & Blanc, 1879)
G6B + G8	*Pyramidula cephalonica* (Westerlund, 1898)
G7	*Pyramidula* sp2*

* additional sampling is required to more fully delineate these putative species

specimens showing the morphology of *P. umbilicata* and some specimens resembling *P. pusilla* (Gittenberger and Bank, 1996; Martínez-Ortí et al., 2007). The topotype of *P. umbilicata* (Wales, UK) and topotype of *P. pusilla* (near Dijon, France) also grouped within G1. According to Gittenberger and Bank (1996), the name *P. pusilla* (Vallot, 1801) is a valid name and has priority over *P. umbilicata* (Montagu, 1803). G1 should therefore be named *P. pusilla*. The species is distributed across the entire sampling area, including the Iberian Peninsula, British Isles, France, Italian Peninsula, the Alps, Carpathians, Dinaric Alps, and the Balkan Peninsula, Anatolia and Crimean Peninsula. This species is the most different in H/D and UD/D shell dimensions. Accordingly, shell shape varies from low to moderately low conical. Umbilicus size ranges from medium-width to wide.

G2A. Validated by molecular and ecological methods. Specimens from SE France (type locality) are similar to the lectotype of *P. rupestris* (Gittenberger and Bank, 1996). In consequence, this species should be called *P. rupestris*. The species is present in the Iberian Peninsula and southern France. According to H/D and UD/D, shell shape ranges from moderately low to high conical, and umbilicus size from narrow to medium-width. The highest conical shells are similar to those of G5.

G2B. This group was delimited as a distinct species by all the molecular validation analyses. According to the niche modeling procedure, significant differences were also detected between the ENMs of G2B and those of the remaining species, with the exception of G1 versus G2B. Considering the ecological data, G2B could be conspecific with G1 (=*P. pusilla* present work). However, the phylogenetic analyses joined it with G2A (=*P. rupestris* present work). Morphologically, specimens mainly resemble *P. pusilla* and *P. umbilicata* morphotypes (Bank and Gittenberger, 1996). Hartmann (1842) proposed the name *Delomphalus rupestris saxatilis* Hartmann, 1842 (p. 122. Plate. 37. Figs. 4-6) for the low and moderately low conical shells of *Pyramidula* from the Swiss Alps, separating them from the conical shells of *Delomphalus rupestris rupestris*. The former is the first valid name available to designate the putative species G2B. Considering the morphology and distribution of G2B and the data and figures published by Hartmann (1842), we associate the name *Pyramidula saxatilis* with this taxon. The species is present mainly in the Alps, but also in Carpathians and the North Italian Peninsula. Shell shape ranges from low to moderately low conical, and umbilicus size from narrow to medium-width. In some areas, the distribution ranges of *P. pusilla* (G1) and *P. saxatilis* (G2B) overlap, being both species morphologically similar. Shell characters measured for this study did not found significant differences in the relative height of the shells between the two species, although some differences may exist regarding the relative width of the umbilicus. More exhaustive studies are needed to find diagnostic characters for both species.

G5. Well differentiated from the other species of the complex by molecular and ecological methods. The putative species shows three phylogroups (G5A, G5B and G5C) that were not validated as different species by SpedeSTEM, and no ecological differences exist between them. Considering that both lines of evidence (molecular and ecological)

indicate clustering of the three candidate species, we consider G5 a single putative species. Phylogenetic analysis clustered mainly *P. rupestris* and *P. jaenensis* morphospecies within this group. Nevertheless, all samples from Andalucía including topotypes from Jaén (Clessin, 1882) belong to this group. Hence, we consider that G5 should be named *P. jaenensis*. Its distribution range encompasses the Iberian Peninsula, North Africa, Menorca, Sicily, Sardinia, southern France, Italy, Croatia (one sample) and Austria (one sample). Variation in shell shape in *P. jaenensis* is greater than previously thought (Gittenberger and Bank, 1996; Martínez-Ortí et al., 2007) ranging from moderately low to high conical, and the umbilicus from narrow to medium size. No significant differences were found for H/D and UD/D between *P. jaenensis* (G5) and *P. rupestris* (G2A), G6A or G6B.

G6A. Validated by molecular and ecological methods. Specimens assigned to putative species G6A are the only ones with disjunct coiling of the body whorl and also show a distribution in the Aegean islands. Thus, considering the species validation results along with shell morphology and distributions of the samples grouped within this phylogroup, we consider that they belong to *P. chorismenostoma*. The unique shape of its shell readily distinguishes this species.

G6B-G8. Clustering of the two candidate species G6B and G8 into a single putative species was validated by molecular (only SpedeSTEM) and ecological methods. Moreover, shell morphological differences were not detected between them. The distribution of specimens assigned to putative species G6B-G8 spans from Croatia southwards to Greece, with one sample from western Turkey. Thus, according to its distribution range and shell similarity with the species *P. cephalonica* (see Bank and Gittenberger, 1996; Welter-Schultes, 2009), we consider G6B-G8 corresponds to the species *P. cephalonica*.

G3, G4 and **G7.** Only molecular species validation methods could be applied to these three phylogroups. BPP and spedeSTEM were congruent in validating the three candidate species. However, our conclusion is based on a single line of evidence such that more taxonomic criteria and more samples are needed to accurately delimit these species which are distributed along the eastern margin of our sampling area. Recent literature for areas of the Former Soviet Union still only refers to one species, *P. rupestris* (Sysoev and Schileyko, 2009). However, a recent study of the genus in eastern Europe, Central Asia and adjacent territories of Balashov and Gural-Sverlova (2011), suggests the existence of at least two morphologically distinct species. In addition, populations from Israel and Turkey showing the *P. rupetris* morphotype, with a high spire and narrow umbilicus, have been considered by some authors as a different species denominated *P. hierosolymitana* (Schütt, 2005; Heller, 2009). Among these three putative species, G3 and G7 inhabit in Turkey. But only G3 shows conical shells with narrow umbilicus, resembling *P. hierosolymitana*. Therefore, we provisionally propose the name *P.* cf. *hierosolymitana* for G3.

The objective of the present work was to unravel the taxonomy of the genus *Pyramidula* in its western distribution range, focusing on Europe and adjacent areas. Another similar

study would be necessary to clarify the taxonomy of the genus in Asia, since at least six species have been described based solely in morphological characters. Regarding G4 and G7 putative species, both species are present in the eastern margin of our sampling area, and having analyzed only a few individuals, we consider that more samples from the eastern distribution area should be analyzed to draw taxonomic conclusion for them. We think that with the scarce current data we do not have enough information to accurately describe and nominate them. Provisionally, they have been named as *Pyramidula* sp1 (G4) and *Pyramidula* sp2 (G7).

4.4. Conclusion

Through an objective species delimitation approach including multilocus gene and ecological data, we propose the existence of 9 putative species in the *Pyramidula* species complex inhabiting the western Palaearctic region. According to morphological characters, geographical distributions, and the inclusion of topotypes whenever possible, we were able to define and associate existing taxon names to seven of these nine putative species. Further samples, particularly from eastern regions are needed to fully resolve the taxonomy of this genus. Our shell character analysis revealed that although shell shape could discriminate between some of our putative species they are not valid as unique taxonomic criteria. We also hypothesized that shell shape in this genus could be influenced by environmental factors. Thus, Maxent predicted a suitable habitat characterized by a higher altitude, more rainfall and lower temperatures for specimens with lower shells while opposite habitat conditions offered by a Mediterranean climate were predicted as more suitable for specimens with higher shells. Experimental studies are needed to properly address this issue.

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Supplementary material 1: Measurements and descriptions of the shell characters used to define the six European species of *Pyramidula* based on Gittenberger and Bank (1996) and Martínez-Ortí et al. (2007). "UD/D": umbilicus diameter/maximum diameter; "H": height; "D": maximum diameter; "H/D": height/maximum diameter.

Species	Gittenberger and Bank, 1996				Martínez-Ortí et al., 2007				Shell shape description
	UD/D	H (mm)	D (mm)	Scalarid shell	H/D	UD/D	H (mm)	D (mm)	
P. umbilicata	0.33	up to 1.7	up to 2.9	NO	0.56-0.74	0.27-0.37	1.3-2.05	2.05-2.9	Shell low conical, much broader than high. Umbilicus wide.
P. pusilla	0.25 (0.33 NW Spain)	up to 2.25	up to 2.95	NO	0.68-0.9	0.16-0.35	1.6-2.2	2-2.65	Shell moderately low conical, clearly broader than high.
P. rupestris	<0.25	up to 2.5	up to 2.7	NO	0.87-1.13	0.17-0.26	1.8-2.65	1.75-2.75	Shell conical, from broader than high to clearly higher than broad. Umbilicus narrow.
P. jaenensis	<0.25	up to 2.7	up to 2.2	NO	1.06-1.47	0.13-0.25	2.2-3.15	1.9-2.5	Shell high conical, clearly higher than broad. Umbilicus narrow
P. cephalonica	>0.33	up to 1.8	up to 2.6	NO	-	-	-	-	Shell (low) conical, celarly broader than high. Umbilicus wide open.
P. chorismenostoma	-	up to 3.1	up to 2.2	YES	-	-	-	-	Disjunct coiling shell (the initial apical whorls are coiled and contact each other, while the last ones are increasingly wide apart).

Supplementary material 2: Information on the specimens used in this study: voucher specimens; localities; phylogroups in Figure 2 they belong to; candidate species they belong to based on the discovery approach; proposed species; shell measures (H/D and UD/D); morphospecies they belong to based on shell measures; and GenBank accession numbers for the COI, 16S and 5.8S-ITS2-28S gene fragments. Initials of the voucher specimens refers to the institution they belonged to: EHUMC - Zoology and Animal Cell Biology Department, University of the Basque Country (Spain), MVHN - Museu Valencia d'Historia Natural (Spain), MNCN - Museo Nacional de Ciencias Naturales (Spain), NMC - National Museum Cardiff (UK), NHMC - National History Museum of Crete (Greece), HNHM - Hungarian Natural History Museum (Hungary), and ZMH - Zoological Museum Hamburg (Germany). Superscript asterisks (*) mean that the GenBank accession number was published on other study.

Voucher	Locality	Phylogroup	Candidate species	Proposed species	Shell measures		Mophospecies	Genbank accesion numbers		
					H/D	UD/D		*COI*	*16S rRNA*	*5.8-ITS2-28S*
EHUMC_1128	Asón, Cantabria, Spain	Group 1	G1	*P. pusilla*	NA	NA	NA	KP727147	KP726936	KP727360
EHUMC_1142	Mola de Colldejou, Tarragona, Spain	Group 1	G1	*P. pusilla*	0,65	0,32	*P. umbilicata*	KP727161	KP726950	KP727374
MVHN_070910RU03_1	Rovereto, Italy	Group 1	G1	*P. pusilla*	0,64	0,28	*P. umbilicata*	KP727165	KP726954	KP727378
MVHN_070910RU03_2	Rovereto, Italy	Group 1	G1	*P. pusilla*	0,6	0,3	*P. umbilicata*	KP727166	KP726955	KP727379
MVHN_070910RU09_2	Near Olvena, Huesca, Spain	Group 1	G1	*P. pusilla*	NA	NA	NA	KP727170	KP726959	KP727383
MVHN_070910RU12	Albanyà, Girona, Spain	Group 1	G1	*P. pusilla*	0,7	0,32	*P. umbilicata*	KP727173	KP726962	KP727386
EHUMC_1143	Anguiano, La Rioja, Spain	Group 1	G1	*P. pusilla*	0,65	0,36	*P. umbilicata*	KP727179	KP726968	KP727392
EHUMC_1145	Ponikve, Kvarner, Croatia	Group 1	G1	*P. pusilla*	0,71	0,32	*P. umbilicata*	KP727181	KP726970	KP727394
EHUMC_1146	Mjesto Primislje, Kordun, Croatia	Group 1	G1	*P. pusilla*	0,49	0,29	*P. umbilicata*	KP727182	KP726971	KP727395
EHUMC_1150	Conimbriga, Coímbra, Portugal	Group 1	G1	*P. pusilla*	0,79	0,28	*P. pusilla*	KP727187	KP726976	KP727400
EHUMC_1155	Obidos, Leiria, Portugal	Group 1	G1	*P. pusilla*	0,67	0,31	*P. umbilicata*	KP727192	KP726981	KP727405
EHUMC_1165	Vegacervera, León, Spain	Group 1	G1	*P. pusilla*	0,71	0,27	*P. umbilicata*	KP727202	KP726991	KP727415
EHUMC_1166	Irati, Navarra, Spain	Group 1	G1	*P. pusilla*	0,72	0,32	*P. umbilicata*	KP727203	KP726992	KP727416
EHUMC_1176	Bilecik, Turkey	Group 1	G1	*P. pusilla*	0,67	0,43	*P. umbilicata*	KP727213	KP727002	KP727426
EHUMC_1177	Konya, Turkey	Group 1	G1	*P. pusilla*	0,62	0,31	*P. umbilicata*	KP727214	KP727003	KP727427
MNCN_15.05/49513	Las Majadas, Cuenca, Spain	Group 1	G1	*P. pusilla*	0,65	0,36	*P. umbilicata*	KP727223	KP727012	KP727436
EHUMC_1179	Vandenesse-en-Auxois, Côte-d'Or, France	Group 1	G1	*P. pusilla*	0,7	0,33	*P. umbilicata*	KP727225	KP727014	KP727438
EHUMC_1180	Vandenesse-en-Auxois, Côte-d'Or, France	Group 1	G1	*P. pusilla*	0,74	0,29	*P. umbilicata*	KP727226	KP727015	KP727439
EHUMC_1181	Villefranche-de-Rouergue, Aveyron, France	Group 1	G1	*P. pusilla*	0,73	0,34	*P. umbilicata*	KP727227	KP727016	KP727440
EHUMC_1185	Hontoria del Pinar, Soria, Spain	Group 1	G1	*P. pusilla*	0,61	0,3	*P. umbilicata*	KP727231	KP727020	KP727444
EHUMC_1187	Campania, Salerno, Italy	Group 1	G1	*P. pusilla*	0,74	0,32	*P. umbilicata / P. pusilla*	KP727233	KP727022	KP727446

Voucher	Locality	Phylogroup	Candidate species	Proposed species	Shell measures		Mophospecies	Genbank accesion numbers		
					H/D	UD/D		*COI*	*16S rRNA*	*5.8-ITS2-28S*
EHUMC_1188	Lebeña, Cantabria, Spain	Group 1	G1	*P. pusilla*	0,62	0,34	*P. umbilicata*	KP727234	KP727023	KP727447
EHUMC_1190	Arenas de Cabrales, Asturias, Spain	Group 1	G1	*P. pusilla*	0,62	0,31	*P. umbilicata*	KP727236	KP727025	KP727449
EHUMC_1191	Near Somiedo, Asturias, Spain	Group 1	G1	*P. pusilla*	0,72	0,26	*P. umbilicata / P. pusilla*	KP727237	KP727026	KP727450
EHUMC_1192	Villafeliz de Babia, León, Spain	Group 1	G1	*P. pusilla*	0,62	0,39	*P. umbilicata*	KP727238	KP727027	KP727451
EHUMC_1193	Fuentes Carrionas, Palencia, Spain	Group 1	G1	*P. pusilla*	0,63	0,3	*P. umbilicata*	KP727239	KP727028	KP727452
EHUMC_1194	Bages, Barcelona, Spain	Group 1	G1	*P. pusilla*	0,67	0,32	*P. umbilicata*	KP727240	KP727029	KP727453
EHUMC_1196	Bergadá, Barcelona, Spain	Group 1	G1	*P. pusilla*	0,71	0,29	*P. umbilicata*	KP727242	KP727031	KP727455
EHUMC_1197	Laufen, Switzerland	Group 1	G1	*P. pusilla*	0,62	0,3	*P. umbilicata*	KP727243	KP727032	KP727456
EHUMC_1201	Near Lesachtal, Kärnten, Austria	Group 1	G1	*P. pusilla*	0,62	0,29	*P. umbilicata*	KP727247	KP727036	KP727460
EHUMC_1202	Pieria, Makedonia, Greece	Group 1	G1	*P. pusilla*	0,62	0,32	*P. umbilicata*	KP727248	KP727037	KP727461
EHUMC_1203	Denbighshire, UK	Group 1	G1	*P. pusilla*	0,61	0,35	*P. umbilicata*	KP727249	KP727038	KP727462
EHUMC_1204	Near Llangollen, Anglesey, UK	Group 1	G1	*P. pusilla*	0,6	0,33	*P. umbilicata*	KP727250	KP727039	KP727463
NHMC_50.29715	Tzoumerka mt.,Trikala, Greece	Group 1	G1	*P. pusilla*	0,75	0,34	*P. umbilicata / P. pusilla*	KP727256	KP727045	KP727469
NHMC_50.29312	Falakro mt., Makedonia, Greece	Group 1	G1	*P. pusilla*	0,64	0,35	*P. umbilicata*	KP727268	KP727057	KP727481
NHMC_50.29485	Olympos mt., Makedonia, Greece	Group 1	G1	*P. pusilla*	0,72	0,29	*P. umbilicata / P. pusilla*	KP727269	KP727058	KP727482
NHMC_50.31790	Gkiona mt., Sterea Ellada, Greece	Group 1	G1	*P. pusilla*	0,60	0,34	*P. umbilicata / P. cephalonica*	KP727270	KP727059	KP727483
NHMC_50.32098	Vardousia mt., Sterea Ellada, Greece	Group 1	G1	*P. pusilla*	NA	NA	NA	KP727271	KP727060	KP727484
NHMC_50.35549	Tzoumerka mt., Ioannina - Ipeiros, Greece	Group 1	G1	*P. pusilla*	0,71	0,31	*P. umbilicata / P. pusilla*	KP727279	KP727068	KP727492
NHMC_50.35728	Near Milea, Ioannina - Ipeiros, Greece	Group 1	G1	*P. pusilla*	NA	NA	NA	KP727280	KP727069	KP727493
EHUMC_1205	Mount Penna, Tuscany, Italy	Group 1	G1	*P. pusilla*	0,64	0,31	*P. umbilicata*	KP727282	KP727071	KP727495
EHUMC_1207	Siena, Italy	Group 1	G1	*P. pusilla*	0,68	0,27	*P. umbilicata / P. pusilla*	KP727284	KP727073	KP727497
HNHM_99350	Korab Mts, Diber, Albania	Group 1	G1	*P. pusilla*	0,73	0,30	*P. umbilicata*	KP727290	KP727079	KP727503
HNHM_99346	Kendervice Mts., Albania	Group 1	G1	*P. pusilla*	NA	NA	NA	KP727291	KP727080	KP727504
EHUMC_1208	Vallyvaughan, Clare, Ireland	Group 1	G1	*P. pusilla*	0,66	0,34	*P. umbilicata*	KP727293	KP727082	KP727506
EHUMC_1210	Vysoké Tatry Mts., Slovakia	Group 1	G1	*P. pusilla*	0,69	0,30	*P. umbilicata / P. pusilla*	KP727295	KP727084	KP727508
EHUMC_1211	Belianske Tatry Mts., Slovakia	Group 1	G1	*P. pusilla*	0,60	0,32	*P. umbilicata*	KP727296	KP727085	KP727509
EHUMC_1214	Moravia, Javoricko env., Czech Republic	Group 1	G1	*P. pusilla*	0,62	0,34	*P. umbilicata*	KP727299	KP727088	KP727512
EHUMC_1215	Muránská, Murán env., Slovakia	Group 1	G1	*P. pusilla*	0,63	0,31	*P. umbilicata*	KP727300	KP727089	KP727513
EHUMC_1216	Moravia, Rudice env., Czech Republic	Group 1	G1	*P. pusilla*	0,73	0,33	*P. umbilicata / P. pusilla*	KP727301	KP727090	KP727514

EHUMC_1217	Slovensky kras NP, Silica env., Slovakia	Group 1	G1	*P. pusilla*	0,58	0,28	*P. umbilicata*	KP727302	KP727091	KP727515
EHUMC_1219	Logarska, Slovenia	Group 1	G1	*P. pusilla*	0,72	0,29	*P. umbilicata / P. pusilla*	KP727304	KP727093	KP727517
NMC_1980.15.1	Near Fahamore, Kerry, Ireland	Group 1	G1	*P. pusilla*	0,61	0,35	*P. umbilicata*	KP727313	KP727102	KP727526
EHUMC_1229	Aquila di Arroscia, Liguria, Italy	Group 1	G1	*P. pusilla*	0,67	0,33	*P. umbilicata*	KP727323	KP727112	KP727536
EHUMC_1231	Roccaforte Ligure, Piedmont, Italy	Group 1	G1	*P. pusilla*	0,79	0,25	*P. pusilla*	KP727325	KP727114	KP727538
EHUMC_1232	Crocefieschi, Liguria, Italy	Group 1	G1	*P. pusilla*	0,78	0,27	*P. pusilla*	KP727326	KP727115	KP727539
EHUMC_1234	Stazzema, Tuscany, Italy	Group 1	G1	*P. pusilla*	0,64	0,33	*P. umbilicata*	KP727328	KP727117	KP727541
EHUMC_1235	Sigillo, Umbria, Italy	Group 1	G1	*P. pusilla*	0,65	0,29	*P. umbilicata*	KP727329	KP727118	KP727542
HNHM_98874_1	Gümüshane, Turquía	Group 1	G1	*P. pusilla*	0,63	0,38	*P. cephalonica*	KP727352	KP727141	KP727565
HNHM_98875	Erzurum, Turquía	Group 1	G1	*P. pusilla*	0,63	0,37	*P. umbilicata / P. cephalonica*	KP727354	KP727143	KP727567
EHUMC_1236	Mount Angar-Burun, Crimea, Ukraine	Group 1	G1	*P. pusilla*	0,64	0,29	*P. umbilicata*	KP727355	KP727144	KP727568
EHUMC_1238	Carros, N of Nice, France	Group 1	G1	*P. pusilla*	NA	NA	NA	KP727357	KP727146	KP727570
EHUMC_1129	Encio-Obarenes, Burgos, Spain	Group 2	G2-A	*P. rupestris*	0,95	0,19	*P. rupestris*	KP727148	KP726937	KP727361
EHUMC_1132	Marsella - Toulon, Var, France	Group 2	G2-A	*P. rupestris*	0,99	0,26	*P. rupestris*	KP727151	KP726940	KP727364
EHUMC_1136	East Provenza, France	Group 2	G2-A	*P. rupestris*	0,98	0,2	*P. rupestris*	KP727155	KP726944	KP727368
EHUMC_1137	Arles, Provenza, France	Group 2	G2-A	*P. rupestris*	1,12	0,21	*P. jaenensis*	KP727156	KP726945	KP727369
EHUMC_1138	Arles, Provenza, France	Group 2	G2-A	*P. rupestris*	1,09	0,21	*P. jaenensis*	KP727157	KP726946	KP727370
MVHN_070910RU01	Estany de Montcortés, Lleida, Spain	Group 2	G2-A	*P. rupestris*	0,79	0,27	*P. pusilla*	KP727162	KP726951	KP727375
MVHN_070910RU04	Relleu, Alicante, Spain	Group 2	G2-A	*P. rupestris*	0,97		NA	KP727167	KP726956	KP727380
MVHN_070910RU11	Ricote, Murcia, Spain	Group 2	G2-A	*P. rupestris*	0,92	0,2	*P. rupestris*	KP727171	KP726960	KP727384
MVHN_210610DG05	Portell, Castellón, Spain	Group 2	G2-A	*P. rupestris*	1	0,22	*P. jaenensis*	KP727172	KP726961	KP727385
MVHN_070910RU15	Bellús, Valencia, Spain	Group 2	G2-A	*P. rupestris*	1,02	0,2	*P. jaenensis*	KP727176	KP726965	KP727389
MVHN_070910RU17	Near Benasque, Huesca, Spain	Group 2	G2-A	*P. rupestris*	0,77	0,24	*P. pusilla*	KP727178	KP726967	KP727391
MVHN_231210CS01	Alcoi, Alicante, Spain	Group 2	G2-A	*P. rupestris*	1,04	0,21	*P. jaenensis*	KP727186	KP726975	KP727399
EHUMC_1158	Betpouey, France	Group 2	G2-A	*P. rupestris*	NA	0,2	NA	KP727195	KP726984	KP727408
EHUMC_1159	Riglos, Huesca, Spain	Group 2	G2-A	*P. rupestris*	0,86	0,24	*P. pusilla*	KP727196	KP726985	KP727409
EHUMC_1160	Pals, Girona, Spain	Group 2	G2-A	*P. rupestris*	1,15	0,21	*P. jaenensis*	KP727197	KP726986	KP727410
EHUMC_1162	Valderejo, Álava, Spain	Group 2	G2-A	*P. rupestris*	0,85	0,31	*P. pusilla*	KP727199	KP726988	KP727412
EHUMC_1163	Valderejo, Álava, Spain	Group 2	G2-A	*P. rupestris*	0,89	0,23	*P. pusilla / P.rupestris*	KP727200	KP726989	KP727413
EHUMC_1164	Anento, Zaragoza, Spain	Group 2	G2-A	*P. rupestris*	0,81	0,25	*P. pusilla*	KP727201	KP726990	KP727414
EHUMC_1167	Portilo del Busto, Burgos, Spain	Group 2	G2-A	*P. rupestris*	0,83	0,25	*P. pusilla*	KP727204	KP726993	KP727417
EHUMC_1168	Arnedillo, La Rioja, Spain	Group 2	G2-A	*P. rupestris*	1,01	0,2	*P. jaenensis*	KP727205	KP726994	KP727418
EHUMC_1169	Valdenoceda, Burgos, Spain	Group 2	G2-A	*P. rupestris*	0,73	0,27	*P. umbilicata*	KP727206	KP726995	KP727419
EHUMC_1171	Cassis, France	Group 2	G2-A	*P. rupestris*	1,06	0,17	*P. jaenensis*	KP727208	KP726997	KP727421
MNCN_15.05/50925	Liétor, Albacete, Spain	Group 2	G2-A	*P. rupestris*	1,05	0,23	*P. jaenensis*	KP727218	KP727007	KP727431
MNCN_15.05/49951	Alcalá de Júcar, Albacete, Spain	Group 2	G2-A	*P. rupestris*	1,01	0,21	*P. jaenensis*	KP727219	KP727008	KP727432
MNCN_15.05/51665	Minglanilla, Cuenca, Spain	Group 2	G2-A	*P. rupestris*	1,08	NA	NA	KP727222	KP727011	KP727435
EHUMC_1183	Berdejo, Zaragoza, Spain	Group 2	G2-A	*P. rupestris*	0,85	NA	NA	KP727229	KP727018	KP727442
EHUMC_1189	Lebeña, Cantabria, Spain	Group 2	G2-A	*P. rupestris*	0,82	0,21	*P. pusilla*	KP727235	KP727024	KP727448

Voucher	Locality	Phylogroup	Candidate species	Proposed species	Shell measures		Mophospecies	Genbank accesion numbers		
					H/D	UD/D		*COI*	*16S rRNA*	*5.8-ITS2-28S*
EHUMC_1195	Bages, Barcelona, Spain	Group 2	G2-A	*P. rupestris*	0,89	0,31	*P. pusilla*	KP727241	KP727030	KP727454
EHUMC_1199	Tiscar, Granada, Spain	Group 2	G2-A	*P. rupestris*	0,83	0,21	*P. pusilla*	KP727245	KP727034	KP727458
EHUMC_1222	Duilhac-sous-Peyrepertuse, Aude, France	Group 2	G2-A	*P. rupestris*	0,74	0,24	*P. pusilla*	KP727316	KP727105	KP727529
EHUMC_1223	La Preste, France	Group 2	G2-A	*P. rupestris*	0,72	0,24	*P. pusilla*	KP727317	KP727106	KP727530
EHUMC_1224	Gombreny-Montgrony, Girona, Spain	Group 2	G2-A	*P. rupestris*	0,95	0,26	*P. rupestris*	KP727318	KP727107	KP727531
EHUMC_1225	Lillet-Castellar d'Nug, Barcelona, Spain	Group 2	G2-A	*P. rupestris*	0,87	0,28	*P. pusilla*	KP727319	KP727108	KP727532
EHUMC_1228	Portell de Cosp, Tarragona, Spain	Group 2	G2-A	*P. rupestris*	0,88	0,24	*P. pusilla / P.rupestris*	KP727322	KP727111	KP727535
MVHN_070910RU02_1	Malcesine, Verona, Italia	Group 2	G2-B	*P. saxatilis*	0,77	0,3	*P. pusilla*	KP727163	KP726952	KP727376
MVHN_070910RU16	Passo di campolongo, Italy	Group 2	G2-B	*P. saxatilis*	0,72	0,27	*P. pusilla*	KP727177	KP726966	KP727390
EHUMC_1144	Johnsbach, Austria	Group 2	G2-B	*P. saxatilis*	0,71	0,26	*P. pusilla*	KP727180	KP726969	KP727393
EHUMC_1170	The Alps, France	Group 2	G2-B	*P. saxatilis*	0,69	0,32	*P. umbilicata / P. pusilla*	KP727207	KP726996	KP727420
EHUMC_1198	Gobbera, Italy	Group 2	G2-B	*P. saxatilis*	0,65	0,27	*P. umbilicata*	KP727244	KP727033	KP727457
EHUMC_1209	Veľká Fatra Mts., Slovakia	Group 2	G2-B	*P. saxatilis*	0,68	0,32	*P. umbilicata / P. pusilla*	KP727294	KP727083	KP727507
EHUMC_1213	San Marino castle, San Marino	Group 2	G2-B	*P. saxatilis*	0,79	0,27	*P. pusilla*	KP727298	KP727087	KP727511
EHUMC_1218	Logarska, Slovenia	Group 2	G2-B	*P. saxatilis*	0,72	0,23	*P. umbilicata / P. pusilla*	KP727303	KP727092	KP727516
NMC_2001.054.0054	Near Stuben, Tirol, Austria	Group 2	G2-B	*P. saxatilis*	0,63	0,34	*P. umbilicata*	KP727310	KP727099	KP727523
NMC_2001.054.0030	Bludenz, Austria	Group 2	G2-B	*P. saxatilis*	0,67	0,28	*P. umbilicata*	KP727311	KP727100	KP727524
EHUMC_1220	Eschenlohe-Oberau, Bayern, Germany	Group 2	G2-B	*P. saxatilis*	0,77	0,26	*P. pusilla*	KP727314	KP727103	KP727527
EHUMC_1149	Artvin, Turkey	Group 3	G3	*P. cf. hierosolymitana*	0,89	0,25	*P. pusilla / P.rupestris*	KP727185	KP726974	KP727398
EHUMC_1172	Isparta, Uluborlu, Turkey	Group 3	G3	*P. cf. hierosolymitana*	0,95	0,19	*P. rupestris*	KP727209	KP726998	KP727422
EHUMC_1173	Artvin, Kinaliçam, Turkey	Group 3	G3	*P. cf. hierosolymitana*	0,7	0,32	*P. umbilicata*	KP727210	KP726999	KP727423
EHUMC_1174	Torul, Turkey	Group 3	G3	*P. cf. hierosolymitana*	0,83	0,25	*P. pusilla*	KP727211	KP727000	KP727424
HNHM_98874_2	Gümüshane, Turkey	Group 3	G3	*P. cf. hierosolymitana*	0,79	0,29	*P. pusilla*	KP727353	KP727142	KP727566
NHMC_50.31818_1	Gkiona mt., Sterea Ellada, Greece	Group 4	G4	*Pyramidula* sp1	1,02	NA	NA	KP727272	KP727061	KP727485
NHMC_50.31818_2	Gkiona mt., Sterea Ellada, Greece	Group 4	G4	*Pyramidula* sp1	0,84	NA	NA	KP727273	KP727062	KP727486
NHMC_50.23326_1	Petratis gorge, Cyprus	Group 4	G4	*Pyramidula* sp1	0,84	NA	NA	KP727277	KP727066	KP727490
NHMC_50.23326_2	Petratis gorge, Cyprus	Group 4	G4	*Pyramidula* sp1	0,89	NA	NA	KP727278	KP727067	KP727491
HNHM_98855_2	Epirus, Ioannina, Grecia	Group 4	G4	*Pyramidula* sp1	0,90	NA	NA	KP727339	KP727128	KP727552
EHUMC_1130	Alaior, Menorca, Spain	Group 5	G5-A	*P. jaenensis*	0,91	0,25	*P. rupestris*	KP727149	KP726938	KP727362
EHUMC_1131	Alaior, Menorca, Spain	Group 5	G5-A	*P. jaenensis*	0,93	0,27	*P. rupestris*	KP727150	KP726939	KP727363
EHUMC_1135	East Provenza, France	Group 5	G5-A	*P. jaenensis*	0,79	0,25	*P. pusilla*	KP727154	KP726943	KP727367
EHUMC_1139	Arles, Provenza, France	Group 5	G5-A	*P. jaenensis*	0,92	0,22	*P. rupestris*	KP727158	KP726947	KP727371
EHUMC_1178	Oliena, Sardinia, Italy	Group 5	G5-A	*P. jaenensis*	0,9	0,26	*P. rupestris*	KP727224	KP727013	KP727437

NMC_1985.199.2	Near Vranja, Croatia	Group 5	G5-A	*P. jaenensis*	0,86	0,23	*P. pusilla*	KP727252	KP727041	KP727465
EHUMC_1206	Gualdo Tadino, Umbria, Italy	Group 5	G5-A	*P. jaenensis*	0,86	0,24	*P. pusilla*	KP727283	KP727072	KP727496
EHUMC_1212	Lunzer Mitter - Obersee, Austria	Group 5	G5-A	*P. jaenensis*	0,80	0,31	*P. pusilla*	KP727297	KP727086	KP727510
EHUMC_1221	Vers-Pont-du-Gard, Gard, France	Group 5	G5-A	*P. jaenensis*	0,83	NA	NA	KP727315	KP727104	KP727528
EHUMC_1227	Codolar, Tarragona, Spain	Group 5	G5-A	*P. jaenensis*	0,88	0,25	*P. pusilla / P.rupestris*	KP727321	KP727110	KP727534
EHUMC_1230	Aquila di Arroscia, Liguria, Italy	Group 5	G5-A	*P. jaenensis*	0,85	0,25	*P. pusilla*	KP727324	KP727113	KP727537
EHUMC_1233	Portovenere, Liguria, Italy	Group 5	G5-A	*P. jaenensis*	0,86	0,26	*P. pusilla*	KP727327	KP727116	KP727540
EHUMC_1237	Madonie mountains, Sicily, Italy	Group 5	G5-A	*P. jaenensis*	NA	NA	NA	KP727356	KP727145	KP727569
EHUMC_1140	Valle de Abdalajis, Málaga, Spain	Group 5	G5-B	*P. jaenensis*	1,18	0,19	*P. jaenensis*	KP727159	KP726948	KP727372
EHUMC_1141	Torcal de Antequera, Málaga, Spain	Group 5	G5-B	*P. jaenensis*	1,05	0,27	*P. jaenensis*	KP727160	KP726949	KP727373
MVHN_070910RU05	Ciudad encantada, Cuenca, Spain	Group 5	G5-B	*P. jaenensis*	0,91	0,22	*P. rupestris*	KP727168	KP726957	KP727381
MVHN_070910RU13	Venta del Moro, Valencia, Spain	Group 5	G5-B	*P. jaenensis*	1,07	0,22	*P. jaenensis*	KP727174	KP726963	KP727387
MVHN_060610IJ01	Cazorla, Jaén, Spain	Group 5	G5-B	*P. jaenensis*	1,01	0,24	*P. jaenensis*	KP727175	KP726964	KP727388
EHUMC_1156	Torrecella en Cameros, La Rioja, Spain	Group 5	G5-B	*P. jaenensis*	1	0,24	*P. jaenensis*	KP727193	KP726982	KP727406
EHUMC_1157	Fuentetoba, Soria, Spain	Group 5	G5-B	*P. jaenensis*	0,94	0,25	*P. rupestris*	KP727194	KP726983	KP727407
EHUMC_1161	Alhama, Granada, Spain	Group 5	G5-B	*P. jaenensis*	1,37	0,16	*P. jaenensis*	KP727198	KP726987	KP727411
MNCN_15.05/52048	Peñalén, Guadalajara, Spain	Group 5	G5-B	*P. jaenensis*	0,99	0,22	*P. rupestris*	KP727215	KP727004	KP727428
MNCN_15.05/50730	Zaorejas, Guadalajara, Spain	Group 5	G5-B	*P. jaenensis*	0,73	0,27	*P. umbilicata / P. pusilla*	KP727216	KP727005	KP727429
MNCN_15.05/52013	Valtablado, Guadalajara, Spain	Group 5	G5-B	*P. jaenensis*	0,88	0,31	*P. rupestris*	KP727217	KP727006	KP727430
MNCN_15.05/49858	Cueva de los Chorros, Albacete, Spain	Group 5	G5-B	*P. jaenensis*	0,82	0,26	*P. pusilla*	KP727220	KP727009	KP727433
MNCN_15.05/51720	Cuenca, Cuenca, Spain	Group 5	G5-B	*P. jaenensis*	1,01	0,23	*P. jaenensis*	KP727221	KP727010	KP727434
EHUMC_1184	Sto Domingo de Silos, Burgos, Spain	Group 5	G5-B	*P. jaenensis*	0,79	0,3	*P. pusilla*	KP727230	KP727019	KP727443
NMC_1984.392.1	Near Souk Tleta Taghramet, Morocco	Group 5	G5-B	*P. jaenensis*	0,83	0,25	*P. pusilla*	KP727253	KP727042	KP727466
MVHN_120911LB03	Jabalcón, Granada, Spain	Group 5	G5-B	*P. jaenensis*	1,22	0,23	*P. jaenensis*	KP727285	KP727074	KP727498
MVHN_1917	Sierra de Alcaraz, Albacete, Spain	Group 5	G5-B	*P. jaenensis*	0,87		NA	KP727292	KP727081	KP727505
NMC_1984.394.1	Near Souk Tleta Taghramet, Morocco	Group 5	G5-B	*P. jaenensis*	0,79	0,28	*P. pusilla*	KP727307	KP727096	KP727520
NMC_1984.384.1	Near Sefliane, Morocco	Group 5	G5-B	*P. jaenensis*	0,86	0,26	*P. pusilla*	KP727308	KP727097	KP727521
EHUMC_1226	Irurtzun, Navarra, Spain	Group 5	G5-B	*P. jaenensis*	0,90	0,29	*P. pusilla*	KP727320	KP727109	KP727533
EHUMC_1133	Marsella - Toulon, Var, France	Group 5	G5-C	*P. jaenensis*	0,76	0,31	*P. pusilla*	KP727152	KP726941	KP727365
EHUMC_1134	Marsella - Toulon, Var, France	Group 5	G5-C	*P. jaenensis*	0,85	0,27	*P. pusilla*	KP727153	KP726942	KP727366
MVHN_070910RU02_2	Malcesine, Verona, Italia	Group 5	G5-C	*P. jaenensis*	NA	NA	NA	KP727164	KP726953	KP727377
MVHN_070910RU09_1	Near Olvena, Huesca, Spain	Group 5	G5-C	*P. jaenensis*	0,74	0,28	*P. pusilla*	KP727169	KP726958	KP727382
EHUMC_1151	Conimbriga, Coímbra, Portugal	Group 5	G5-C	*P. jaenensis*	0,85	0,21	*P. pusilla*	KP727188	KP726977	KP727401
EHUMC_1152	Conimbriga, Coímbra, Portugal	Group 5	G5-C	*P. jaenensis*	1,07	0,18	*P. jaenensis*	KP727189	KP726978	KP727402
EHUMC_1153	Conimbriga, Coímbra, Portugal	Group 5	G5-C	*P. jaenensis*	1,04	0,23	*P. jaenensis*	KP727190	KP726979	KP727403
EHUMC_1154	Arrabida, Setubal, Portugal	Group 5	G5-C	*P. jaenensis*	0,95	0,2	*P. rupestris*	KP727191	KP726980	KP727404
EHUMC_1182	Olvena, Huesca, Spain	Group 5	G5-C	*P. jaenensis*	0,82	NA	NA	KP727228	KP727017	KP727441

Voucher	Locality	Phylogroup	Candidate species	Proposed species	Shell measures		Mophospecies	Genbank accesion numbers		
					H/D	UD/D		*COI*	*16S rRNA*	*5.8-ITS2-28S*
EHUMC_1186	Berlanga de Duero, Soria, Spain	Group 5	G5-C	*P. jaenensis*	0,9	0,32	*P. pusilla* / *P.rupestris*	KP727232	KP727021	KP727445
EHUMC_1200	Toscolano, Brescia, Italy	Group 5	G5-C	*P. jaenensis*	0,7	0,24	*P. pusilla*	KP727246	KP727035	KP727459
NMC_1986.306.32	Near Sidi-Kacem, Morocco	Group 5	G5-C	*P. jaenensis*	0,82	0,28	*P. pusilla*	KP727254	KP727043	KP727467
NMC_1986.327.01	Near Tounfite, Morocco	Group 5	G5-C	*P. jaenensis*	0,71	0,32	*P. umbilicata* / *P. pusilla*	KP727305	KP727094	KP727518
NMC_1984.249.1	Near Arbaoun, Algeria	Group 5	G5-C	*P. jaenensis*	0,88	0,27	*P. pusilla*	KP727306	KP727095	KP727519
NMC_1992.080.299	Djebel Zaghouan, Tunisia	Group 5	G5-C	*P. jaenensis*	0,81	0,31	*P. pusilla*	KP727309	KP727098	KP727522
NHMC_50.23749	Astypalaia island, Greece	Group 6	G6-A	*P. chorismenostoma*	0,86	0,29	*P. chorismenostoma*	KP727255	KP727044	KP727468
NHMC_50.27302	Chios island, Greece	Group 6	G6-A	*P. chorismenostoma*	1,00	0,26	*P. chorismenostoma*	KP727257	KP727046	KP727470
NHMC_50.25701	Mikro Kouneli islet, Greece	Group 6	G6-A	*P. chorismenostoma*	0,86	0,30	*P. chorismenostoma*	KP727258	KP727047	KP727471
NHMC_50.32095	Folegandros island, Greece	Group 6	G6-A	*P. chorismenostoma*	0,91	0,27	*P. chorismenostoma*	KP727259	KP727048	KP727472
NHMC_50.26702	Pserimos islet, Greece	Group 6	G6-A	*P. chorismenostoma*	1,05	0,27	*P. chorismenostoma*	KP727260	KP727049	KP727473
NHMC_50.26697	Telendos islet, Greece	Group 6	G6-A	*P. chorismenostoma*	0,90	0,24	*P. chorismenostoma*	KP727261	KP727050	KP727474
NHMC_50.26187_1	Astypalaia island, Grrece	Group 6	G6-A	*P. chorismenostoma*	0,85	0,29	*P. chorismenostoma*	KP727262	KP727051	KP727475
NHMC_50.26187_2	Astypalaia island, Grrece	Group 6	G6-A	*P. chorismenostoma*	0,96	0,26	*P. chorismenostoma*	KP727263	KP727052	KP727476
NHMC_50.34163	Crete island, Greece	Group 6	G6-A	*P. chorismenostoma*	0,90	0,30	*P. chorismenostoma*	KP727274	KP727063	KP727487
NHMC_50.22417_2	Near Kalyvia, Ioannina, Greece	Group 6	G6-B	*P. cephalonica?*	0,88	0,31	*P. pusilla*	KP727265	KP727054	KP727478
HNHM_99351	Rumija Mts, Stari Bar, Montenegro	Group 6	G6-B	*P. cephalonica?*	0,78	0,29	*P. pusilla*	KP727286	KP727075	KP727499
HNHM_99349	Rumija Mts. Near Stari Bar, Montenegro	Group 6	G6-B	*P. cephalonica?*	0,80	0,26	*P. pusilla*	KP727288	KP727077	KP727501
HNHM_99347	Lesitse Mts, Evros, Greece	Group 6	G6-B	*P. cephalonica?*	0,82	0,31	*P. pusilla*	KP727289	KP727078	KP727502
HNHM_98860	Epirus, Ioannina, Grecia	Group 6	G6-B	*P. cephalonica?*	0,76	0,28	*P. pusilla*	KP727344	KP727133	KP727557
HNHM_98861	Epirus, Ioannina, Grecia	Group 6	G6-B	*P. cephalonica?*	0,82	0,29	*P. pusilla*	KP727345	KP727134	KP727558
ZMH_86507/999_1	Imereti, Kutaisi, Georgia	Group 7	G7	*Pyramidula* sp2	0,72	0,29	*P. umbilicata* / *P. pusilla*	KP727330	KP727119	KP727543
ZMH_86507/999 _2	Imereti, Kutaisi, Georgia	Group 7	G7	*Pyramidula* sp2			NA	KP727331	KP727120	KP727544
ZMH_100219/5_1	Krasnodar, Mostovsky, Russia	Group 7	G7	*Pyramidula* sp2	0,66	0,30	*P. umbilicata*	KP727332	KP727121	KP727545
HNHM_98871	Trabzon, Turkey	Group 7	G7	*Pyramidula* sp2	0,73	0,33	*P. umbilicata* / *P. pusilla*	KP727349	KP727138	KP727562
HNHM_98872	Trabzon, Turkey	Group 7	G7	*Pyramidula* sp2	0,70	0,31	*P. umbilicata* / *P. pusilla*	KP727350	KP727139	KP727563
HNHM_98873	Gümüshane, Turkey	Group 7	G7	*Pyramidula* sp2	0,71	0,30	*P. umbilicata* / *P. pusilla*	KP727351	KP727140	KP727564
EHUMC_1147	Grohovo, Kvaner, Croatia	Group 8	G8	*P. cephalonica*	0,77	0,33	*P. pusilla*	KP727183	KP726972	KP727396
EHUMC_1148	Grohovo, Kvaner, Croatia	Group 8	G8	*P. cephalonica*	0,73	0,34	*P. pusilla*	KP727184	KP726973	KP727397
NMC_1985.250.1	Near Zlatica, Montenegro	Group 8	G8	*P. cephalonica*	0,77	0,31	*P. pusilla* / *P. cephalonica*	KP727251	KP727040	KP727464
NHMC_50.22417_1	Near Kalyvia, Ioannina, Greece	Group 8	G8	*P. cephalonica*	0,77	0,29	*P. pusilla*	KP727264	KP727053	KP727477
NHMC_50.32106_1	Samos island, Greece	Group 8	G8	*P. cephalonica*	NA	NA	NA	KP727266	KP727055	KP727479

NHMC_50.32106_2	Samos island, Greece	Group 8	G8	*P. cephalonica*	0,85	NA	NA	KP727267	KP727056	KP727480
HNHM_99348	Lovcen Mts, near Kotor, Montenegro	Group 8	G8	*P. cephalonica*	0,77	0,32	*P. pusilla*	KP727287	KP727076	KP727500
NMC_1985.219.2	Near Zvecanje, Hrvatska, Croatia	Group 8	G8	*P. cephalonica*	0,81	0,31	*P. pusilla*	KP727312	KP727101	KP727525
HNHM_98850	Near Spileo, Grevena, Grecia	Group 8	G8	*P. cephalonica*	0,70	0,28	*P. umbilicata*	KP727334	KP727123	KP727547
HNHM_98853_1	Thessaly, Trikala, Grecia	Group 8	G8	*P. cephalonica*	0,77	0,26	*P. pusilla*	KP727335	KP727124	KP727548
HNHM_98853_2	Thessaly, Trikala, Grecia	Group 8	G8	*P. cephalonica*	0,83	0,29	*P. pusilla*	KP727336	KP727125	KP727549
HNHM_98854	Epirus, Ioannina, Grecia	Group 8	G8	*P. cephalonica*	0,75	0,27	*P. pusilla*	KP727337	KP727126	KP727550
HNHM_98855_1	Epirus, Ioannina, Grecia	Group 8	G8	*P. cephalonica*	0,85	0,28	*P. pusilla*	KP727338	KP727127	KP727551
HNHM_98857	Epirus, Ioannina, Grecia	Group 8	G8	*P. cephalonica*	0,72	0,29	*P. umbilicata* / *P. pusilla*	KP727340	KP727129	KP727553
HNHM_98858	Epirus, Ioannina, Grecia	Group 8	G8	*P. cephalonica*	0,84	0,29	*P. pusilla*	KP727341	KP727130	KP727554
HNHM_98859_1	Epirus, Ioannina, Grecia	Group 8	G8	*P. cephalonica*	0,83	0,32	*P. pusilla*	KP727342	KP727131	KP727555
HNHM_98859_2	Epirus, Ioannina, Grecia	Group 8	G8	*P. cephalonica*	0,79	0,31	*P. pusilla*	KP727343	KP727132	KP727556
HNHM_98863	Epirus, Ioannina, Grecia	Group 8	G8	*P. cephalonica*	0,85	0,27	*P. pusilla*	KP727346	KP727135	KP727559
HNHM_98866	Pogradec, Lin, Albania	Group 8	G8	*P. cephalonica*	0,70	0,34	*P. umbilicata*	KP727347	KP727136	KP727560
HNHM_98868	Pukë, Mertur, Albania	Group 8	G8	*P. cephalonica*	0,82	0,28	*P. pusilla*	KP727348	KP727137	KP727561
EHUMC_1175	Amasaya, Turkey			*Pyramidula* sp?	0,68	0,27	NA	KP727212	KP727001	KP727425
NHMC_50.30001_1	Vouni Panagias, Cyprus			*Pyramidula* sp?	0,87	0,30	NA	KP727275	KP727064	KP727488
NHMC_50.30001_2	Vouni Panagias, Cyprus			*Pyramidula* sp?	0,77	0,33	NA	KP727276	KP727065	KP727489
NHMC_50.26377	Samaan mountains near Mushabak, Syria			*Pyramidula* sp?	0,72	0,31	NA	KP727281	KP727070	KP727494
ZMH_100219/5_2	Krasnodar, Mostovsky, Russia			*Pyramidula* sp?	0,65	0,24	NA	KP727333	KP727122	KP727546
		Outgroup – *Chondrina avenacea*						FJ171544*	DQ305071*	AY014032*
MVHN_2159	Stockholm, Sweden	Outgroup – *Arianta arbustorum*						KP727358	KJ458486*	KJ458584*
EHUMC_1003	Bakio, Bizkaia, Spain	Outgroup – *Cochlicella acuta*						KP727359	KJ458503*	KJ458599*

Supplementary material 3: Primers used for amplification and sequencing.

Gene	Primer	Sequence	Reference
COI	LCO1490 (5')	5' GGTCAACAAATCATAAAGATATTGG 3'	Folmer et al. (1994)
	HC02198 (3')	5' TAAACTTCAGGGTGACCAAAAAATCA 3'	Folmer et al. (1994)
16S rRNA	16sar (5')	5' CGCCTGTTTATCAAAAACAT 3'	Palumbi et al. (1991)
	16sbr (3')	5' CCGGTCTGAACTCAGATCACGT 3'	Palumbi et al. (1991)
5.8S-ITS2	LSU-1 (5')	5' CTAGCTGCGAGAATTAATGTGA 3'	Wade et al. (2006)
	LSU-3 (3')	5' ACTTTCCCTCACGGTACTTG 3'	Wade et al. (2006)
28S	LSU-2mod (5')	5' TCTCAGGAGTCGGGTTGTTT 3'	Razkin et al. (2015)
	LSU-5 (3')	5' GTTAGACTCCTTGGTCCGTG 3'	Wade et al. (2006)

Supplementary material 4: Results of Xia's test based on simulations with 1,000 replicates and 32 OTUs. Iss = Index of substitution saturation; Iss.cSym and Iss.cAsym = critical substitution saturation index for a symmetrical or asymmetrical real tree, respectively. Significant substitution saturation exists in the data set when Iss > Iss.c.

	Iss	Iss.cSym	P	Iss.cAsym	P
COI 1	0.034	0.689	0	0.373	0
COI 2	0.004	0.689	0	0.373	0
COI 3	0.301	0.694	0	0.383	0.0064
16S rRNA	0.045	0.682	0	0.354	0
5.8S-ITS2	0.026	0.687	0	0.357	0
28S	0.002	0.72	0	0.394	0

Supplementary material 5: Lengths of the fragments sequenced (maximum and minimum) before and after alignment, number of polymorphic sites and evolutionary model selected using the Akaike information criteria implemented in jModeltest for the different partitions of the ingroup.

Gene	Minimum lenght	Maximum lenght	Aligned length	Polymorphic sites	Best model	Pinvar	Gamma
COI	621	621	621	207	TVM+I+G	0.5790	0.9900
16S rRNA	325	338	363	85	TIM1+G	-	0.2650
5.8S-ITS2	561	600	668	57	GTR+G	-	0.4780
28S	831	840	841	12	TrN+I	0.7610	-
Total	2348	2382	2493	361	TVM+I+G	0.5360	0.3910

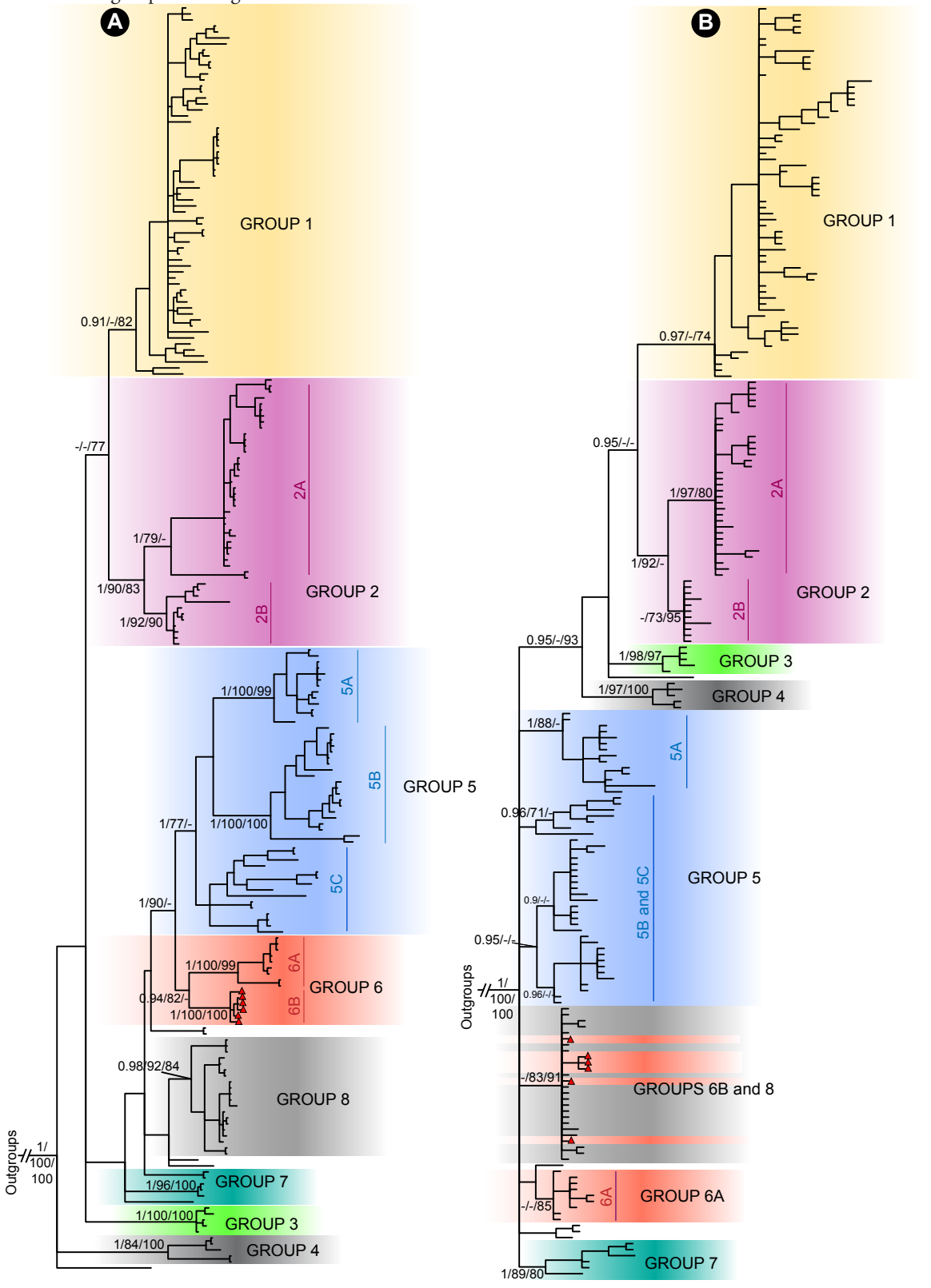
Supplementary material 6: Alignment of the concatenated data set with all analyzed genes (*COI – 16S rRNA – 5.8S (partial) – ITS2 – 28S*), outgroups included.

The file is available at: https://www.dropbox.com/s/jmhxdlzh3thr4ck/SUP_6_Alignment.nxs?dl=0

Supplementary material 7:

A. Phylogenetic tree of *Pyramidula* based on Bayesian inference (BI) analysis of the concatenated data set including mitochondrial *COI* and *16S rRNA* sequences. Numbers correspond to BI posterior probabilities, and ML and MP bootstrap values, respectively. The tree was coloured to distinguish eight major supported clades and the internal clades of some groups as in Figure 2.

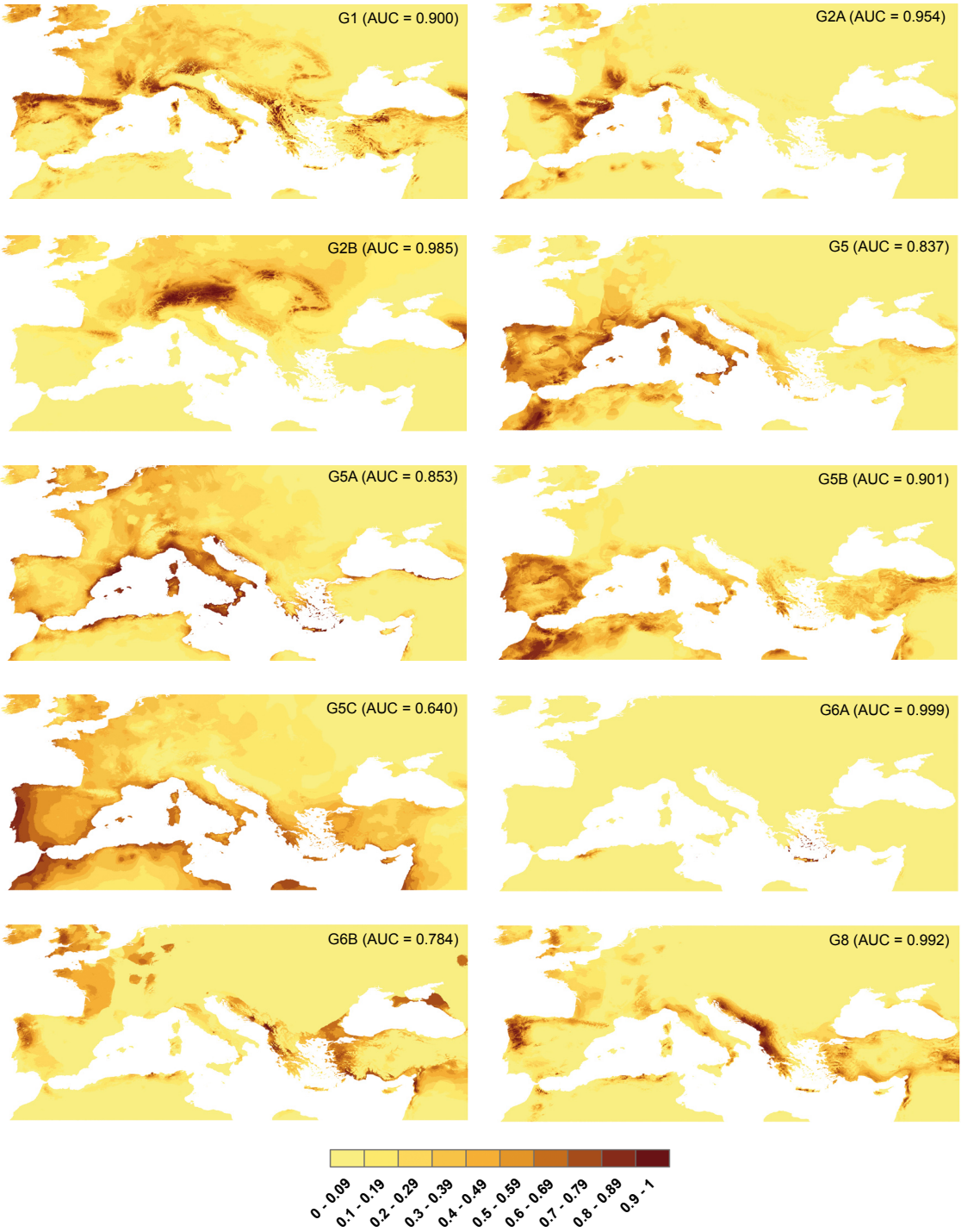
B. Phylogenetic tree of *Pyramidula* based on Bayesian inference (BI) analysis of the concatenated data set including nuclear *5.8S, ITS2* and *28S* sequences. Numbers correspond to BI posterior probabilities, and ML and MP bootstrap values, respectively. The tree was coloured to distinguish eight major supported clades and the internal clades of some groups as in Figure 2.



Supplementary material 8: SpedeSTEM results. k = number of nodes; AIC = Akaike information criterion; Δi = Akaike differences; modelLik = model likelihood; ωi = model probabilities.

Species tree	k	ln	AIC	Δi	modelLik	ωi
(G4,G3G5BG5CG8G6BG6AG5AG7G2AG2BG1)	1	-1507.294	3016.587	694.082	0.000	0.000
(G4,(G3G5BG5CG8G6BG6AG5AG7,G2AG2BG1))	2	-1282.427	2568.855	246.349	0.000	0.000
(G4,(G3G5BG5CG8G6BG6AG5AG7,(G2AG2B,G1)))	3	-1259.238	2524.475	201.970	0.000	0.000
(G4,((G3,G5BG5CG8G6BG6AG5AG7),(G2AG2B,G1)))	4	-1235.566	2479.133	156.627	0.000	0.000
(G4,((G3,(G5BG5CG8G6BG6AG5A,G7)),(G2AG2B,G1)))	5	-1211.932	2433.863	111.358	0.000	0.000
(G4,((G3,(G7,(G5BG5CG8G6BG6A,G5A))),(G2AG2B,G1)))	6	-1187.021	2386.042	63.536	0.000	0.000
(G4,((G3,(G7,(G5A,(G5BG5C,G8G6BG6A)))),(G2AG2B,G1)))	7	-1162.026	2338.052	15.547	0.000	0.000
(G4,((G3,(G7,(G5A,(G5BG5C,(G8G6B,G6A))))),(G2AG2B,G1)))	8	-1156.917	2329.834	7.328	0.026	0.015
(G4,((G1,(G2A,G2B)),(G3,(G7,(G5A,(G5BG5C,(G8G6B,G6A)))))))	**9**	**-1152.253**	**2322.506**	**0.000**	**1.000**	**0.585**
(G4,((G1,(G2A,G2B)),(G3,(G7,(G5A,((G5B,G5C),(G8G6B,G6A)))))))	10	-1151.972	2323.944	1.438	0.487	0.285
(G4,((G3,(G7,(G5A,((G5B,G5C),(G6A,(G8,G6B)))))),(G1,(G2A,G2B))))	11	-1151.877	2325.754	3.248	0.197	0.115

Supplementary material 9: Habitat suitability maps created by Maxent for each candidate species. The AUC value for each model is displayed in the corresponding map. Colour modifications were made using ArcGIS software.



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PAPER III

Species limits, interspecific hybridization and phylogeny in the cryptic land snail complex *Pyramidula*: the power of RADseq data

Oihana Razkin, Gontran Sonet, Karin Breugelmans, María J. Madeira, Benjamín J. Gómez-Moliner and Thierry Backeljau

Molecular phylogenetics and evolution (under review)

Abstract

Restriction site-associated DNA sequencing (RADseq) was used to jointly assess phylogenetic relationships, interspecific hybridization and species delimitation in the cryptic, non-model land snail complex *Pyramidula*. A robust phylogeny was inferred using a matrix of concatenated sequences of almost 1,500,000 bp long, containing > 97,000 polymorphic sites. Maximum likelihood analyses fully resolved the phylogenetic relationships among species and drastically improved phylogenetic trees obtained from mtDNA and nDNA gene trees (COI, 16S rRNA, 5.8S rRNA, ITS2 and 28S rRNA sequence data). The best species delimitation scenario was selected on the basis of 875 unlinked single nucleotide polymorphisms, showing that nine *Pyramidula* species should be distinguished in Europe. Applying D-statistics provided no or weak evidence of interspecific hybridization among *Pyramidula,* except for some evidence of gene flow between two species.

Keywords

Restriction site-associated DNA sequencing, phylogeny, interspecific hybridization, species delimitation, *Pyramidula*

1. Introduction

The inference of phylogenetic relationships among closely related, recently diverged, non-model species is a challenging problem (Maddison and Knowles, 2006), because either there are no good phylogenetic markers available or those that are available contain insufficient phylogenetic signal. Moreover, different gene trees of closely related taxa may show conflicting topologies due to interspecific gene flow or incomplete lineage sorting (Maddison, 1997; Wendel and Doyle, 1998; Degnan and Rosenberg, 2009). The inference of species trees from multilocus data can help to distinguish these two processes (Kubatko, 2009; Yu et al., 2011). Unfortunately, obtaining multiple informative markers for non-model species is not a straightforward task (Schlötterer, 2004; Thomson et al., 2008).

Current high-throughput sequencing technologies allow gathering large scale genome-wide data at moderate to low costs. As such, they are increasingly applied to address phylogenetic problems (Emerson et al., 2010; Eaton and Ree, 2013; Wagner et al., 2013; Hipp et al., 2014; Takahashi et al., 2014). This is particularly true for Restriction-site associated DNA sequencing (RADseq; Baird et al., 2008; Davey and Blaxter, 2010), a technique that can be applied to non-model organisms for which there is no reference genome data available. RADseq can identify thousands of genetic markers across the genome in many individuals and it reduces the representation of the genome by sampling only at specific sites defined by restriction enzymes (Davey and Blaxter, 2010).

RADseq allows (1) creating phylogenetic datasets of unprecedented size (Eaton and Ree, 2013; Eaton, 2014; Escudero et al., 2014; Hipp et al., 2014; Takahashi et al., 2014), (2) genotyping thousands of SNP throughout the genome (Baird et al., 2008), (3) detecting hybridization and introgression among non-model organisms (Twyford and Ennos, 2011; Eaton and Ree, 2013), and (4) applying new methods for inferring species trees and species delimitation (Leaché et al., 2014). Therefore, RADseq may be a promising tool to assess species limits and phylogenetic relationships in closely related taxa for which traditional DNA sequence approaches have failed to provide well-supported solutions.

The land snail genus *Pyramidula* Fitzinger, 1833 is a challenging case for the application of RADseq. It is a non-model species complex distributed over almost all of Europe, the Mediterranean area, Central Asia and Japan (Gómez-Moliner, 1988; Welter-Schultes, 2012). It comprises small species (diameter < 3 mm) with widely umbilicated, trochoid shells (Gittenberger and Bank, 1996). *Pyramidula* spp. inhabit limestone rocks (Gittenberger and Bank, 1996; Kerney, 1999; Martínez-Ortí et al., 2007) from sea level to altitudes of 3800 m (Schileyko and Balashov, 2012). During most of the 20th century, the genus was considered to contain one single species, *Pyramidula rupestris* (Draparnaud, 1801), until Gittenberger and Bank (1996) suggested that in Europe at least six species should be distinguished on conchological basis. However, the diagnostic shell characters highly relied on size and shape features, i.e. phenotypic features that may be plastic and hence that may be affected by environmental factors (Goodfriend, 1986; Heller, 1987; Harley et al., 2009; Stankowski, 2011).

In order to resolve the taxonomy of the *Pyramidula* complex, Razkin et al. (under review) applied an integrative taxonomic and species delimitation approach to analyse 211 specimens of *Pyramidula* collected throughout Europe and adjacent Mediterranean areas. Phylogenetic relationships and species boundaries were inferred on the basis of a multilocus DNA sequence dataset and niche modelling. In this way, nine putative *Pyramidula* species were distinguished in the western Palaearctic region. However, the phylogenies based on mitochondrial (mtDNA) and nuclear (nDNA) markers showed several unsupported branches and incongruent topologies. This latter observation was tentatively interpreted as the result of incomplete lineage sorting. Hence, further work involving more molecular markers was needed to assess whether, and to what extent, processes like interspecific gene flow or incomplete lineage sorting have shaped the phylogenetic relationships among *Pyramidula* species.

The present study addresses the problematic issues in the study of Razkin et al. (under review) by applying RADseq technology in order to 1) reconstruct the phylogenetic relationships within *Pyramidula*, 2) re-assess the delimitation of nine species, and 3) test whether interspecific gene flow or incomplete lineage sorting are responsible for the discordant mtDNA and nDNA gene trees of *Pyramidula*.

2. Materials and methods

2.1. Taxon sampling

We selected 25 individuals of *Pyramidula* from the study of Razkin et al. (under review) representing the nine putative species: *P. pusilla* (n = 4), *P. rupestris* (n = 3), *P. jaenensis* (n = 4), *P. chorismenostoma* (n = 1), *P. cephalonica* (n = 4), *P. saxatilis* (n = 2), *P.* cf. *hierosolymitana* (n = 2), *Pyramidula* sp1 (n = 1) and *Pyramidula* sp2 (n = 2). Two remaining individuals were conchologically characterized as *P. cephalonica*, but their mtDNA sequences were more similar to *P. chorismenostoma* than to *P. cephalonica*, and thus suggested possible interspecific hybridization or incomplete lineage sorting. The selection of the individuals was based on their geographic spread (Figure 1). Locality information is provided in Table 1. Genomic DNA extracts were already available from Razkin et al. (under review).

2.2. RAD sequencing

Two RAD libraries were prepared following the protocol of Baird et al. (2008) and Etter et al. (2011). We used a Qubit fluorimeter 2.0 (Life Technologies) to check that each DNA extract contained a minimum of 250 ng of genomic DNA per 35 µl. Each individual sample was digested for 60 min at 37 ºC with restriction enzyme SbfI (New England Biolabs; NEB). P1 adapters (IDT) containing a sample specific molecular identifier were ligated to each digested DNA sample. These barcoded samples were pooled in two libraries and purified using DNA Clean & Concentrator™-5 (Zymo Research). Each library was sheared to an optimal size of 400 bp (samples of 50 µl in microTUBE AFA Fiber Screw-Cap for 60 s using a focused-ultrasonicator M220, Covaris) followed by a size selection on

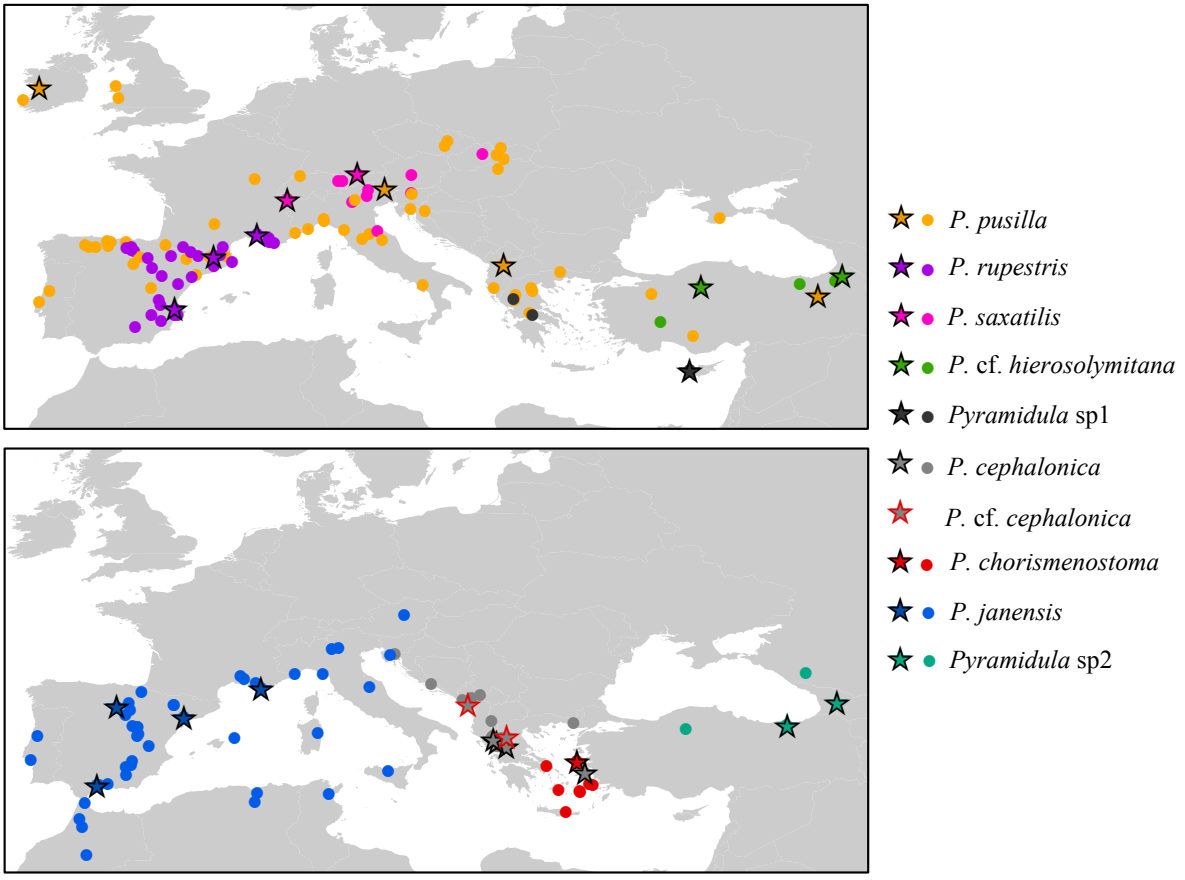

Figure 1: Geographic origins of the samples of *Pyramidula*. Stars refer to the samples analyzed using RADseq data in the present study. Dots refer to samples analyzed in Razkin et al. (under review) and provide a rough idea of the distribution of each species.

gel. Libraries were then blunted with Quick Blunting Kit (NEB), followed by an A-tailing step. Afterwards, P2 adapters (IDT) were ligated and PCR amplification was performed using P1 and P2 primers with Phusion High-Fidelity Master Mix (Thermo-scientific). We used a Qubit fluorimeter 2.0 (Life Technologies) to check that the amount of DNA in the final amplified library increased by 1.5x and a 2100 Bioanalyzer system with a High Sensitivity DNA kit (Agilent Technologies) to check that the final fragment size was in the expected range of 300-400 bp. Each library was run in a single paired-end Illumina MiSeq lane flow cell (v2 kit 2 x 250 bp) at The GenePool facility (University of Edinburgh). We used the reads 1 (250 bp).

2.3. RADseq data processing

For the data analysis, we followed the pipeline implemented in the software *pyRAD* v3.0 (Eaton and Ree, 2013; Eaton, 2014). *pyRAD* assembles RADseq data into groups of similar sequences that will be considered orthologs and treated as different loci without using a reference sequence (pipeline "*de novo*"). Compared to *Stacks* (Catchen et al., 2011), which

Table 1: Information on the specimens used in this study, on the RAD tags sequenced here and the Genbank accession numbers from Razkin et al. (under review): RAD tags: reads that passed quality filtering; cluster: number of clusters at 90% similarity; mean depth: mean depth of clusters with depth of coverage ≥ 5; consensus loci: number of loci with depth ≥ 5 and passed paralog filter; loci in final dataset: number of loci available for 18 taxa or more.

Voucher number	Species	Locality	RAD tags	Clusters	Mean depth	Consensus loci	Loci in final data set	Genbank COI	Genbank 16S	Genbank nuclear
EHUMC_1201	*P. pusilla*-a	Near Lesachtal, Kärnten, Austria	302,136	38,308	11.428	15,021	4,856	KP727247	KP727036	KP727460
HNHM_99350	*P. pusilla*-b	Korab Mts, Diber, Albania	360,865	58,723	11.378	16,209	5,074	KP727290	KP727079	KP727503
EHUMC_1208	*P. pusilla*-c	Vallyvaughan, Clare, Ireland	380,449	39,592	13.927	14,612	5,097	KP727293	KP727082	KP727506
HNHM_98875	*P. pusilla*-d	Erzurum, Turkey	284,484	40,553	10.803	13,746	4,714	KP727354	KP727143	KP727567
EHUMC_1138	*P. rupestris*-a	Arles, Provence, France	429,798	35,144	15.229	18,796	5,512	KP727157	KP726946	KP727370
MVHN_070910RU15	*P. rupestris*-b	Bellús, Valencia, Spain	307,041	31,028	12.357	15,895	5,198	KP727176	KP726965	KP727389
EHUMC_1225	*P. rupestris*-c	Lillet-Castellar d'Nug, Barcelona, Spain	414,475	30,865	16.005	15,559	5,420	KP727319	KP727108	KP727532
EHUMC_1170	*P. saxatilis*-a	The Alps, France	325,337	35,566	13.119	13,861	5,096	KP727207	KP726996	KP727420
EHUMC_1220	*P. saxatilis*-b	Eschenlohe-Oberau, Bayern, Germany	790,027	131,046	17.181	23,306	5,631	KP727314	KP727103	KP727527
EHUMC_1149	*P.* cf. *hieroslymitana*-a	Artvin, Turkey	410,993	29,065	15.458	15,622	5,514	KP727185	KP726974	KP727398
HNHM_98874_2	*P.* cf. *hieroslymitana*-b	Gümüshane, Turkey	287,216	30,419	12.592	11,972	4,956	KP727353	KP727142	KP727566
NHMC_50.23326_1	*Pyramidula* sp1-a	Petratis gorge, Cyprus	182,297	32,269	9.143	9,275	3,557	KP727277	KP727066	KP727490
EHUMC_1133	*P. jaenensis*-a	Marsella - Toulon, Var, France	500,246	34,469	16.756	19,860	5,817	KP727152	KP726941	KP727365
EHUMC_1140	*P. jaenensis*-b	Valle de Abdalajis, Málaga, Spain	201,257	28,041	10.591	9,576	4,260	KP727159	KP726948	KP727372
EHUMC_1184	*P. jaenensis*-c	Sto Domingo de Silos, Burgos, Spain	247,143	22,880	12.373	11,054	4,817	KP727230	KP727019	KP727443
EHUMC_1227	*P. jaenensis*-d	Codolar, Tarragona, Spain	278,517	35,283	12	12,119	4,937	KP727321	KP727110	KP727534
NHMC_50.27302	*P. chorismenostoma*-a	Chios island, Greece	154,784	31,131	8.937	6,917	3,035	KP727257	KP727046	KP727470
ZMH_86507/999 _2	*Pyramidula* sp2-a	Imereti, Kutaisi, Georgia	425,231	45,050	14.413	16,546	5,497	KP727331	KP727120	KP727544
HNHM_98871	*Pyramidula* sp2-b	Trabzon, Turkey	391,970	48,616	14.165	14,947	5,457	KP727349	KP727138	KP727562
NHMC_50.32106_2	*P. cephalonica*-a	Samos island, Greece	315,065	62,779	10.791	12,441	4,828	KP727267	KP727056	KP727480
HNHM_98853_1	*P. cephalonica*-b	Thessaly, Trikala, Greece	478,386	36,531	16.899	16,731	5,815	KP727335	KP727124	KP727548
HNHM_98858	*P. cephalonica*-c	Epirus, Ioannina, Greece	454,324	41,254	15.086	19,090	5,854	KP727341	KP727130	KP727554
HNHM_98859_2	*P. cephalonica*-d	Epirus, Ioannina, Greece	972,898	62,153	26.331	24,097	5,937	KP727343	KP727132	KP727556
HNHM_99349	*P.* cf. *cephalonica*-e	Rumija Mts. Near Stari Bar, Montenegro	285,760	29,683	11.528	15,392	5,466	KP727288	KP727077	KP727501
HNHM_98861	*P.* cf. *cephalonica*-f	Epirus, Ioannina, Greece	409,134	39,985	15.315	14,814	5,586	KP727345	KP727134	KP727558

does not consider indels and was developed for population level analyses, *pyRAD* was developed to search for homologies across more divergent samples (even among different species) and uses a clustering method (see below) that allows for indel variation.

pyRAD separated the reads of the 25 individual using the sample specific molecular identifier barcodes that were attached during the library preparation. Base calls with a phred quality score below 20 were converted to Ns (undetermined sites) and reads including more than a maximum number of Ns were discarded (several maxima were tested, see below). Each read (250 bp) was reduced to 236 bp after removing the sample specific molecular identifier (8 bp) and restriction sites (6 bp). Filtered reads were clustered and aligned using the two programs that are implemented in the *pyRAD* pipeline, *vsearch* (https://github.com/torognes/vsearch) and *muscle* (Edgar, 2004). Consensus sequences kept information on heterozygous sites as ambiguity codes and those containing more than the allowed number of heterozygous sites (see below) or more than two haplotypes were discarded so as to eliminate paralogs (or repetitive or high copy number DNA regions).

The key parameters of *pyRAD* that can cause an under- or overmerging of clusters, and therefore may lead to misidentification of orthologous sequences, are the "clustering threshold" and the "minimum depth of coverage". The clustering threshold is the minimum percent similarity required by *vsearch* to consider sequences as orthologs. High similarity thresholds lead to a too strict identification of clusters (loci), so that orthologous loci with more variable sequences may be considered as different loci. Conversely, too low similarity thresholds will group orthologous loci with highly variable sequences, but may also include paralogous and other non orthologous loci in the same cluster (locus). The minimum depth of coverage is the minimum number of identical reads required to take a sequence into account. Low values for this parameter may increase the risk to include erroneous sequences in the analysis, while high values increase the risk to exclude orthologous loci with low coverage.

Catchen et al. (2013) stated that the optimal values for the "clustering threshold" and the "minimum depth of coverage" depend on the degree of polymorphism, the amount of sequencing error and the depth of coverage. They suggested testing a range of values for each parameter for each dataset. Therefore, we performed 9 tests in *pyRAD* using three clustering thresholds (85%, 90% and 95%) and three depths of coverage (3, 5 and 7). Three other parameters ("Maximum number of undetermined sites in filtered sequences"; "Maximum number of Ns in a consensus sequence" and "Maximum number of heterozygous sites in a consensus sequence") were adjusted to each clustering threshold following the recommendations of the *pyRAD* manual. In further analyses, we used the output of the test that retained as many orthologous loci as possible so as to reduce the chance to include sequences with errors. Following Viricel et al. (2014) we looked at the resulting matrix length and the number of SNPs obtained with the set of the previously defined *pyRAD* parameters (coverage and similarity threshold). When a plateau was observed (less variation in the matrix length and the number of resulting SNPs for a larger variation in the *pyRAD*

parameters), we used the largest *pyRAD* parameter in the window of this plateau. In his way, we reduce (but not exclude) the chance to include sequences with errors and minimise the risk of including non-orthologous sequences. For the parameter "Minimum taxon coverage" that specifies the minimum number of samples with data for a given locus to be retained in the final dataset, a value of 18 samples was applied for the 9 tests. With the aim to know how the variation of this parameter could affect the phylogenetic analyses, we modified this value (12, 18, 23 and 25) for one selected test.

For the remaining parameters default values were employed.

2.4. Phylogenetic inference

2.4.1. RADseq phylogeny

For the phylogenetic analyses we used the output of *pyRAD* which contained all the loci concatenated into one supermatrix. Maximum likelihood (ML) analyses were performed using RAxML v8.1.11 (Stamatakis, 2014) implemented at the CIPRES Science Gateway (Miller et al., 2010) applying a rapid bootstrapping analysis with 100 bootstrap pseudoreplicates and a general-time-reversible nucleotide substitution model. The analyses were performed for the supermatrix of each of the 11 tests (9 test values for "clustering threshold" and "minimum depth of coverage" and 2 extra tests values for "minimum taxon coverage").

2.4.2. Five-gene phylogeny

We also reconstructed a phylogeny for the same 25 taxa included in the RADseq analysis, but with the COI, 16S rRNA, 5.8S rRNA, ITS2 and 28S rRNA sequence data of Razkin et al. (under review). The results of these analyses were compared with those obtained with the RADseq data.

Sequences were aligned with the online version of Mafft v7 (Katoh and Standley, 2013). We used the Q-INS-i algorithm for rRNA, which considers the secondary structure of RNA (Katoh and Toh, 2008), and the Auto algorithm for COI. Default values were used for the remaining parameters. Numbers of variable sites were inferred using DnaSP v5.10.1 (Librado and Rozas, 2009). ML trees were reconstructed using three datasets: COI+16S (mitochondrial), 5.8S+ITS2+28S (nuclear) and all five fragments concatenated. Each dataset was partitioned according to the genes, and COI was further partitioned according to the three codon positions. ML trees were inferred with RAxML v8.1.11 (Stamatakis, 2014) with bootstrapping over 100 replicates.

Outgroup samples were not available for the RADseq data and hence, all phylogenies were midpoint rooted.

2.5. Species delimitation

We used the Bayes Factor Delimitation* (BFD*) method (Leaché et al., 2014) to compare

several species delimitation scenarios. We first used SNAPP, a package for inferring species trees from unlinked biallelic markers (Bryant et al., 2012), to set up five hypothetical scenarios. Then, we ran BEAST 2 (Bouckaert et al., 2014) to calculate the marginal likelihoods of each scenario under the priors selected in SNAPP. Finally, we used BFD* to make pairwise comparisons among the marginal likelihoods obtained for the different scenarios.

2.5.1. SNAPP- prior selection

The dataset obtained with the *pyRAD* analysis (clustering threshold of 0.9 with a minimum depth of coverage of 5 = c90m5) was selected with the criteria described in 2.3 and was used to carry out SNAPP analyses. We did not allow for missing data (minimum taxon coverage = 25) and we obtained a subset of 368 SNPs that came from different reads and that we considered as unlinked. If multiple SNPs were found in a locus, *pyRAD* sampled SNP sites randomly. In order to increase the number of unlinked SNPs, we also performed SNAPP analyses on another dataset (clustering threshold of 0.9 with a minimum depth of coverage of 3 = c90m3, using minimum taxon coverage = 25), yielding 875 SNPs.

For the ancestral population sizes a variety of theta values were tested, all of them with gamma distribution priors: Gamma (2, 200), Gamma (2, 2000) and Gamma (2, 20000) (Leaché et al., 2014). Priors were set to default values or calculated according to the manual. Each analysis was run in BEAST 2 (Bouckaert et al., 2014) for 1 million generations with sampling every 1000 generations. The first 10% iterations were discarded as burnin and convergence was confirmed by examining log files (ESS > 200).

Different prior distribution on theta yielded identical posterior probabilities (PP) in the species tree. The Gamma value (2, 2000) was retained for subsequent analysis. We found no topological and branch length differences between the analyses of c90m5 and c90m3. Therefore, we only reported the results of the largest dataset (c90m3) which was used in subsequent analyses for BFD*.

2.5.2. BFD* - comparison between scenarios

Marginal likelihoods of each scenario were estimated in BEAST 2 by conducting a path sampling method with 48 steps, each one consisting of 400,000 generations with a burnin value of 10%. Bayes Factors were calculated as twice the difference of the log marginal likelihoods of the two models used in the comparison (base scenario and alternative scenario). A positive Bayes Factor supports the base scenario, whereas negative values support the alternative model.

Our base scenario was the recognition of the nine species proposed by Razkin et al. (under review): *P. pusilla*, *P. rupestris*, *P. jaenensis*, *P. chorismenostoma*, *P. cephalonica*, *P. saxatilis*, *P.* cf. *hieroslymitana*, *Pyramidula* sp1 and *Pyramidula* sp2 (Figure 2a). Since it was not computationally feasible to test all possible scenarios we tested four alternative scenarios. In two of them we clustered closely related species based on the results of the phylogenetic

inference: Clustering 1 (Figure 2b), in which *P. rupestris* + *P. saxatilis* were clustered as a single species and Clustering 2 (Figure 2c), in which *P. jaenensis* + *P. chorismenostoma* + *P. cephalonica* were clustered as a single species. In the other two scenarios we split the most widely distributed species into two putative sister species: Split 1 and Split 2. In Split 1 (Figure 2d) *P. pusilla* was divided into two putative species (*P. pusilla*_west, clustering the samples of the Atlantic region and *P. pusilla*_east, clustering the samples of the Mediterranean region of the distribution range of *P. pusilla*). In Split 2 (Figure 2e) *P. jaenensis* was divided into two putative species (*P. jaenensis*_east, clustering the samples from the Iberian Peninsula and *P. jaenensis*_west, clustering the samples outside the Iberian Peninsula, with the Pyrenees as geographic barrier).

2.6. Test for interspecific hybridization

We used the D-statistic (Green et al., 2010; Durand et al., 2011) to evaluate whether hybridization has occurred between species in *Pyramidula*. Assuming that we have a four taxon tree with topology (((P1, P2), P3), O), under the null hypothesis of no hybridization, the two non-concordant allele patterns (ABBA, where P2 and P3 share the allele B; and BABA, where P1 and P3 share the allele B) are expected to occur with equal frequencies, so that D (the difference between the numbers of ABBA and BABA counts) = 0. Significant deviation of D from 0 rejects the null hypothesis of incomplete lineage sorting, suggesting that hybridization has occurred between P3 and either P1 or P2.

Eaton and Ree (2013) presented an extension of the D-statistic (called "partitioned D-statistic test") which allows for a better detection of interspecific hybridization and enables to infer the directionality of gene flow (i.e. from P2/P1 into P3, from P3 into P1/P2, or in both directions). This partitioned D-statistic is based on a five-taxon tree (((P1, P2), ($P3_1$, $P3_2$)), O), where $P3_1$ and $P3_2$ are two lineages from within the P3 clade. In this case, three D-statistics are estimated: D_1 measures whether the counts non-concordant sites of ABBAA and BABAA are significantly different; D_2 measures whether the counts of ABABA and BAABA are significantly different; and D_12 measures whether the counts of ABBBA and BABBA are significantly different. While D_1 and D_2 reflect the signal of gene flow involving $P3_1$ and $P3_2$ respectively, D_12 reflects interspecific gene flow involving the branch of the most recent common ancestor of $P3_1$ and $P3_2$. D_12 indicates whether gene flow existed from P3 into P1/2, or in the opposite direction: if hybridization occurred from P3 into P1/2, both $P3_1$ and $P3_2$ lineages should share derived alleles resulting in a significant D_12. Conversely, a non-significant D_12 means that gene flow occurred from P1/2 into $P3_1$ or $P3_2$.

In order to evaluate the existence of ancestral hybridization between species in *Pyramidula*, each terminal was selected as follows in the four-taxon D-statistic test: P1 and P2 were two sister species in our trees; P3 was selected as a sister-species of clade P1P2; a different species was used as outgroup (O). Since different individuals can represent the same terminal taxon of P1, P2 and P3, all possible combinations of individuals for a given tree

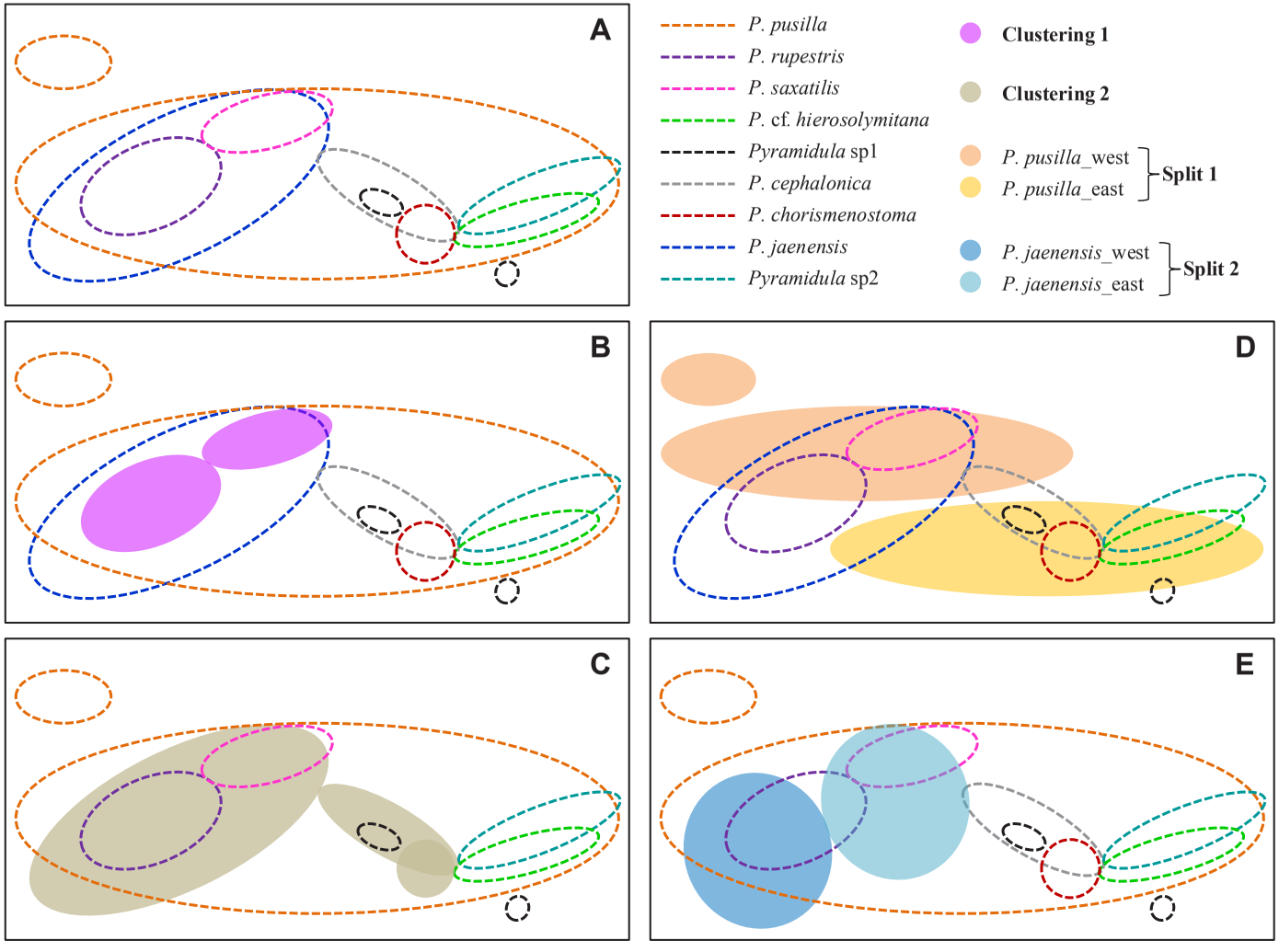

Figure 2: (a) Base species delimitation model and (b-e) alternative species delimitation models: (b) Clustering 1: *P. rupestris* + *P. saxatilis*; (c) Clustering 2: *P. jaenensis* + *P. chorismenostoma* + *P. cephalonica*. (d) Split 1: *P. pusilla* = *P. pusilla*_west + *P. pusilla*_east; and (e) Split 2: *P. jaenensis* = *P. jaenensis*_west + *P. jaenensis*_east. Circles refer to approximate distribution areas of species and colors of each species are defined at the top right.

were tested. For those four-taxon D-statistic tests that rejected the null hypothesis of no gene flow, partitioned D-statistic tests were performed in order to infer the directionality of gene flow. Taxa for each terminal were the same as in the four-taxon D-statistic tests but adding two individuals of the same species to P3 ($P3_1$ and $P3_2$). This partitioned D-statistic test was only performed in those cases where two P3 lineages were available.

All tests were performed in *pyRAD* (http://dereneaton.com/software) including heterozygous sites and employing 1000 bootstrap iterations to estimate the value and standard deviation of D. Significance was determined by converting the resultant Z-scores into a two-tailed *P*-value. The standard Bonferroni correction for multiple comparisons was applied.

3. Results

3.1. RADseq data processing

The Illumina MySeq yielded an average of 655,063 reads per sample (first reads) ranging from 292,674 to 1.6 x 10^6. After phred quality filtering, it was reduced to an average of 383,593 reads per sample, ranging from 154,784 to 972,898 (Table 1). The size of the nine *pyRAD* output data matrices and the number of SNPs they included decreased with increasing "minimum depth coverage" and "similarity threshold". This trend was clear when increasing the similarity threshold from 0.90% to 0.95%, but hardly visible below 0.90% (Figure 3). The length of the matrices of the concatenated sequences ranged from 857,081 to 2,272,846 positions; and the numbers of SNPs ranged from 54,954 to 159,712 (Figure 3).

Taking into account that the length of the nine *pyRAD* output matrices and their content

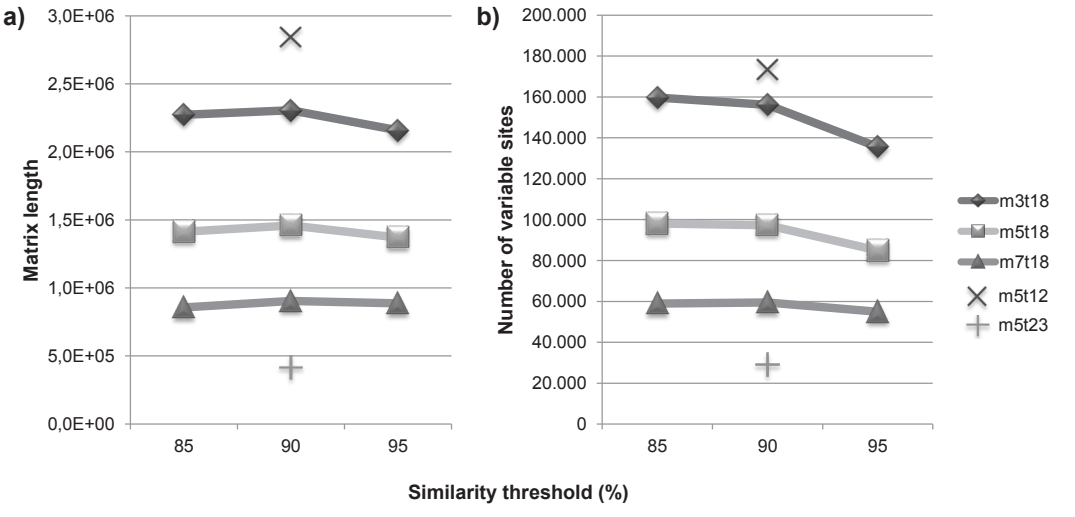

Figure 3: Length of the concatenated sequence dataset (a) and total number of single nucleotide polymorphisms (SNPs) (b) resulting from nine pyRAD analyses where three clustering similarity thresholds and three minimum depth of coverages ('m') were applied. Other two pyRAD analyses are also represented where two different minimum taxon coverage values ('t') were applied.

in SNPs varied very little when decreasing the similarity threshold from 0.90% to 0.85% (Figure 3), we assumed that decreasing the similarity threshold below 90% was not needed to substantially improve the identification of orthologous loci. Yet, for the "minimum depth of coverage" there were huge differences between tests for the length of matrices and number of SNPs, probably because some of our samples had a very low coverage. We selected a minimum depth of coverage of 5 in order to reduce the chances to include sequencing errors and, at the same time, to include a maximum of loci with a sequenced with a low depth. The "minimum taxon coverage" parameter influenced drastically the length of the matrices, the number of SNPs and missing data obtained with a clustering threshold set to 0.90% and a minimum depth of coverage set to 5 (2,846,408, 1,459,651 and 415,737 positions, 173,440, 97,269 and 29,079 SNPs and 28.87%, 15.98 and 4.85% missing data, respectively for minimum taxon coverage of 12, 18 and 23).

3.2. Phylogenetic inference

3.2.1. RADseq phylogeny

The nine RADseq matrices yielded the same tree topology with maximal support for all clades (Figure 4a). The tests made with three different values for the minimum taxon coverage also showed the same topology with maximal support for all clades, except for one clade with a bootstrap support value of 95% (Figure 4a).

The nine species delimited by Razkin et al. (under review) received maximal support in the RADseq trees (Figure 4a) and were grouped into two main clades. The first clade included *P. cephalonica, P. chorismenostoma, P. jaenensis, P.* cf. *hierosolymitana, Pyramidula* sp1 and *Pyramidula* sp2, with *P. chorismenostoma* and *P. jaenensis* being sister taxa forming a clade with *P. cephalonica* and *Pyramidula* sp2. The nodes where *P.* cf. *hierosolymitana* and *Pyramidula* sp1 branched off were situated deeper in the tree. The second clade grouped the remaining species, *P. pusilla, P. rupestris* and *P. saxatilis*, with the latter two being sister species.

The two *P.* cf. *cephalonica* individuals (e, f) that showed patterns of incomplete lineage sorting in the study of Razkin et al. (under review) are sister-taxa and as such they clustered with all other *P. cephalonica* specimens.

3.2.2. Five-gene phylogeny

Basic sequence information of these data is provided in Table 2. *P. pusilla*, *P. rupestris* and *P.* cf. *hierosolymitana* were the only species that were recovered as well-supported clades in each of the three datasets (COI+16S, 5.8S+ITS2+28S, all concatenated) (Figure 4b-d). The monophyly of *P. saxatilis* was supported by the COI+16S and concatenated datasets, whereas the monophyly of *Pyramidula* sp2 was supported by the 5.8S+ITS2+28S and the concatenated datasets. *P. jaenensis* was never supported, but neither rejected, and *Pyramidula* sp2 was not resolved in the COI+16S tree. The position and monophyly of *P. cephalonica* was variously resolved: in the COI+16S and concatenated trees, *P.* cf. *cephalonica* e-f were

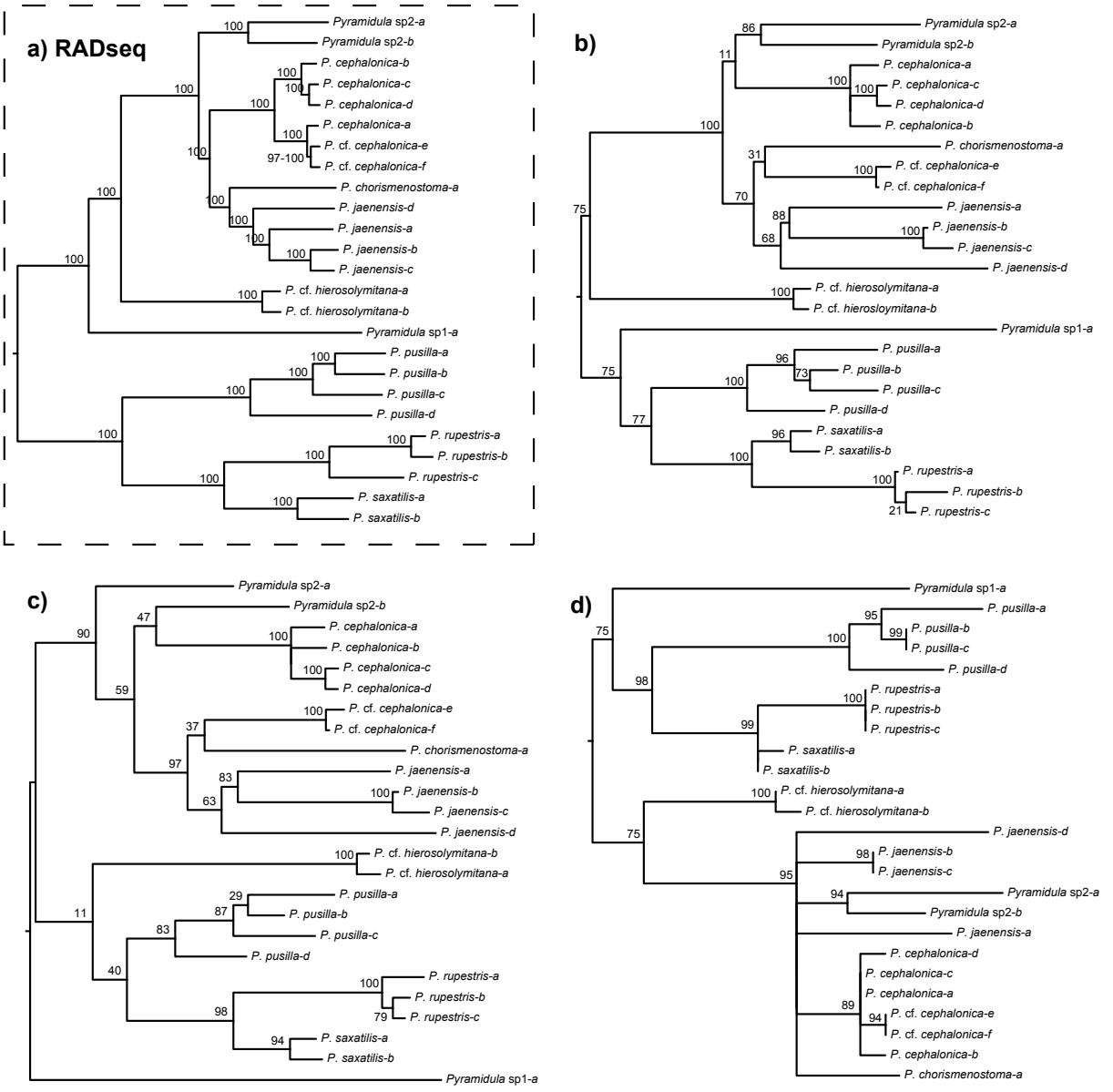

Figure 4: Phylogenetic trees inferred by maximum-likelihood analyses of: (a) the RADseq supermatrix using clustering threshold = 0.90%, minimum depth coverage = 5 and minimum taxon coverage = 18. (b) the concatenated mtDNA (COI + 16S) and nDNA (5.8S + ITS2 + 28S) sequences. (c) the concatenated mtDNA sequences. (d) the concatenated nDNA sequences.

grouped together in a clade with *P. jaenensis* and *P. chorismenostoma*. However, in the 5.8S+ITS2+28S tree, *P. cephalonica* a-f formed a well-supported clade. In the three trees *P. cephalonica, P. chorismenostoma, P. jaenensis* and *Pyramidula* sp2 formed a well-supported clade. *P. pusilla, P. rupestris* and *P. saxatilis* were grouped together in the 5.8S+ITS2+28S and concatenated datasets. The three trees strongly supported the sister relationship between *P. rupestris* and *P. saxatilis*. In the 5.8S+ITS2+28S and all concatenated trees *Pyramidula* sp1 grouped with *P. cephalonica, P. chorismenostoma, P. jaenensis* and *Pyramidula* sp2, while in the COI+16S tree *Pyramidula* sp1 was not grouped to any other species being situated deeper in the tree. The positions of *P. jaenensis*, *Pyramidula* sp2 and *P. chorismenostoma* were not resolved in 5.8S+ITS2+28. The deeper nodes were poorly resolved in COI+16S tree.

3.3. Species delimitation

3.3.1. Species tree

The consensus species tree inferred for the base species delimitation model suggested by Razkin et al. (under review) is shown in Figure 5. This species tree was obtained using 875 unlinked biallelic markers in SNAPP. Relationships between species in the recovered topology were consistent with those estimated using ML of the concatenated rmatrix of

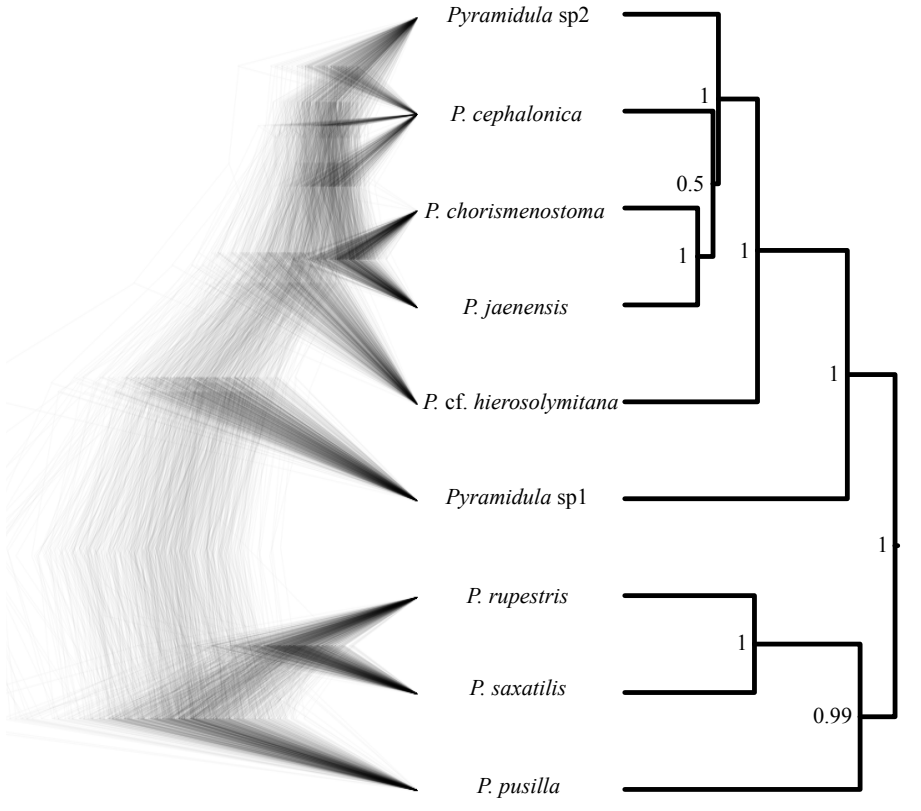

Figure 5: Densitree diagram representing all species trees obtained from SNAPP (left) and consensus topology with posterior probability values (right).

Table 3: Results for BFD* species delimitation: ML (marginal likelihood); BF (Bayes factor).

Model	Description	Species number	ML	BF	Rank
0. Base scenario	Species delimited in Razkin et al. (under review) (Figure 2-a)	9	-4505,8	-	1
1. Clustering 1	*P. rupestris* +*P. saxatilis* (Figure 2-b)	8	-4656,7	301,8	4
2. Clustering 2	*P. jaenensis* + *P. chorismenostoma* + *P. cephalonica* (Figure 2-c)	7	-4806,7	601,8	5
3. Split 1	*P. pusilla* = *P. pusilla*_west + *P. pusilla*_east (Figure 2-d)	10	-4524,1	36,6	3
4. Split 2	*P. jaenensis* = *P. jaenensis*_west + *P. jaenensis*_east (Figure 2-e)	10	-4515,5	19,4	2

RADseq data (Figure 4a), except for the most recent common ancestor of *P. cephalonica*, *Pyramidula* sp2 and *P. chorismenostoma* + *P. jaenensis* that was not recovered (PP = 0.5). For the remaining nodes high PP were found (> 0.99).

3.3.2. Comparisons between species delimitation scenarios

The tested species delimitation models are represented in Figure 2 and the results are summarized in Table 3. The base scenario with nine species (Razkin et al., under review) was favoured over the alternative delimitation models. The two models clustering closely related species into a single species were consistently rejected. The other two models in which widely distributed species were split into two species were also rejected, although their marginal likelihood values were closer to that of the base scenario.

3.4. Test for intraspecific hybridization

3.4.1. Four-taxon D-statistics test

All D-statistics were based on loci obtained by *pyRAD* using a minimum depth of coverage of 5 and similarity threshold at 90%. Eight D-statistic tests were run for distinct four-taxon subtrees and repeated over all possible combinations of individuals. ummary results are shown in Table 4 and full results of each replicate (unique combinations of individuals) are available in Supplementary material 1.

We first tested for gene flow between *P. pusilla* and either *P. rupestris* or *P. saxatilis* (test 1). This test found 8 significant replicates out of 24 between *P. pusilla* and *P. saxatilis* using a significance level of $\alpha = 0.05$, but one significant replicate out of 24 between the two taxa with a significance level of $\alpha = 0.01$. Tests 2-5 examined whether *P. cephalonica, P.* cf. *hierosolymitana, Pyramidula* sp1 and *Pyramidula* sp2 hybridized with either *P. chorismenostoma* or *P. jaenensis*. No significant differences were detected in the counts of non-concordant allele patterns in any of the replicates of tests 2 and 3. Tests 4 and 5 provided some significant replicates of gene flow between *P. chorismenostoma* and *Pyramidula* sp1 (test 4: 2 out of 4 with $\alpha = 0.05$), and between *P. chorismenostoma* and *Pyramidula* sp2

Table 4: Results of the four taxon D-statistic tests showing the range of Z-scores ("Range Z"); number of significant replicates ("Nsig/n"); main allele pattern in significant replicates ("Allele pattern (sigD)"); species involved in introgression; range of number of available RAD loci ("Range nloci"); and range of percentage of non concordant sites over all possible replicates ("Range pdisc").

TEST	P1	P2	P3	O	Range Z	Nsig/n (α= 0.05, 0.01)	Allele pattern (sigD)	Species involved in introgression	Range nloci	Range pdisc
1 - 4taxon	*P. rupestris*	*P. saxatilis*	*P. pusilla*	*P.* sp1	(0.47, 2.63)	**8/24, 1/24**	ABBA	*P. pusilla* - *P. saxatilis*	(2272, 3040)	(0.1, 0.12)
2 - 4taxon	*P. chorismenostoma*	*P. jaenensis*	*P. cephalonica*	*P. pusilla*	(0.05, 1.51)	0/24, 0/24			(1716, 2704)	(0.12, 0.14)
3 - 4taxon	*P. chorismenostoma*	*P. jaenensis*	*P.* cf. *hierosolymitana*	*P. pusilla*	(0.03, 1.67)	0/8, 0/8			(1755, 2510)	(0.06, 0.07)
4 - 4taxon	*P. chorismenostoma*	*P. jaenensis*	*P.* sp1	*P. pusilla*	(0.84, 2.2)	**2/4**, 0/4	BABA	*P. chorismenostoma* - *P.* sp1	(1302, 1675)	(0.07, 0.09)
5 - 4taxon	*P. chorismenostoma*	*P. jaenensis*	*P.* sp2	*P. pusilla*	(0.98, 2.64)	**2/8, 1/8**	BABA	*P. chorismenostoma* - *P.* sp2	(1889, 2540)	(0.11, 0.12)
6 - 4taxon	*P. cephalonica*	*P. chorismenostoma*	*P.* cf. *hierosolymitana*	*P. pusilla*	(0.2, 2.8)	**6/12, 1/12**	BABA	*P. cephalonica* - *P.* cf. *hierosolymitana*	(1913, 2494)	(0.07, 0.09)
7 - 4taxon	*P. cephalonica*	*P. chorismenostoma*	*P.* sp1	*P. pusilla*	(0.17, 0.58)	0/6, 0/6			(1420, 1664)	0.09
8 - 4taxon	*P. cephalonica*	*P. chorismenostoma*	*P.* sp2	*P. pusilla*	(1.6, 2.8)	**9/12, 1/12**	BABA	*P. cephalonica* - *P.* sp2	(2072, 2515)	(0.15, 0.16)

(test 5: 2 out of 8 with α = 0.05 and 1 out of 8 with α = 0.01), respectively. Finally, tests 6-8 explored whether gene flow occurred between *P.* cf. *hierosolymitana* or *Pyramidula* sp1 or *Pyramidula* sp2 and *P. cephalonica* or *P. chorismenostoma*. No significant results were detected for test 7. Tests 6 and 8 provided some significant replicates of gene flow between *P. cephalonica* and *P.* cf. *hierosolymitana* (tests 6: 6 out of 12 with α = 0.05 and 1 out of 12 with α = 0.01), and between *P. cephalonica* and *Pyramidula* sp2 (test 8: 9 out of 12 with α = 0.05 and 1 out of 12 with α = 0.01). After Bonferroni correction for multiple comparisons none of the tests remained significant.

3.4.2. Partitioned D-statistics

Partitioned D-statistic tests were only performed for those four-taxon D-statistic tests that showed some signal of ancestral interspecific gene (tests 1, 4, 5, 6 and 8, before Bonferroni correction). Since two different individuals from P3 were needed ($P3_1$ and $P3_2$) test 4 could not be done (Table 5, Supplementary material 1). Generally more than 100 sites for each allele pattern were obtained for D_12, but fewer than 60 sites for each llele pattern in D_1 and D_2 for all replicates, so reducing their statistical power.

For test 1, D_12 provided the strongest evidence of gene flow between *P. saxatilis* and the most recent common ancestor of *P. pusilla* a-d, since 13 of the 36 replicates were significant for α = 0.05 and 4 out of 36 for α = 0.01. D_1 was significant for a few replicates suggesting gene flow between both *P. pusilla – P. saxatilis* and *P. pusilla – P. rupestris*. Also D_2 was significant for a few replicates indicating a possible gene flow between *P. pusilla* and *P. rupestris.* Having obtained a significant D_12 (but not being both D_1 and D_2 significant) means that gene flow may have occurred from *P. saxatilis* into both *P. pusilla* lineages selected as $P3_1$ and $P3_2$. Another possible scenario is that the ancestor of the two lineages of *P. pusilla* hybridized with *P. saxatilis.* The significant replicates in D_1 and D_2 indicating a possible gene flow between *P. pusilla* and *P. rupestris* are too weak to take into account since few non-concordant sites were available in D_1 and D_2 tests, and also because none of the four-taxon D-statistic tests found any signal of gene flow between *P. pusilla* and *P. rupestris*. For the remaining tests (5, 6 and 8) very few replicates yielded significant evidence of gene flow between species (Table 5). After Bonferroni correction for multiple comparisons only two replicates from test 1 remained significant (shown in Supplementary material 1).

4. Discussion

This study provides the very first illustration of jointly applying phylogenetic inference, testing for interspecific hybridization and species delimitation using RADseq data to successfully resolve longstanding taxonomic and phylogenetic problems in a non-model cryptic species complex.

4.1. Phylogenetic inference

The mtDNA (Figure 4c) and nDNA (Figure 4d) trees on their own did not support

Table 5: Results of the partitioned D-statistic tests showing the range of Z-scores ("Range Z"); number of significant replicates ("Nsig/n"); main allele pattern in significant replicates ("Allele pattern (sigD)"); range of number of available RAD loci ("Range nloci"); and range of percentage of non concordant sites over all possible replicates ("Range pdisc").

TEST	P1	P2	P3_1	P3_2	O	D_12			D_1			D_2			Range nloci	Range pdisc
						Range Z	Nsig/n (α= 0.05, 0.01)	Allele pattern (sigD)	Range Z	Nsig/n (α= 0.05, 0.01)	Allele pattern (sigD)	Range Z	Nsig/n (α= 0.05, 0.01)	Allele pattern (sigD)		
1 - part	*P. rupestris*	*P. saxatilis*	*P. pusilla*$_1$	*P. pusilla*$_2$	*P.* sp1	(0.63, 2.87)	**13/36, 4/36**	ABBBA (13)	(0.05, 4.06)	**4/36, 2/36**	BABAA (2), ABBAA (2)	(0.1, 4.47)	**4/36, 2/36**	BAABA (4)	(1860, 2551)	(0.11, 0.13)
5 - part	*P. chorismenostoma*	*P. jaenensis*	*P.* sp2$_1$	*P.* sp2$_2$	*P. pusilla*	(1.58, 2.22)	**1/4,** 0/4	BABBA (1)	(1.23, 2.89)	**3/4, 1/4**	BABAA (3)	(0.16, 2.4)	0/4, 0/4		(1757, 2284)	(0.12, 0.13)
6 - part	*P. cephalonica*	*P. chorismenostoma*	*P.* cf. *hierosolymitana*$_1$	*P.* cf. *hierosolymitana*$_2$	*P. pusilla*	(0.37, 2.47)	**1/6,** 0/6	BABBA (1)	(0.1, 0.38)	0/6, 0/6		(0.7, 2.86)	**1/6, 1/6**	BAABA (1)	(1834, 2138)	(0.08, 0.09)
8 - part	*P. cephalonica*	*P. chorismenostoma*	*P.* sp2$_1$	*P.* sp2$_2$	*P. pusilla*	(1.58, 2.38)	**1/6,** 0/6	BABBA (1)	(0.04, 0.78)	0/6, 0/6		(0.24, 1.29)	0/6, 0/6		(1926, 2250)	(0.17, 0.18)

the nine putative species of *Pyramidula* suggested by Razkin et al. (under review). The combination of mtDNA and nDNA fragments increased support values (Figure 4b), but still some species and interspecific relationships were not fully resolved in the five-genes-trees. The RADseq approach, however, yielded a fully resolved tree (Figure 4a) with (near) maximally supported nodes confirming the monophyly of the nine species suggested by Razkin et al. (under review). The uncertain position of *Pyramidula* sp1 for mtDNA and nDNA gene trees was successfully resolved with the RADseq phylogeny. While the nDNA tree included *Pyramidula* sp1 in the major clade containing *P. pusilla, P. rupestris* and *P. saxatilis*, the tree based on mtDNA fragments did not support its relationships with other species. The RADseq tree included *Pyramidula* sp1 with full support in a different clade clustering *P. cephalonica, P. jaenensis, P. chorismenostoma* and *Pyramidula* sp2. The number of nucleotides in the combined dataset of mtDNA and nDNA was 2,451 bp with 287 polymorphic sites, whereas the RADseq dataset for the tree construction comprized 1,459,651 bp with 97,269 polymorphic sites; hence, the increased number of markers of the RADseq data helped to fully resolve the phylogenetic relationships between species. This is in line with other recent studies using reduced representation genomic data to resolve phylogenetic relationships among closely related species (Escudero et al., 2014; Hipp et al., 2014; Takahashi et al., 2014).

4.2. Interspecific hybridization

Although interspecific hybridization is well documented in terrestrial molluscs (*Cerion*: Galler and Gould, 1979; Woodruff and Gould, 1987; Woodruff, 1989; *Partula*: Johnson et al., 1993; *Cepaea:* Johnson, 1976; *Albinaria*: Schilthuizen and Lombaerts, 1994), our analyses of *Pyramidula* provided only weak (and after Bonferroni correction nearly no) evidence of gene flow between species. The strongest signal of ancestral interspecific hybridization, based on four taxon and partitioned D-statistic tests, was found between *P. pusilla* and *P. saxatilis*. Hybridization implies contact between both species. The distribution of *P. pusilla* is the widest among the studied species and overlaps with the distribution of almost all other species. In addition, the niche modeling analyses of Razkin et al. (under review), suggested that *P. saxatilis* is the only species whose ecological niche model does not significantly differ from that of *P. pusilla*. Besides, the species tree (Figure 5) suggests longer diversification processes in the group of *P. pusilla, P. saxatilis* and *P. rupestris* than in those of the remaining species. Thus, *P. pusilla* and *P. saxatilis* may have shared the same niche in the same area at some time, making admixture possible. Indeed, other studies on land snails have suggested hybridization at occasional contact zones or following diversification events (Douris et al., 2007).

The partitioned D-test provided no conclusive evidence with respect to the directionality of eventual gene flow between *P. pusilla* and *P. saxatilis*. Replicates of test 1 in which D_12 yielded significant differences in non-concordant allele patterns suggested gene flow between *P. pusilla* and *P. saxatilis*. However, in these replicates, the test did not find any

significant D_1 and D_2. These results can be explained by two different scenarios. The first one is that the direction of gene flow occurred from *P. pusilla* into *P. saxatilis*: the ancestor of the two *P. pusilla* lineages, or a lineage which diverged from this ancestor, could introgress into *P. saxatilis*. The alternative scenario is that gene flow passed from *P. saxatilis* into both *P. pusilla* lineages. Therefore, we cannot conclude in which direction eventual gene flow occurred.

The remaining species showed non-significant or very weak signals of ancestral interspecific gene flow and therefore, incomplete lineage sorting may explain the differences in the non-concordant allele patterns. The species tree (Figure 5) suggests that *P. cephalonica, P. chorismenostoma* and *P. jaenensis* underwent more rapid radiations, being hence good candidates for incomplete lineage sorting (Whitfield and Lockhart, 2007).

4.3. Species delimitation

Razkin et al. (under review) suggested nine putative species in the *Pyramidula* species complex in Europe. We tested four species delimitation scenarios using RADseq data and Bayes factor delimitation. These results confirmed that the base scenario of 9 putative species was the most plausible one. The four alternative scenarios included two clustering and two split scenarios. The marginal likelihood values obtained by the two split scenarios were close to the best scenario (base scenario). One explanation could be that arbitrarily splitting species is the most difficult scenario to distinguish from the true model if split populations are connected by moderate to high gene flow (Leaché et al., 2014).

In both, the present study and the study of Razkin et al. (under review), the construction of a species tree was necessary in the procedure of species delimitation. Razkin et al. (under review) constructed the species tree for the dataset of four genes using *BEAST implemented in BEAST v 1.8 (Drummond and Rambaut, 2007). This Bayesian method for tracing multispecies coalescense coestimates multiple gene trees embedded in a shared species tree (Heled and Drummond, 2010). Several nodes in the species tree obtained in Razkin et al. (under review) were not supported. In contrast, in the present study the species tree was inferred in SNAPP (Bryant et al., 2012). This method estimates the likelihood of species tree topology also based on a multispecies coalescent model, but using unlinked biallelic markers instead of sampling gene trees. The resulting species tree recovered all nodes with high posterior supports (> 0.99), except for the clustering of *P. cephalonica, P. chorismenostoma* and *P. jaenensis.* As suggested above, these species could have undergone a rapid radiation and hence, their phylogenetic relationships can be more difficult to resolve. The remaining part of the tree topology was fully supported, making its resolution more complete than that obtained using five gene fragments in Razkin et al. (under review). The topology obtained in the species tree supported the results of the phylogenetic inference on the concatenated RADseq dataset. These results demonstrated the benefit of using potentially unlinked markers to reconstruct a species tree. Since analyzing a large number of gene trees under the coalescent model is computationally challenging, using multiple

unlinked biallelic markers becomes a feasible alternative to infer species trees.

4.4. Implications for *Pyramidula*

RADseq resolved taxonomic issues for a cryptic species complex of the land snail *Pyramidula* that were undecided in the approach of Razkin et al. (under review) using morphological, niche modelling and DNA data. Analyzing mtDNA and nDNA trees separately enabled inspecting incongruence patterns. While the nDNA tree grouped all *P. cephalonica* samples (a-f) together, the mtDNA tree grouped *P. cephalonica* a-d samples and *P.* cf. *cephalonica* e-f samples separately. Although not supported (nor contradicted) in the mtDNA tree of the present study, the grouping of *P.* cf. *cephalonica* e-f with *P. chorismenostoma* was supported in the mtDNA tree of Razkin et al. (under review) (where more samples of both *P.* cf. *cephalonica* and *P. chorismenostoma* could be included). This incongruence between nDNA and mtDNA phylogenies, together with the distribution and morphology of the analyzed samples suggested the possibility of interspecific gene flow between *P. cephalonica* and *P. chorismenostoma*, or incomplete lineage sorting. The phylogenetic RADseq trees fully supported the grouping of all *P. cephalonica* samples (a-f) and, taking into account that the morphology and distribution of *P.* cf. *cephalonica* e-f concur with that of *P. cephalonica* a-d, we conclude that *P.* cf. *cephalonica* e-f and *P. cephalonica* a-d are conspecific. Interspecific hybridization tests did not find significant signals of ancestral interspecific gene flow between *P. cephalonica* and *P. chorismenostma*. Besides, branch lengths of the species tree suggested that the clade clustering *P. cephalonica, P. chorismenostoma* and *P. jaenensis* underwent rapid radiations, making their admixture more difficult. Gene flow levels are higher in those areas where species live in sympatry (Ballard and Whitlock, 2004) but in this case the samples analyzed of *P.* cf. *cephalonica* e-f and *P. chorismenostoma* were not sympatric, making the hypothesis of recent hybridization less plausible. All these information jointly suggested that incongruence between the mtDNA and nDNA gene trees regarding *P. cephalonica* and *P. chorismenostoma* may be due to incomplete lineage sorting of the mtDNA, due to a rapid speciation process (Avise, 2000). Incomplete lineage sorting has also been effectively documented in other molluscs (Periwinkles: Wilding et al. 2000; *Oncomelaria hupensis:* Wilke et al. 2005; pyrgulinid microgastropods: Schreiber et al. 2011).Although some shell characters could discriminate between some of the species in *Pyramidula*, they are not able to differentiate all of them, and hence, they are not valid as unique taxonomic criteria (Razkin et al., 2015). Particularly *P. pusilla* and *P. saxatilis* cannot be distinguished based on the relative height of the shell (height/diameter). Therefore, shell morphology would not be useful to infer interspecific gene flow between both species.

Two decades after Gittenberger and Bank (1996) defined the morphological species of *Pyramidula* in Europe splitting *Pyramidula rupestris* on conchological basis; we conclude that the present study could define the phylogenetic species and fully resolve their phylogenetic relationships using high throughput sequencing. However, a similar study including samples from Asia would be needed in order to elucidate the taxonomy of the remaining *Pyramidula* species.

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Supplementary material

Supplementary material 1: Results of the four taxon D-statistic tests and partitioned D-statistic tests of all replicates. Significant replicates are highlighted.

The file is available at: https://www.dropbox.com/s/hiok3idw3yb1kxa/SUP_1_Dtests.xlsx?dl=0

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PAPER IV

Evolutionary patterns of the western species of *Pyramidula* (Gastropoda, Stylommatophora)

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In preparation

Abstract

The study of the evolutionary history of the land snail *Pyramidula* is particularly interesting because it comprises cryptic species which can be delimited by means of genetic data and ecological niche requirements. On the one hand, closely related species with well differentiated ecological niches are good candidates to study their response to climatic fluctuations. On the other hand, unravelling the processes leading to the phenotypic evolution of cryptic taxa remains challenging because both adaptive and non-adaptive processes may be involved. With phylogenetic and distributional information of 264 individuals belonging to seven *Pyramidula* species from Europe and adjacent areas, the present study aimed to: (1) study historical biogeography of the western species of *Pyramidula*, reconstructing ancestral areas; (2) evaluate individual responses on the range of species during climatic fluctuations; and (3) explore the evolution of shell shape, reconstructing ancestral states and exploring adaptive forces. We found that different migration patterns shaped the diversification of the species in *Pyramidula*; that individual species responded with diverse range dynamics to climate fluctuations; and that cryptic species and the current variability on shell shape may have been the results of several adaptive and non-adaptive processes.

Keywords

Historical biogeography, ancestral reconstruction, ecological niche modeling, phenotypic evolution, *Pyramidula*

1. Introduction

The study of the evolutionary processes underlying species diversification is crucial to understand the variety of ecological, morphological and behavioural traits that species posses (Wiens et al., 2010). Historical biogeography deals with the geographic distributions of organisms over evolutionary time scales (Crisci et al., 2003). Ancestral geographic ranges of the species and past biogeographic events can be inferred by using information on phylogenetic relationships and present distribution data (Lomolino et al., 2010). Indeed, methods to reconstruct ancestral geographical distributions are increasing rapidly, as well as the development of new software packages (Yu et al., 2015).

Focusing in a recent evolutionary time scale, it is well established that climate cycles during the Quaternary have had a crucial influence in shaping current species distributions (Hewitt, 2000, 2004; Hofreiter and Stewart, 2009). Traditionally, the most accepted scenario has been that during glacial periods species ranges were restricted to southern glacial refugia and they expanded northward during interglacial periods, under more favourable conditions (Hewitt, 2000). This scenario was complicated by the suggestion that cryptic northern refugia had existed for some temperate species (Willis et al., 2000; Stewart and Lister, 2001). Recent works have suggested that either glacial or interglacial refugia may have existed depending on individualistic species' response to climate changes, according to their adaptations and environmental tolerances (Stewart et al., 2010). Therefore, glacial southern refugia should be considered as the traditional ones for temperate species; while interglacial polar refugia might have kept cold-adapted species at higher latitudes, during interglacial periods (Stewart et al., 2010). Thus, temperate species would have expanded during interglacial periods and cold-adapted species during glacial periods.

Currently, the identification of refugia and expansion/contraction patterns can be inferred by applying the ecological niche modelling (ENM) procedure: ecological niche models (ENMs), which are produced using the present distribution and environmental data, can be projected onto historical landscapes [e.g. Last Glacial Maximum (LGM) or Last Inter Glacial (LIG)] (Marske et al., 2011; Hidalgo-Galiana et al., 2014; Anadón et al., 2015; Feuda et al., 2015). This approach also enables inspecting how current climate change may affect species' distributions under different climate models for the future (Bystriakova et al., 2014). ENM has proven to be an important tool for phylogeography, becoming particularly useful for those species with no fossil data (Hugall et al., 2002; Carstens and Richards, 2007). Based on niche conservatism, the tendency of species to retain their niches and related ecological traits over time (Harvey and Pagel, 1991), it is expected that closely related taxa show differentiated responses to climatic fluctuations.

Unravelling how phenotypic differentiation and variability occurs is another issue of evolutionary biology. On the one hand, adaptive radiation is considered to be the main force producing phenotypic divergence as the novel species adapt to different ecological niches (Gavrilets and Losos, 2009). On the other hand, niche conservatism might maintain phenotypic traits within species. Cryptic species, morphologically similar organisms, are

of particular interest to study phenotypic evolution (Smith et al., 2011) because similar phenotypes may have originated under both adaptive and non-adaptive processes (Schluter, 2000).

Pyramidula is a cryptic species complex of land snails distributed over almost all of Europe, the Mediterranean area, Central Asia and Japan (Gómez-Moliner, 1988; Welter-Schultes, 2012). Historically, species have been morphologically defined, based on highly correlated shell characters (Gittenberger and Bank, 1996; Martínez-Ortí et al., 2007). Nine species have been delimited for the western Palaearctic region on the basis of multilocus DNA and niche modelling by Razkin et al. (under review-a). This study showed that ecological niches observed for each species were significantly different between them. Shell characters were also studied, concluding that they were not valid as unique taxonomic criterium and that they could have been influenced by environmental factors. All aforementioned characteristics makes the study of the evolutionary history of *Pyramidula* interesting, because (1) it comprises closely related species with well differentiated ecological niches, being good candidates to show independent responses to climatic fluctuations; and (2) species are cryptic for shell shape, character that may have been influenced by adaptive forces.

The present study was designed to: (1) study historical biogeography of the western species of *Pyramidula*, reconstructing ancestral areas; (2) evaluate individual responses on the range of species during climatic fluctuations; and (3) study the evolution of shell shape, reconstructing ancestral states and exploring adaptive forces.

2. Methodology

2.1. Sample data

Seven out of the nine species delimited in Razkin et al. (under review-a) were included in the analyses. The other two species were not included due to low number of specimens with morphological and geographical information. A total of 264 individuals of *Pyramidula* were included in the analyses. Sequence data of COI, 16S rRNA and 5.8S (partial) - ITS2 - 28S (partial), and measures of shell characters from Razkin et al. (under review-a) were employed (voucher numbers of the used specimens are accessible in Supplementary material 1).

2.2. Phylogenetic analyses

Sequences of 190 individuals were aligned with Mafft v7 online version (Katoh, 2002; Katoh and Standley, 2013) as this method has been described to perform better than other pairwise alignment methods (Golubchik et al., 2007). We used the Q-INS-i algorithm for rRNA, which considers the secondary structure of RNA (Katoh and Toh, 2008), and default values were assigned to the remaining parameters. Evolutionary models were estimated independently for each of the gene partitions using jModelTest v2.1.1 (Darriba et al., 2012) applying the Akaike Information Criterion (AIC). *Chondrina avenacea* was

used as outgroup.

Phylogenetic relationships between species were already determined in Razkin et al. (under review-b), where Restriction-site associated DNA sequencing (RADseq) technology was employed. Therefore, we only conducted a Bayesian phylogenetic analysis using BEAST 1.8 (Drummond et al., 2012) in order to obtain the required trees for subsequent analyses. Two independent runs were performed using all gene fragments. Substitution models were adjusted to the models obtained with jModeltest and SRD06 model was selected for COI. Yule process was employed for the speciation prior. Since no calibration point was available for *Pyramidula* and the estimation of divergence times was not aim of this study, a strict clock to accommodate rate variation among lineages was applied. States were screened every 10,000 steps over a total chain length of 33,000,000 steps, being the 10% of them discarded as burn-in. Information in the set of post burn-in states was checked using Tracer v1.6 (Rambaut et al., 2014) not allowing ESS values < 200. All post burn-in trees were combined in order to obtain a total of 2,000 trees. The maximum clade credibility tree was obtained from TreeAnnotator v1.8.

2.3. Biogeographic analyses

2.3.1. Ancestral area reconstruction

The geographic evolution of the genus in its western distribution range was inferred performing two alternative reconstruction methods, both of them implemented in the software Reconstruct Ancestral States in Phylogenies (RASP; Yu et al., 2015): Statistical Dispersal-Vicariance Analysis (S-DIVA; Yu et al., 2010) and Dispersal-Extinction-Cladogenesis (DEC; Ree and Smith, 2008) (the implementation in RASP of the model of Lagrange (Smith, 2009)). A maximum of two areas was allowed at each node and biologically unlikely ranges were excluded. The 2,000 trees and the maximum clade credibility tree obtained from BEAST were used.

Seven distribution areas were considered: A) the eastern part of the sampling area, including Anatolia, the area surrounding the Black Sea and Cyprus; B) Balkan Peninsula, Aegean Islands and Crete; C) Italian Peninsula and adjacent islands; D) Central Europe; E) Iberian Peninsula and Balearic Islands; F) North Africa and G) British Islands.

2.3.2. Range dynamics in climate changes

In order to explore to what extent distribution ranges of different species in *Pyramidula* were modified or may change in response to climate changes, ENM procedure was applied to different climatic conditions. Apart from the georeferenced specimens used for phylogenetic analyses, localities of other 74 specimens were included in this section (Supplementary material 2). Their species identification was determined from unpublished COI sequence data. A total of 256 locality points were included: *P. cephalonica*, n=21; *P. chorismenostoma*, n=36; *P. jaenensis*, n=60; *P. pusilla*, n=86; *P. rupestris*, n=38 and *P. saxatilis*, n=15. ENM was not performed for *Pyramidula* sp2 because only six locality points were available.

Maxent v.3.3.3 (Phillips et al., 2006) software was used to create ENMs of each species as it performs better than other methods (Phillips et al., 2006; Wisz et al., 2008). Environmental layers were downloaded from the WorldClim database (Hijmans et al., 2005; http://www.worldclim.org/) at a 2.5 arc-minutes resolution. So as not to overparameterize niche models, we included the variables selected in Razkin et al. (under review-a) for *Pyramidula* where a Pearson correlation coefficient cutoff of 0.75 was applied. The selection consisted of 10 climatic variables: isothermality (bio3), temperature seasonality (bio4), maximum temperature warmest month (bio5), mean temperature wettest quarter (bio8), annual precipitation (bio12), precipitation wettest quarter (bio13), precipitation driest month (bio14), precipitation wettest quarter (bio16), precipitation warmest quarter (bio18) and precipitation coldest quarter (bio19).

We ran Maxent applying a random test percentage of 25%, choosing the logistic output format and creating response curves. The area under the ROC curve (AUC) of the tested data was used as a measure of the present time model's predictive power (Phillips et al., 2006). Values above 0.75 are considered as potentially useful (Elith, 2000). ENMs were projected into Last Glacial Maximum (LGM; 21 ky BP) and future (2070 AD, average for 2061-2080; based on representative concentration pathway (RCP) 6.0) conditions. All projections were downloaded from WorldClim. In order to account for uncertainty of the projections, two alternative climatic models were used for LGM and future: the Community Climate System Model (CCSM) and the Model for Interdisciplinary Research on Climate (MIROC). The importance and effects of each bioclimatic variable were evaluated exploring percent contribution, permutation importance and response curves generated by Maxent.

2.4. Shell shape evolution

2.4.1. Ancestral state reconstruction

We performed ancestral state reconstruction for one morphological shell character, the relative height (H/D = height / maximum diameter). This character describes the general shell shape (conical *vs.* low) and it has been used in several evolutionary studies (Cain, 1977, 1978; Cain and Cowie, 1978; Cook and Jaffar, 1984; Goodfriend, 1986; Harley et al., 2009). Analyses were performed in Mesquite v2.75 (Maddison and Maddison, 2011) using the maximum clade credibility tree obtained from BEAST. Reconstructions were performed with a squared parsimony model applying a Brownian motion model.

2.4.2. Hypotheses testing for shell shape evolution

Razkin et al. (under review-a) concluded that shell shape in *Pyramidula* may be influenced by environmental factors. Therefore, we performed Pearson's correlation tests between shell shape measures used in section 2.4.1 and environmental variables used in 2.3.2. Pearson tests were performed in two levels: (a) using information of all *Pyramidula* individuals together; and (b) using information of individuals of the different species separately. Significant

correlations were considered the result of adaptive processes of the shell shape in response to environmental factors. In this case, two alternative hypotheses were postulated:

i) Adaptive radiation + niche conservatism: phenotypic divergence occurred in association with ecological divergence under adaptive radiation; but based on niche conservatism, further adaptive forces did not take place within delimited species. Hence, we assume that the variability on shell shape within species is not due to adaptive response to environmental factors. Taking into account this hypothesis, Pearson tests "a" should be significant and Pearson tests "b" should be not significant.

ii) Adaptive radiation + adaptive forces within species: microenvironmental variability would also affect phenotypic adaptation within species and this would explain the existing variability on shell shape within species. Taking into account this hypothesis, all Pearson tests (both, "a" and "b" levels) should find significant correlations.

3. Results

3.1. Phylogenetic inference

The data set comprised 190 representatives of the genus, with 2493 aligned characters. More information about the sequences of each gene and the complete data set are shown in Supplementary material 3.

The maximum clade credibility tree obtained from Beast is shown in Figure 1. Relationships between species were fully supported. Compared to the phylogeny obtained in Razkin et al. (under review-b) based on RADseq data, only one of the relationships was found to be different: RADseq data closely related *P. cephalonica* to the clade grouping *P. chorismenostoma* and *P. jaenensis*, while the topology obtained from BEAST clustered *P. cephalonica* with *Pyramidula* sp2. Anyway, in both phylogenetic inferences the four species of the clade I (*P. cephalonica, P. chorismenostoma, P. jaenensis* and *Pyramidula* sp2) formed a monophyletic group. Species of the clade II also were monophyletic, including *P. rupestris, P. saxatilis* and *P. pusilla*. The first two species were closely related forming a sister clade to *P. pusilla.*

3.2. Biogeographic analyses

3.2.1. Ancestral area reconstruction

Ancestral area reconstruction for 13 major nodes is shown in Figure 1. The results obtained for the inferred ancestral areas using S-DIVA and DEC were not identical, but they always coincided in their optimal solutions. At species level, both methods found the same and unique area for the ancestor of *Pyramidula* sp2 (A), *P. cephalonica* (B) and *P. chorismenostoma* (B). The inhabiting area for the ancestor of *P. jaenensis* was inferred to be mainly CE; for the ancestor of *P. rupestris* DE or E; and for *P. saxatilis* CD or D. For *P. pusilla* both methods suggested several possible ancestral areas, mainly BD and D. The ancestor of the clade I was inferred to have originated mainly in BC or B. For the clade II, S-DIVA inferred that the ancestral area was D, but DEC suggested several areas (all of them including D). The most

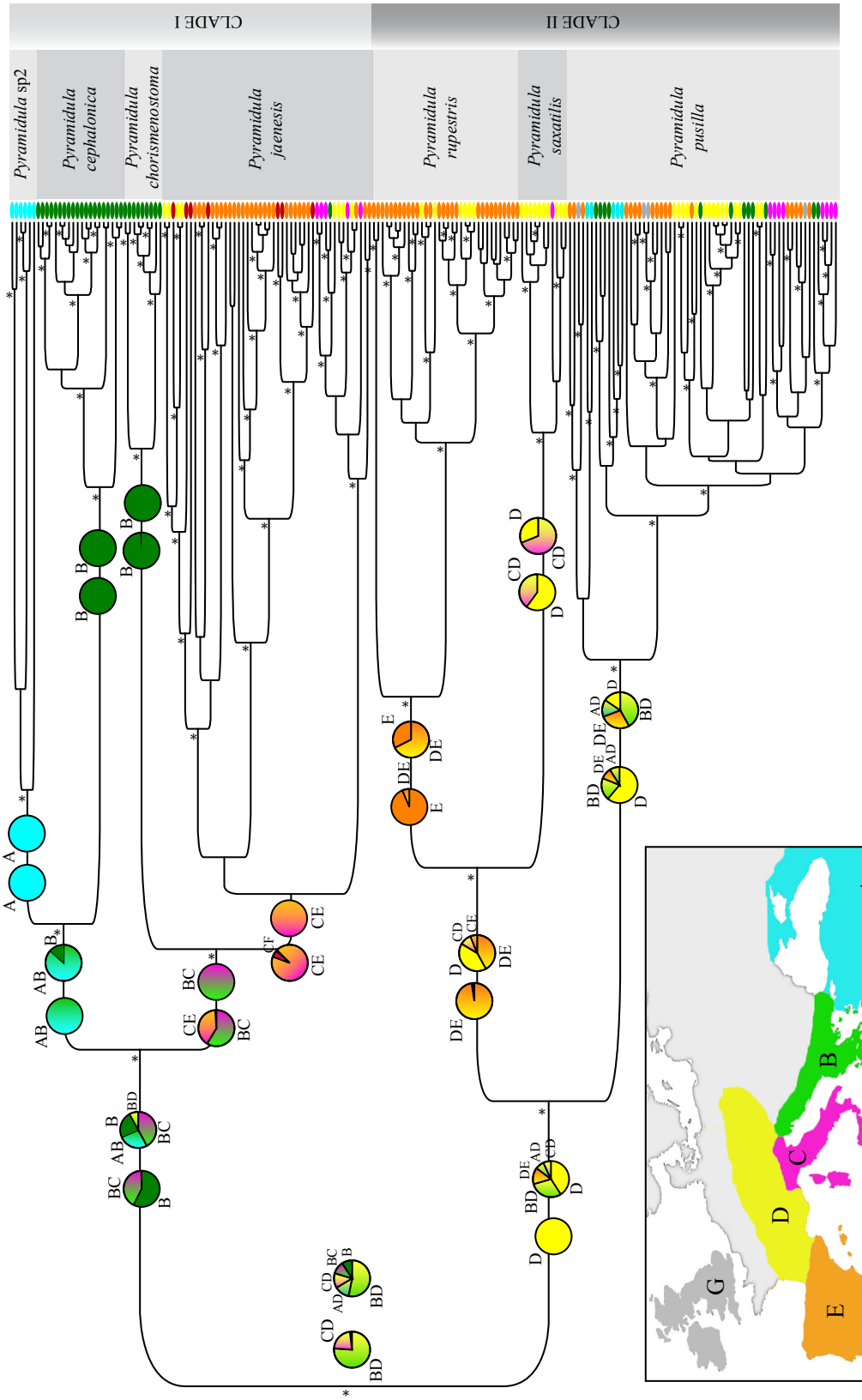

Figure 1: Maximum clade credibilty tree inferred in BEAST, based on COI, 16S rRNA and 5.8S - ITS2 - 28S sequence data. Posterior probabilities > 0.95 are indicated with an asterisk (*). Pie charts in the nodes indicate reconstruction of ancestral areas (left: S-DIVA, right: DEC). Colors and letters of pie charts refer to the areas described in the map of the left bottom and in the text (section 2.3.1).

probable ancestral area of the ingroup was inferred to be BD.

3.2.2. Range dynamics in climate changes

ENMs created for present time showed good prediction ability, obtaining AUC values always higher than 0.85 (Table 1). Percentages of relative contributions of environmental variables in the ENMs for each species were different (Table 1). On average, the two variables with the highest explanatory power for the genus were temperature seasonality (bio4) and precipitation of the warmest quarter (bio18). Response curves of each species to bio4 and bio18 are shown in Figure 2. The response curves of bio4 showed that *P. cephalonica, P. chorismenostoma, P. jaenensis* and *P. rupestris* reached the maximum probability of presence when temperature seasonality (calculated as standard deviation) range between 5,5 ºC and 6,5 ºC, while the probability of presence for *P. pusilla* and *P. saxatilis* decrease above the value of 3 ºC. The response curves of bio18 showed that *P. cephalonica, P. corismenostoma, P. jaenensis* and *P. rupestris* reached the maximum probability of presence between 0 mm and 125 mm, while the probability of presence for *P. pusilla* and *P. saxatilis* kept increasing until 600 mm.

ENMs for the LGM, present time and future for the six species are shown in Figures 3-8. LGM and future ENMs were created under two alternative climatic projections (CCSM and MIROC). To avoid including too many maps in the main text, the maps displayed for LGM and future were created using average probabilities of both projections. All original ENMs based on CCSM and MIROC projections are available in Supplementary material 4.

Table 1: Percentages of the relative contribution of the environmental variables to the ecological niche model of each species and AUC evaluation value for each model. Bio3 = isothermality, bio4 = temperature seasonality, bio5 = maximum temperature warmest month, bio8 = mean temperature wettest quarter, bio12 = annual precipitation, bio13 = precipitation wettest quarter, bio14 = precipitation driest month, bio16 = precipitation wettest quarter, bio18 = precipitation warmest quarter and bio19 = precipitation coldest quarter.

Variable	*P. cephalonica*	*P. chorismenostoma*	*P. jaenensis*	*P. pusilla*	*P. rupestris*	*P. saxatilis*	Genus (average)
bio3	0	24	9,1	2,7	7,5	0,2	7,25
bio4	11,9	18,6	42,8	16,9	63,8	0,8	25,8
bio5	4,1	0,3	4,7	12,5	0	3,6	4,2
bio8	3,8	0,8	6,7	16,1	0,9	0,7	4,83
bio12	0	0	18	33,6	1,3	0	8,82
bio13	0	6,7	2,4	2,9	3,5	0	2,58
bio14	11,9	4,2	5,6	9,1	0	0	5,13
bio16	0,2	0	0	0	0	0	0,03
bio18	3,2	38	5,3	2,1	12,9	93,9	25,9
bio19	64,8	7,5	5,5	4	10,2	0,8	15,47
AUC	0,981	0,998	0,854	0,868	0,936	0,971	

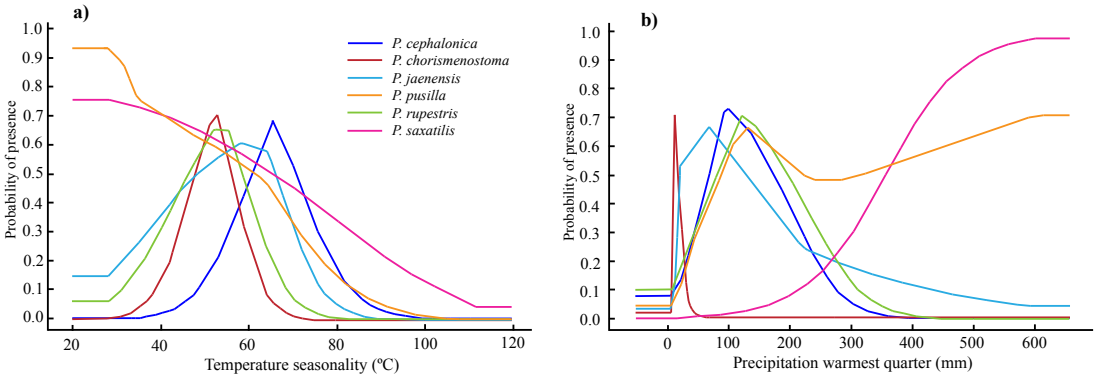

Figure 2: Response curves of *Pyramidula* species to a) temperature seasonality, and b) precipitation of the warmest quarter.

ENM of *P. cephalonica* (Figure 3): the potential distribution during the LGM was confined to a north-western area of the Balkans and the north-eastern coast of the Black Sea. Current model found larger suitable areas expanding southwards in the Balkan Peninsula and towards western Turkey. The prediction for the future was similar to the model of the present time.

ENM of *P. chorismenostoma* (Figure 4): the projection of the species distribution to the LGM did not find suitable areas with high probabilities of presence, obtaining different results for CCSM and MIROC models (Supplementary material 4b). For the present time the potential distribution expanded to all Aegean islands and Crete. In the future potential distribution suitable areas were restricted to the central Aegean islands and central and eastern Crete.

ENM of *P. jaenensis* (Figure 5): the projection to the LGM found suitable areas mainly in the coastal areas of the western Mediterranean Basin, Balearic Islands and central Iberian Peninsula. Potential distribution for the model of the present expanded towards the central mountain ranges of the Iberian Peninsula, the SW Iberian Peninsula and NW Africa. For the future, potential distribution reduced mainly to the northern Iberian Peninsula, WN Africa and the south and west of France.

ENM of *P. pusilla* (Figure 6): suitable areas in the LGM occupied a large part of the study area, including the land bridge of NW Europe, which joined Europe and the British Islands. NE Europe and southern areas found the lowest probability of presence. For the present, the potential distribution was found along the main mountain ranges of Europe as well as in the Atlantic Lusitanic region (including British Islands, Portugal, NW Iberian Peninsula and west of France). These suitable areas decreased in the projection to the future.

ENM of *P. rupestris* (Figure 7): suitable areas during the LGM were confined to southern France and eastern Iberian Peninsula. The model for the present found similar suitable areas, but the potential distribution expanded to areas in the North Iberian Peninsula. The

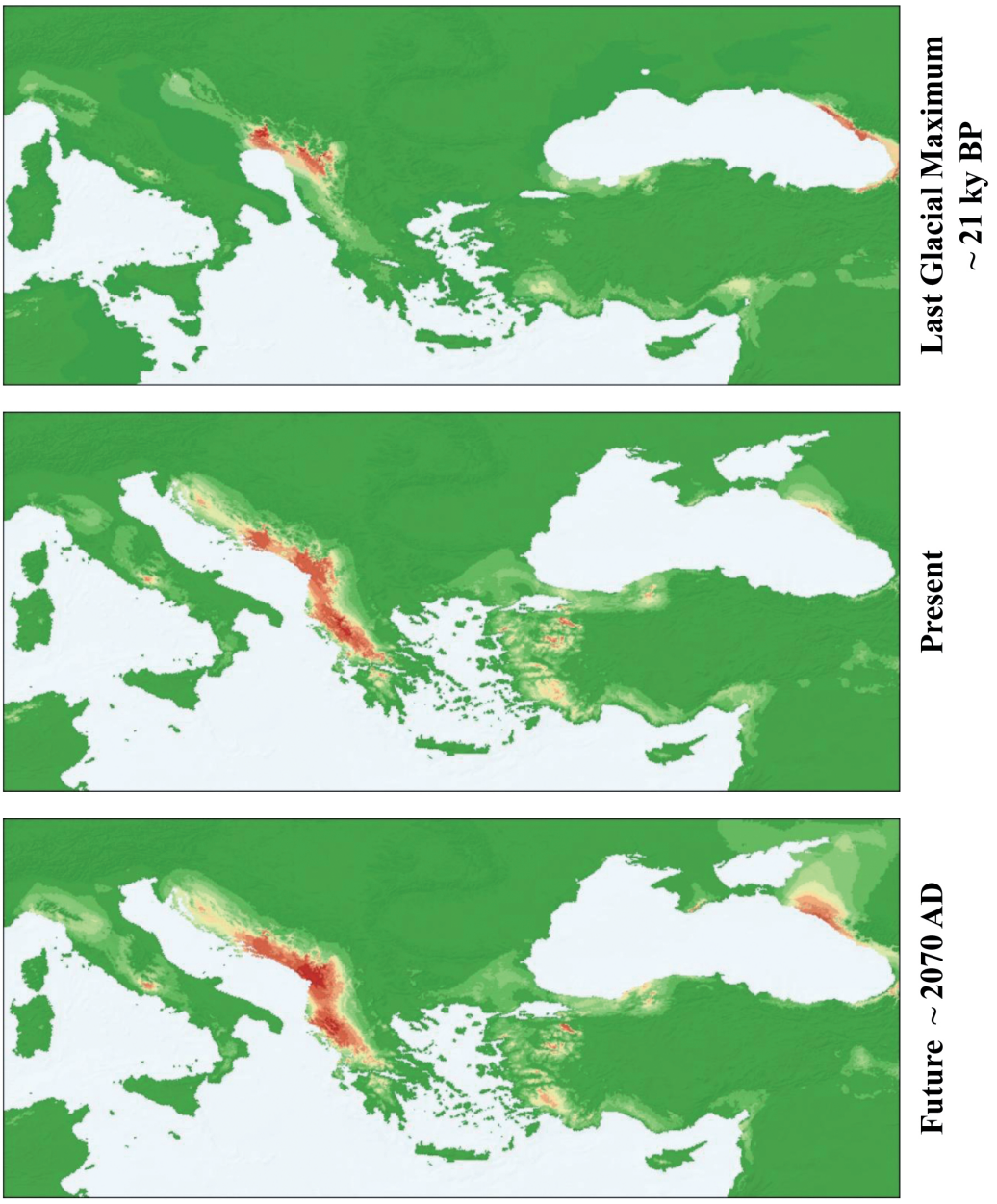

Figure 3: Ecological Niche Models for *P. cephalonica* for the Last Glacial Maximum (average probabilities of MIROC and CCSM models), present-day conditions and future based on RCP 6.0 (average probabilities of MIROC and CCSM models). Reddish colours indicate better-predicted conditions.

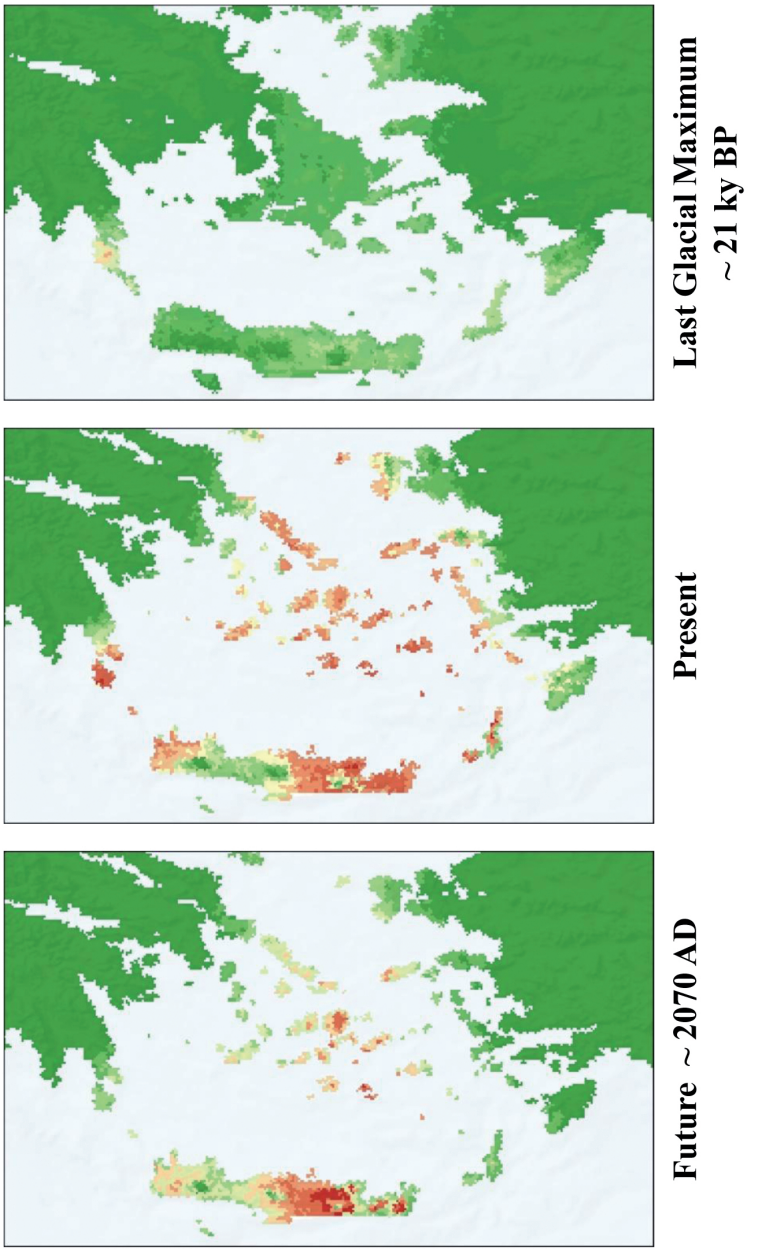

Figure 4: Ecological Niche Models for *P. chorismenostoma* for the Last Glacial Maximum (average probabilities of MIROC and CCSM models), present-day conditions and future based on RCP 6.0 (average probabilities of MIROC and CCSM models). Reddish colours indicate better-predicted conditions.

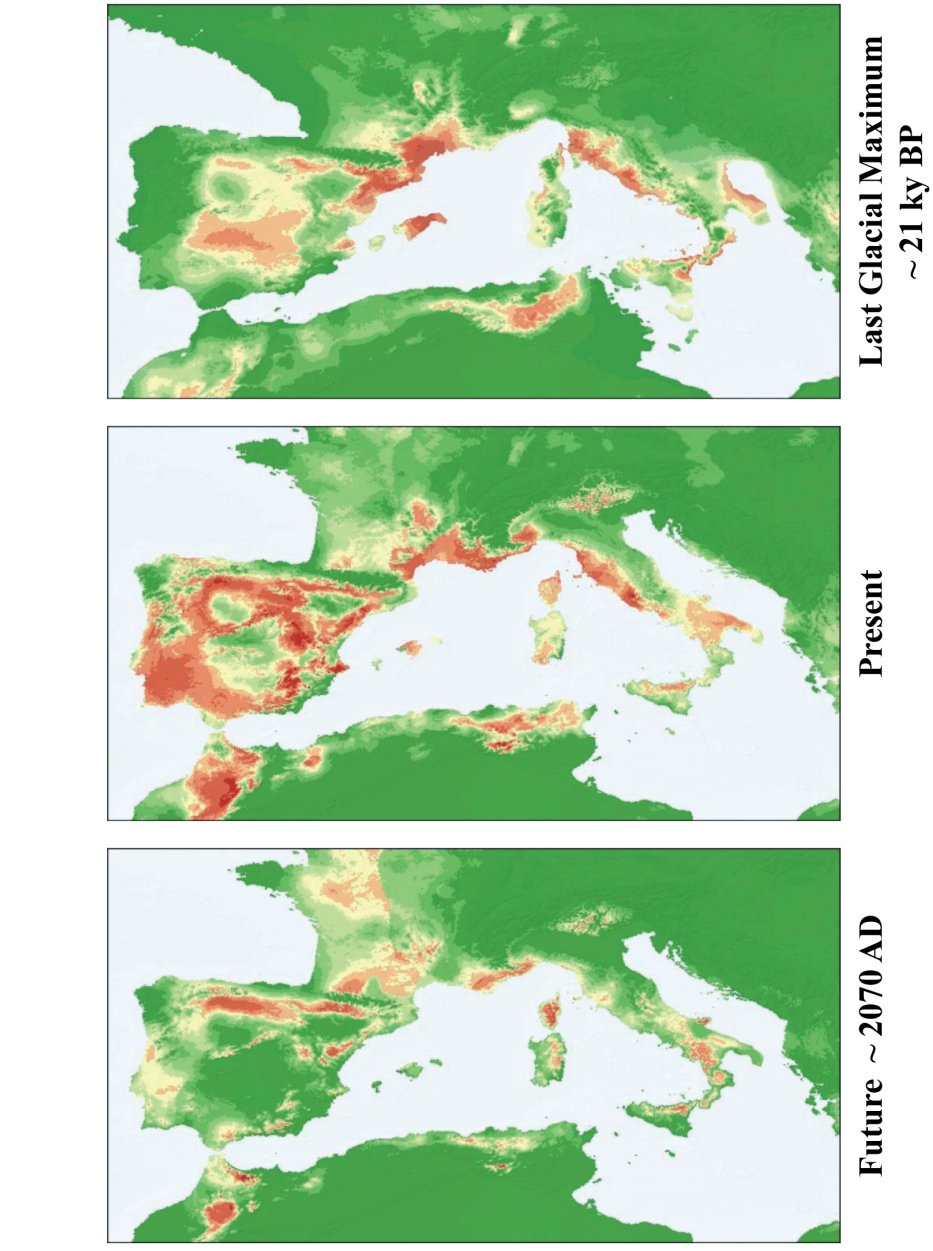

Figure 5: Ecological Niche Models for *P. janensis* for the Last Glacial Maximum (average probabilities of MIROC and CCSM models), present-day conditions and future based on RCP 6.0 (average probabilities of MIROC and CCSM models). Reddish colours indicate better-predicted conditions.

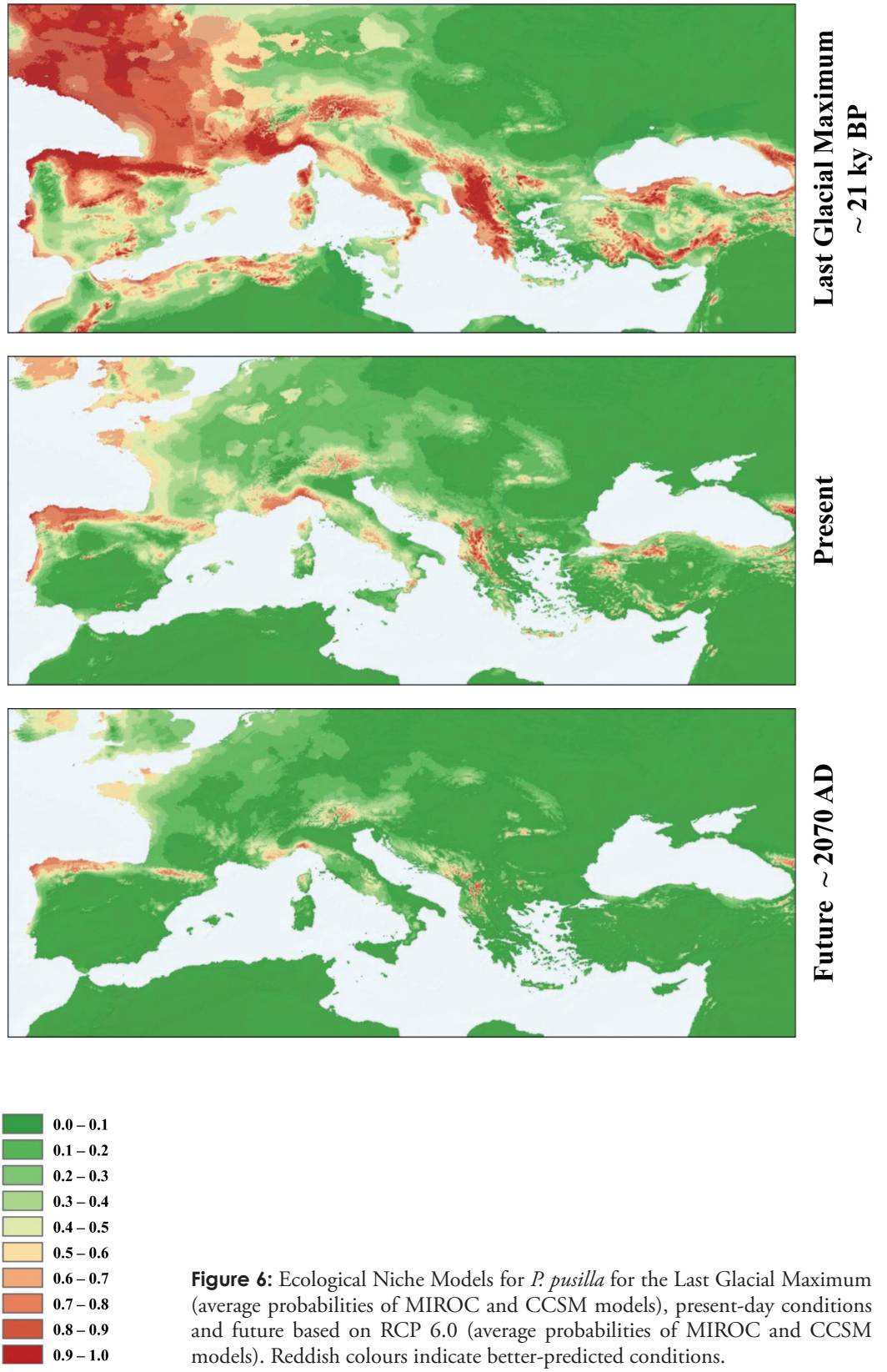

Figure 6: Ecological Niche Models for *P. pusilla* for the Last Glacial Maximum (average probabilities of MIROC and CCSM models), present-day conditions and future based on RCP 6.0 (average probabilities of MIROC and CCSM models). Reddish colours indicate better-predicted conditions.

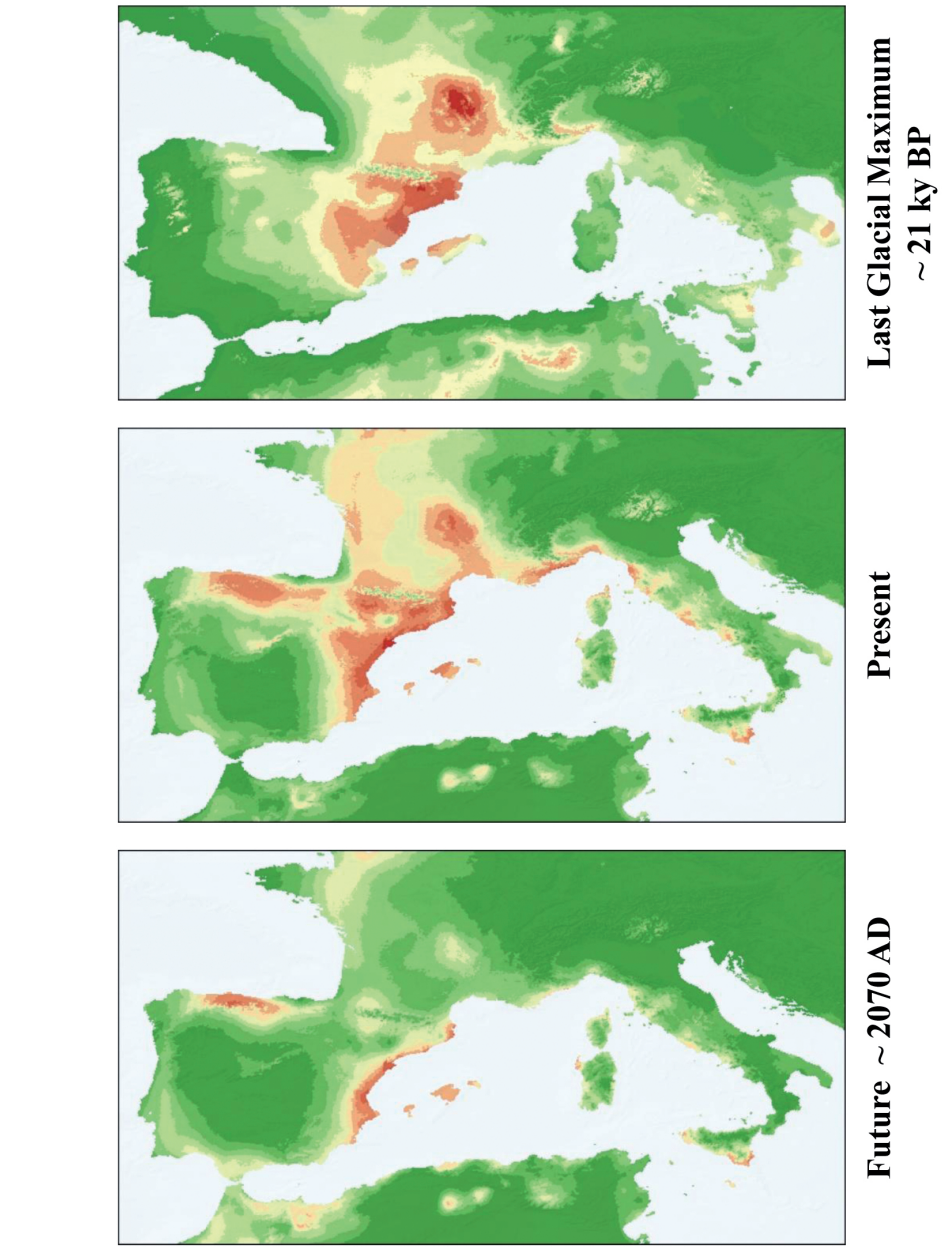

Figure 7: Ecological Niche Models for *P. rupestris* for the Last Glacial Maximum (average probabilities of MIROC and CCSM models), present-day conditions and future based on RCP 6.0 (average probabilities of MIROC and CCSM models). Reddish colours indicate better-predicted conditions.

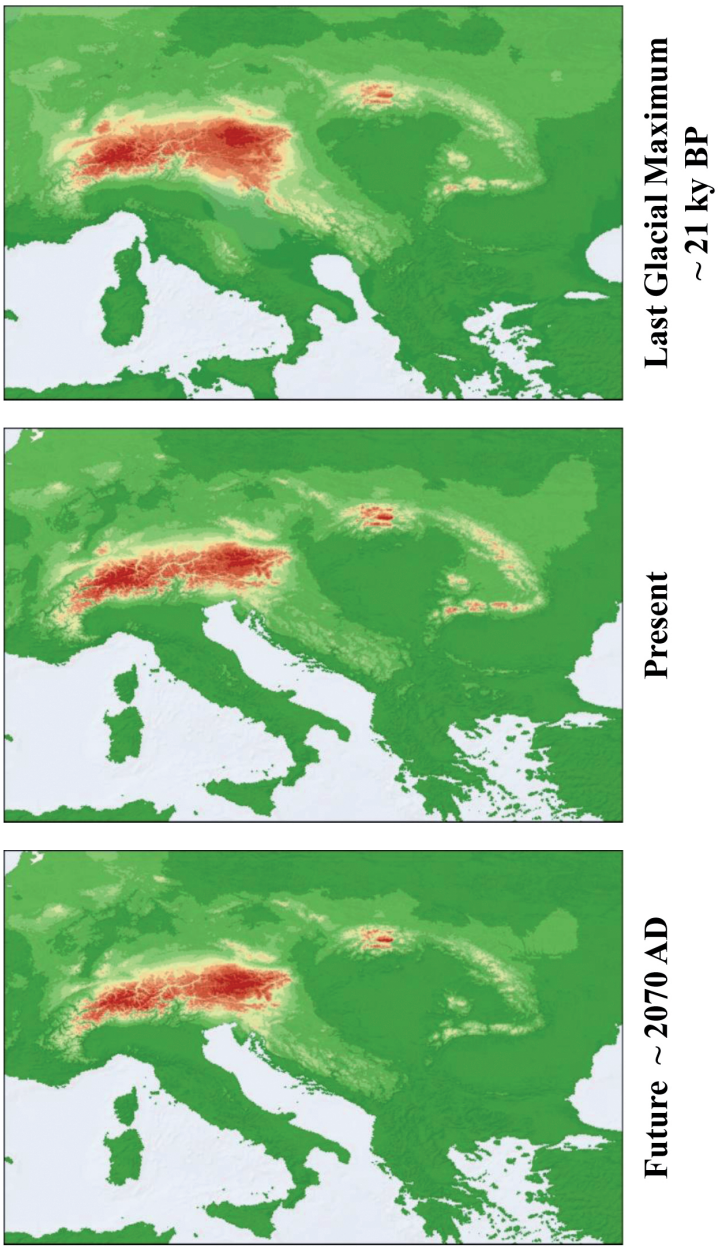

Figure 8: Ecological Niche Models for *P. saxatilis* for the Last Glacial Maximum (average probabilities of MIROC and CCSM models), present-day conditions and future based on RCP 6.0 (average probabilities of MIROC and CCSM models). Reddish colours indicate better-predicted conditions.

projection to the future only found suitable areas in the north and the Mediterranean coast of the Iberian Peninsula.

ENM of *P. saxatilis* (Figure 8): The most suitable areas for all the distribution models were located in the Alps and the Carpathians. The largest habitat suitability was found in the projection of the LGM.

3.3. Shell shape evolution

3.3.1. Ancestral state reconstruction

Character state reconstruction found differences in the shell shape of the ancestors of each species (Figure 9). The ancestral state of *Pyramidula* sp2, *P. saxatilis* and *P. pusilla* was low or moderately low conical, always ≤ 0.75 (*Pyramidula* sp2, H/D = 0.75; *P. saxatilis,* H/D = 0.75 and *P. pusilla,* H/D = 0.68), while the ancestral state of *P. chorismenostoma*, *P. jaenensis* and *P. rupestris* was approximately conical, > 0.85 (*P. chorismenostoma,* H/D = 0.89; *P. jaenensis,* H/D = 0.86 and *P. rupestris,* H/D = 0.87). The ancestor of *P. cephalonica* was moderately low conical (H/D = 0.82). Shell shape descriptions based on H/D measures are available in Razkin et al (under review-a).

3.3.2. Hypotheses testing for shell shape evolution

Results of Pearson tests are summarized in Table 2. Using information of all individuals together (a), pairwise comparisons found significant correlations between shell shape and eight environmental variables. The three variables that obtained the highest Pearson's correlation coefficient values (r) were bio5 (maximum temperature warmest month), bio14 (precipitation driest month) and bio18 (precipitation warmest quarter). The H/D ratio increases with the increment of bio5 and decreases with the increment of bio14 and bio18. Analyzing each species separately (b) did not yield significant correlations between shell shape and environmental variables for all species, excepting *P. rupestris*, in which there was a significant correlation between shell morphology and seven environmental variables. The three variables that obtained the highest Pearson's correlation coefficient values (r) were bio5 (maximum temperature warmest month), bio8 (mean temperature wettest quarter) and bio14 (precipitation driest month). The H/D ratio increases with the increment of bio5 and bio8 (positive r) and it decreases with the increment of bio14 (negative r).

4. Discussion

4.1. Biogeographic analyses

4.1.1. Ancestral area reconstruction

Historical biogeographic inferences are nowadays successfully performed with the combination of divergence time estimations and ancestral area reconstructions (Santos-Gally et al., 2012; Beaulieu et al., 2013; Kaliszewska et al., 2015; Psonis et al., 2015). Divergence time estimations from sequence data are being widely applied using the recently

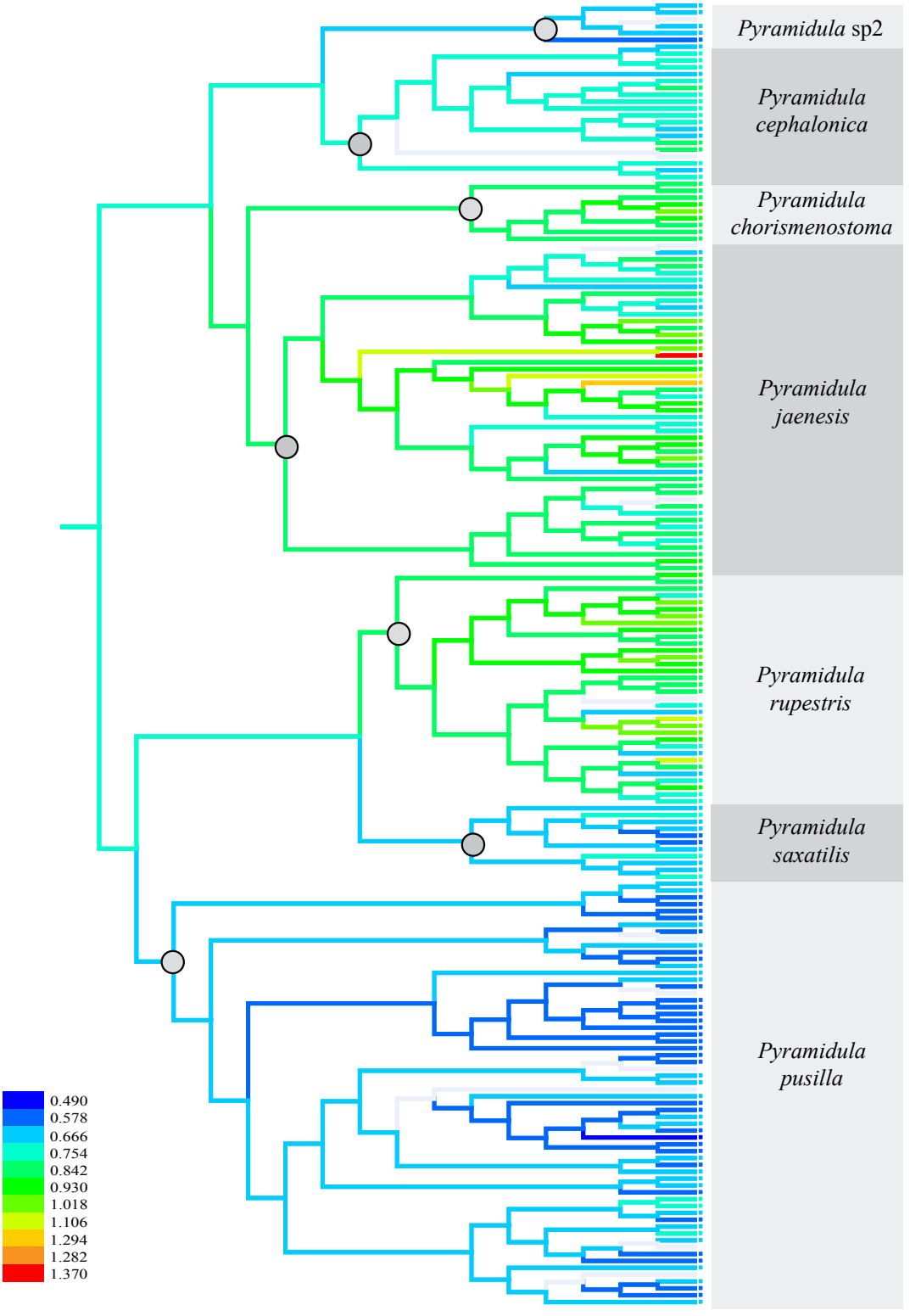

Figure 9: Ancestral state reconstruction of the relative shell height based on maximum clade credibility tree inferred in BEAST. Branches are colour coded to represent different values of the relative shell height (H/D). Terminals and branches without colour indicate unknown values.

Table 2: Relationship between relative shell heigth (H/D) and bioclimatic variables according to Pearson's correlation method. Bold numbers indicate significant p values.

	Pyramidula	*P. cephalonica*	*P. chorismenostoma*	*P. jaenensis*	*P. pusilla*	*P. rupestris*	*P. saxatilis*
bio3 – H/D	**r = 0.32**	r =-0.02	r =0.81	r =0.15	r =-0.016	r =0.19	r =-0.24
	p = 2,02E-02	p = 0,9180	p = 0,0139	p = 0,3242	p = 0,9094	p = 0,2999	p = 0,4685
bio4 – H/D	r = -0.18	r =-0.11	r =0.41	r =-0.043	r =-0.14	r =0.25	r =0.20
	p = 0,0160	p = 0,6334	p = 0,3184	p = 0,7788	p = 0,2964	p = 0,1709	p = 0,5510
bio5 – H/D	**r = 0.50**	r =-0.02	r =0.57	r =0.19	r =-0.02	**r =0.59**	r =0.43
	p = 2,29E-09	p = 0,9093	p = 0,1420	p = 0,2284	p = 0,8795	**p = 0,0004**	p = 0,1816
bio8 – H/D	**r = 0.21**	r =-0.31	r =-0.18	r =-0.33	r =-0.10	**r =0.59**	r =0.17
	p = 0,0065	p = 0,1680	p = 0,6696	p = 0,0289	p = 0,4516	**p = 0,0004**	p = 0,6124
bio12 – H/D	r = **-0.39**	r =-0.16	r =0.45	r =-0.15	r =-0.15	**r =-0.53**	r =-0.33
	p = 1,36E-04	p = 0,4833	p = 0,2653	p = 0,3492	p = 0,2605	**p = 0,0016**	p = 0,3210
bio13 – H/D	**r = -0.24**	r =0.02	r =0.55	r =-0.11	r =0.12	**r =-0.31**	r =-0.46
	p = 0,0017	p = 0,9403	p = 0,1551	p = 0,4815	p = 0,3733	**p = 0,0812**	p = 0,1502
bio14 – H/D	**r = -0.47**	r =-0.25	r =0.27	r =-0.23	r =0.01	**r =-0.62**	r =-0.20
	p = 6,74E-08	p = 0,2841	p = 0,5248	p = 0,1334	p = 0,9385	**p = 0,0002**	p = 0,5537
bio16 – H/D	**r = -0.26**	r =-0.007	r =0.57	r =-0.06	r =0.12	r =-0.42	r =-0.43
	p = 0,0005	p = 0,9761	p = 0,1463	p = 0,6805	p = 0,4029	p = 0,0175	p = 0,1821
bio18 – H/D	**r = -0.48**	r =-0.25	r =-0.10	r =-0.30	r =-0.14	**r =-0.55**	r =-0.51
	p = 1,18E-08	p = 0,2714	p = 0,8216	p = 0,0512	p = 0,3229	**p = 0,0011**	p = 0,1107
bio19 – H/D	r = -0.11	r =0.05	r =0.57	r =0.08	r =021	**r =-0.46**	r =-0.18
	p = 0,1577	p = 0,8446	p = 0,1417	p = 0,6177	p = 0,1211	**p = 0,0079**	p = 0,5905
	a	b					

developed Bayesian approach implemented in BEAST (Drummond et al., 2012). However, they need reliable calibrations (usually based on fossil data or dated biogeographic events) because it is not possible to estimate absolute ages from molecular data alone (Ho and Phillips, 2009). There is no available calibration point for *Pyramidula* and hence, divergence times could not be estimated. It is also possible to use estimates of the mutation rate as a proxy for substitution rates. Unfortunately, the substitution rate for the sequenced genes is unknown for *Pyramidula*. Substitution rates can vary considerably between lineages; for example, substitution rates of the mitochondrial genes for some terrestrial gastropods vary between 1% and 20% nucleotide divergence per million years (Chiba, 1999; Douris et al., 1998; Hayashi and Chiba, 2000; Pfenninger et al., 2003). Therefore, there is no accurate value to infer time divergences for *Pyramidula*, and we focused on the geographical origin of the diversification of the species.

The present study focused only on the species of *Pyramidula* inhabiting Europe and adjacent areas. Therefore, the reconstruction of ancestral areas is limited to the species of *Pyramidula* from Europe and adjacent areas.

The migration patterns of the species in both clades I and II were different (Figure 1). Clade I had a Mediterranean origin and diversification. The inhabiting area for the ancestor of *Pyramidula* sp2 was Anatolia, while for the ancestor of *P. cephalonica* and *P. chorismenostoma* was the Balkan Peninsula. The most probable area for the ancestor of *P. chorismenostoma* and *P. jaenensis* was found to be mainly the Balkan and Italian Peninsulas; and the most probable area for the ancestor of *P. jaenensis* was found to be the south of the Alps and Italian and Iberian Peninsulas. All these suggest that the migration of *P. jaenensis* occurred westward following a Mediterranean route. This result is consistent with other studies on invertebrates and plants for which the dispersial also occurred westward in the Mediterranean region (Lo Presti and Oberprieler, 2009; Micó et al., 2009; Allegrucci et al., 2011; Condamine et al., 2013). Thus, the presence of *P. jaenensis* in Central Europe and North Africa seems to be due to posterior dispersal processes. Indeed, long distance dispersion in *Pyramidula* may have occurred easily since passive dispersal, primarily by birds, have already been documented (Kerney, 1999).

Clade II had its origin and diversification mainly in Central Europe. The most common ancestor of clade II diversified into two lineages; the first one splitted in *P. rupestris* and *P. saxatilis*, and the second one evolved in *P. pusilla*. The first lineage originated in the area containing Central Europe and the Iberian Peninsula. *P. rupestris* might have dispersed towards the Iberian Peninsula, while *P. saxatilis* evolved towards the Alps and the Italian Peninsula. *P. saxatilis* is currently more or less restricted to the Alps with some samples in the Carpathians that could have arrived by posterior dispersal processes. The migration routes followed by *P. pusilla* were more complicated to infer since it is widely distributed in Europe. S-DIVA and DEC found several possible ancestral areas but those with the highest percentage of probability were Central Europe and the Balkans. Therefore, we suggest that *P. pusilla* could have started the speciation process in the Balkans and later expanded

following an east-west migration route. In this study, the Eastern Mediterranean region, including the Balkans, was considered to be the ancestral area of four species: *Pyramidula* sp2, *P. cephalonica*, *P. chorismenostoma* and *P. pusilla*. While the first three species proceeded from ancestors inhabiting the Balkans and adjacent areas; *P. pusilla* might have originated in the Balkans from ancestors that arrived from Central Europe. The persistence of the lineages and species in multiple refugia may have contributed to the maintenance of genetic richness in the Balkan species. The Eastern Mediterranean region, including the Balkans, is considered the centre of diversification of many organisms (Oberprieler, 2005; Micó et al., 2009; Manafzadeh et al., 2014). Indeed, the Eastern Mediterranean region has suffered a recent and intense orogenic activity during the last 16 million years (Krijgsman, 2002), probably allowing vicariance and allopatric speciation events. Future studies with increased sampling in the Balkans are warranted to test these hypotheses. Although we could not infer the ancestral area of the genus, the Balkans and Central Europe were inferred to be important areas for the ancestor of the western species.

4.1.2. Range dynamics in climate changes

ENM showed that the six *Pyramidula* species' responses to climate changes were different. Post-glacial range dynamics (from LGM to present) included expansion and contraction patterns in the species' distributions. The potential distributions of *P. cephalonica, P. chorismenostoma, P. jaenensis* and *P. rupestris* were expanded from LGM to present, while distributions of *P. pusilla* and *P. saxatilis* were contracted. As it has been already suggested, species respond differently to climate changes depending on their adaptations and environmental tolerances (Stewart et al., 2010). In this way, temperate species are confined in southern refugia during glacial periods with a posterior expansion in interglacial periods; and oppositely, cold-adapted species expand during glacial periods, keeping their range at its minimum during interglacial periods (Stewart et al., 2010). Indeed, response curves for bio4 and bio18 environmental variables showed that expanded and contracted species in post-glacial periods yielded different tendencies (Figure 2). The four expanded species showed higher probability of presence with higher temperature seasonality (bio4) and with lower precipitations in the warmest quarter (bio18); while the two contracted species showed higher probability of presence with opposite characteristics. Similar patterns were found for response curves of other variables (not shown). For example, for bio5 (maximum temperature in warmest month) expanded species obtained the maximum probability of presence around 30 ºC, while for contracted species the probability of presence decreased above 5-15 ºC. Taking into account the influence of bio18, we consider that species in *Pyramidula* are also limited by the precipitation and not only by the temperature. Therefore, we can broadly classify the six species into two groups: (1) Temperate species living in semiarid areas ("semiarid and temperate species") = *P. cephalonica, P. chorismenostoma, P. jaenensis* and *P. rupestris*; and (2) cold-adapted species living in areas with high rainfall ("humid and cold-adapted species") = *P. pusilla* and *P. saxatilis*.

Semiarid and temperate species had their distribution ranges in the LGM confined to

small areas in southern refugia (Iberian, Italian and Balkan Peninsulas). But unlike to the classical framework of post-glacial recolonization of northern regions out of the peninsulas (Hewitt, 2000, 2001; Stewart et al., 2010), they expanded following mountain ranges inside peninsulas: *P. jaenensis* expanded following the central and southern mountain ranges of the Iberian Peninsula (Figure 5), *P.* rupestris expanded northward reaching Cantabrian Mountains (Figure 7) and *P. cephalonica* expanded southward following the Dinaric Alps and Pindus Mountains (Figure 3). Altitudinal oscillations in species distribution during the cooling and warming cycles of the Pleistocene have been already suggested for other land snails (Elejalde et al., 2009). ENM of *P. chorismenostoma* for the LGM obtained different results using the two climate models (Supplementary material 4b). The model using CCSM found suitable areas all over the Aegean Islands but in general with low probability of presence, excepting for some coastal areas; while the model using MIROC only found a suitable area with high probability of presence in Kythira Island (connected to continental Greece in the LGM). This was the only area where both models obtained high probability of presence. If this island was the refugium of *P. chorismenosotoma* during the LGM, the species could have afterwards recolonized the Aegean Islands before the complete fragmentation of Cyclades, 9.000 years BP (Van Andel and Shackleton, 1982).

Humid and cold-adapted species had their largest potential distribution in the LGM with a post-glacial contraction, as it has been documented for other cold-adapted taxa (Dalén et al., 2004; Canestrelli et al., 2007; Stewart and Dalén, 2007). *P. pusilla* was the species with the largest difference between the potential distributions during the LGM and present time. During the LGM suitable areas for *P. pusilla* were available widely distributed across Europe and this is probably why nowadays it is the most widespread species in Europe. Although widely distributed, the potential distribution of the present time is restricted to the main mountain ranges of Europe and Atlantic Lusitanic region. High probabilities of presence for *P. saxatilis* in the LGM occurred in the Eastern Alps. Indeed, this area remained non-glaciated during the Pleistocene and it provided refuge for several Alpine plants and land snails (Tribsch and Schönswetter, 2003; Harl et al., 2014). Moreover, in the North-Western Alps and the Western Carpathians more suitable areas were found for the LGM than for the present model, where refugia for other land snails have been documented (Dépraz et al., 2008). But ENMs for the LGM also found suitable climatic conditions along the Alpine glaciers. One hypothesis is that *P. saxatilis* survived in isolated, ice-free areas along the ice sheet, as the persistence of some plants and other snails in areas surrounded by glaciers has already been documented and suggested (Haase, 2003; Parisod and Besnard, 2007; Pfenninger and Pfenninger, 2005; Schönswetter et al., 2005).

We need to keep in mind that ENM only offered suitable climatic conditions and not habitat conditions. *Pyramidula* inhabits limestone rocks and therefore, those potential distribution areas not containing calcareous formations should be removed.

Regarding future ranges of species, ENM also helped forecasting how current climate change may affect species distributions. Considering the climate model projection for

2070 (average for 2061-2080) based on RCP 6.0 conditions, the distributions of five out of the six species were predicted to contract. Contrary to what we would expect under a warmer climate prediction, the semiarid and temperate species *P. jaenensis* and *P. rupestris* were the two species showing the largest reduction in their future potential ranges (Figure 5 and 7). Indeed, if they continue their expansion following the main mountain ranges previously defined, the available area for them will be reduced as the altitude increases. *P. cephalonica* was the only species predicted to be minimally expanded, because its suitable habitat will minimally increase with the climate change.

4.2. Shell shape evolution

Taking into account that the variability of the shell shape in *Pyramidula* was significantly correlated with several climatic variables, we consider that shell shape has been affected by adaptive processes. This correlation was not significant when the variability of the morphology was tested within species. All this suggests that phenotypic divergence occurred together with ecological divergence and resulted in adaptive radiation.

Considering the scenario of adaptive radiation, new species would be adapted morphologically to their environment through natural selection (Gavrilets and Losos, 2009). The reconstruction of ancestral states showed that the H/D ratio of the ancestors of the species ranged from 0.68 to 0.89. Pearson tests showed that bio5 (maximum temperature warmest month), bio14 (precipitation driest month) and bio18 (precipitation warmest quarter) were the most correlated variables to the H/D ratio, suggesting that the increase on temperature and decrease on precipitation increase the H/D ratio. Adaptation to a similar ecological niche may have caused parallel evolution of the morphology in different species (Rundle and Schulter, 2004), resulting in cryptic taxa. Indeed, *Pyramidula* species with similar climatic requirements showed similar shell shapes: humid and cold-adapted species (*P. pusilla* and *P. saxatilis*) obtained the lowest values of H/D for their ancestral state reconstruction, while semiarid and temperate species obtained higher H/D values, remarkably higher for *P. rupestris, P. jaenensis* and *P. chorismenostoma*.

There was no correlation between climatic variables and shell shape within species, suggesting that adaptive forces currently do not play an important role. Therefore, we assume that species in *Pyramidula* retained their ancestral ecological niche (niche conservatism) (Wiens and Graham, 2005), retaining also their main morphology, and that the shell variability that currently exists within species is due to intrinsic polymorphism. Constrained evolution may also have lead to morphological conservatism by means of stabilizing selection. Selecting against extreme morphologies prevents the creation of new phenotypes, and hence, cryptic species will remain similar over time (Schneider and Moritz, 1999; Wiens and Graham, 2005). However, significant correlation did exist between shell shape and considered climate variables for *P. rupestris*. This suggests that this species could be actually undergoing adaptive processes, which could result in a divergence of the shell morphology.

All these hypotheses have been based solely on a morphological character, the relative

height of the shell (H/D), and hence, they should be handled with caution. However, the shell and anatomical morphology in *Pyramidula* are simple (Martínez-Ortí et al., 2007), rendering difficult the study of alternative morphological characters. Besides, the relative height of the shell has been widely studied in evolutionary and adaptive studies of molluscs (Cain, 1977, 1978; Cain and Cowie, 1978; Cook and Jaffar, 1984; Goodfriend, 1986; Harley et al., 2009), suggesting that its plasticity is the result of adaptive responses to several environmental factors (dry conditions, heat stress, balancing mechanism...).

5. Conclusion

Ancestral area reconstructions suggested different migration patterns for the diversification of the two major clades in *Pyramidula*. Species of the first clade, including *P. cephalonica*, *P. chorismenostoma*, *P. jaenensis* and *Pyramidula* sp2, had a Mediterranean origin and diversification, mainly with a westward direction; while species of the second clade, including *P. rupestris*, *P. saxatilis* and *P. pusilla*, originated and diversified mainly across Central Europe. The Eastern Mediterranean region was found to be the ancestral area for several *Pyramidula* species (*P. cephalonica, P. chorismenostoma, P. pusilla* and *Pyramidula* sp2). ENM analyses demonstrated that each *Pyramidula* species responded differently to climate fluctuations: cold-adapted species living in areas with high rainfall suffered contractions in their post-glacial range dynamics, while temperate species living in semiarid areas were expanded. Projections to future climate conditions predicted potential contractions in several species' distributions. Analyses on shell shape evolution suggested that the current variability on shell shape and the crypticity of *Pyramidula* may have been the results of several evolutionary processes, including adaptive radiation, parallel evolution, niche conservatism and constrained evolution.

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Supplementary material

Supplementary material 1: Voucher numbers of the individuals used in this study selected from Razkin et al. (under review-a).

Pyramidula pusilla: EHUMC_1128, EHUMC_1142, MVHN_070910RU03_1, MVHN_070910RU03_2, MVHN_070910RU09_2, MVHN_070910RU12, EHUMC_1143, EHUMC_1145, EHUMC_1146, EHUMC_1150, EHUMC_1155, EHUMC_1165, EHUMC_1166, EHUMC_1176, EHUMC_1177, MNCN_15.05/49513, EHUMC_1179, EHUMC_1180, EHUMC_1181, EHUMC_1185, EHUMC_1187, EHUMC_1188, EHUMC_1190, EHUMC_1191, EHUMC_1192, EHUMC_1193, EHUMC_1194, EHUMC_1196, EHUMC_1197, EHUMC_1201, EHUMC_1202, EHUMC_1203, EHUMC_1204, NHMC_50.29715, NHMC_50.29312, NHMC_50.29485, NHMC_50.31790, NHMC_50.32098, NHMC_50.35549, NHMC_50.35728, EHUMC_1205, EHUMC_1207, HNHM_99350, HNHM_99346, EHUMC_1208, EHUMC_1210, EHUMC_1211, EHUMC_1214, EHUMC_1215, EHUMC_1216, EHUMC_1217, EHUMC_1219, NMC_1980.15.1, EHUMC_1229, EHUMC_1231, EHUMC_1232, EHUMC_1234, EHUMC_1235, HNHM_98874_1, HNHM_98875, EHUMC_1236, EHUMC_1238

Pyramidula rupestris: EHUMC_1129, EHUMC_1132, EHUMC_1136, EHUMC_1137, EHUMC_1138, MVHN_070910RU01, MVHN_070910RU04, MVHN_070910RU11, MVHN_210610DG05, MVHN_070910RU15, MVHN_070910RU17, MVHN_231210CS01, EHUMC_1158, EHUMC_1159, EHUMC_1160, EHUMC_1162, EHUMC_1163, EHUMC_1164, EHUMC_1167, EHUMC_1168, EHUMC_1169, EHUMC_1171, MNCN_15.05/50925, MNCN_15.05/49951, MNCN_15.05/51665, EHUMC_1183, EHUMC_1189, EHUMC_1195, EHUMC_1199, EHUMC_1222, EHUMC_1223, EHUMC_1224, EHUMC_1225, EHUMC_1228

Pyramidula saxatilis: MVHN_070910RU02_1, MVHN_070910RU16, EHUMC_1144, EHUMC_1170, EHUMC_1198, EHUMC_1209, EHUMC_1213, EHUMC_1218, NMC_2001.054.0054, NMC_2001.054.0030, EHUMC_1220

Pyramidula jaenensis: EHUMC_1130, EHUMC_1131, EHUMC_1135, EHUMC_1139, EHUMC_1178, NMC_1985.199.2, EHUMC_1206, EHUMC_1212, EHUMC_1221, EHUMC_1227, EHUMC_1230, EHUMC_1233, EHUMC_1237, EHUMC_1140, EHUMC_1141, MVHN_070910RU05, MVHN_070910RU13, MVHN_060610IJ01, EHUMC_1156, EHUMC_1157, EHUMC_1161, MNCN_15.05/52048, MNCN_15.05/50730, MNCN_15.05/52013, MNCN_15.05/49858, MNCN_15.05/51720, EHUMC_1184, NMC_1984.392.1, MVHN_120911LB03, MVHN_1917, NMC_1984.394.1, NMC_1984.384.1, EHUMC_1226, EHUMC_1133, EHUMC_1134, MVHN_070910RU02_2, MVHN_070910RU09_1, EHUMC_1151, EHUMC_1152, EHUMC_1153, EHUMC_1154, EHUMC_1182, EHUMC_1186, EHUMC_1200, NMC_1986.306.32, NMC_1986.327.01, NMC_1984.249.1, NMC_1992.080.299

Pyramidula chorismenostoma: NHMC_50.23749, NHMC_50.27302, NHMC_50.25701, NHMC_50.32095, NHMC_50.26702, NHMC_50.26697, NHMC_50.26187_1, NHMC_50.26187_2, NHMC_50.34163

Pyramidula sp2: ZMH_86507/999_1, ZMH_86507/999 _2, ZMH_100219/5_1, HNHM_98871, HNHM_98872, HNHM_98873

Pyramidula cephalonica: EHUMC_1147, EHUMC_1148, NMC_1985.250.1, NHMC_50.22417_1, NHMC_50.32106_1, NHMC_50.32106_2, HNHM_99348 , NMC_1985.219.2, HNHM_98850, HNHM_98853_1, HNHM_98853_2, HNHM_98854, HNHM_98855_1, HNHM_98857, HNHM_98858, HNHM_98859_1, HNHM_98859_2, HNHM_98863, HNHM_98866, HNHM_98868

Supplementary material 2: Localities of the new specimens included in Ecological Niche Modeling analyses.

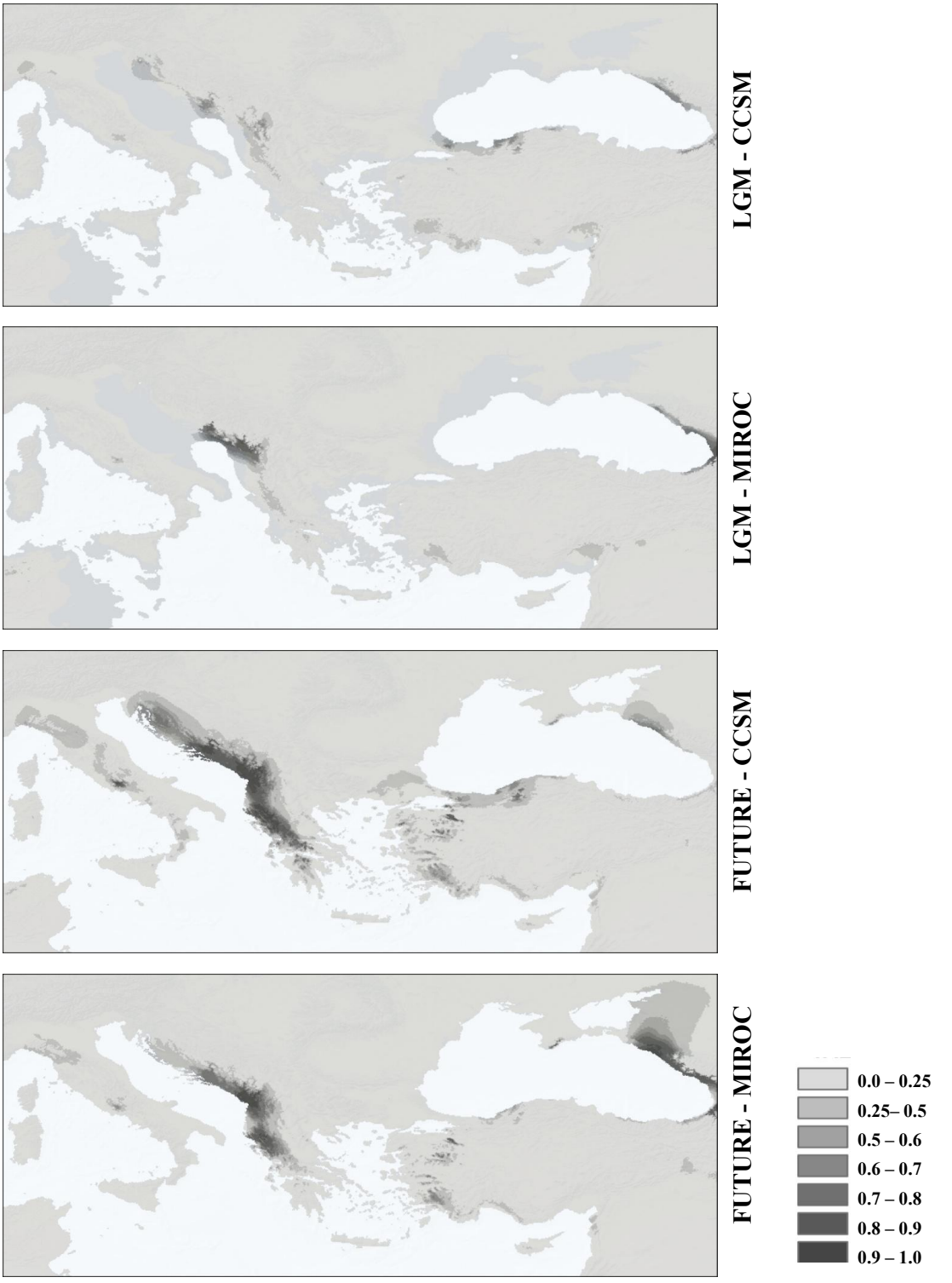
Species	Code	Locality
Pyramidula chorismenostoma	Py186_1	Apollonas, Naxos isl., Greece
	Py187_1	Potamos, Kythira isl., Greece
	Py188_1	Aidonia, Andros isl., Greece
	Py191_1	Charkadio cave, Paros isl., Greece
	Py192_1	Aneratza, Paros isl., Greece
	Py193_1	Megalos Votsos, Fournoi isl., Greece
	Py194_1	Syrna isl., Greece
	Py196_1	Chrysostomos, Ikaria isl., Greece
	Py197_1	Valaxa isl., Greece
	Py201_1	Anydros isl., Greece
	Py202_1	Levitha isl., Greece
	Py203_1	Korakas, Amorgos isl., Greece
	Py204_1	Malta, Sikinos isl., Greece
	Py206_1	Syringas, Syros isl., Greece
	Py207_1	Agia Paraskevi, Syros isl., Greece
	Py211_1	Lastos plateau, Karpathos isl., Greece
	Py225_1	Chodros, Crete, Greece
	Py230_1	Apostolos, Sifos isl., Greece
	Py239	Tilos island, Dodekanissa, Greece
	Py241	Anidro isl., Greece
	Py247	Syros isl., Greece
	Py248	Ikaria isl., Greece
	Py253	Andros isl., Greece
	Py259	Naxos isl., Greece
	Py260	Astypalaia isl., Greece
	Py261	Sifnos isl., Greece
	Py269	Lentas, Crete, Greece
Pyramidula jaenensis	Py019_1	Valle de Abdalajis, Málaga, Spain
	Py043_1	Anguiano, La Rioja, Spain
	Py056_1	Estoi, Faro, Portugal
	Py057_1	Torcal de Antequera, Málaga, Spain
	Py061_1	Itoiz, Navarra, Spain
	Py066_1	Los Alazores, Jaén, Spain
	Py069_1	Jódar, Jaén, Spain
	Py120_1	Capo Figari, Sardinia, Italy
	Py170_1	Near Arbaoun, Sétif, Algeria
	Py234_1	Nabeur, Tunisia

Species	Code	Locality
Pyramidula chorismenostoma	Py186_1	Apollonas, Naxos isl., Greece
	Py187_1	Potamos, Kythira isl., Greece
	Py188_1	Aidonia, Andros isl., Greece
	Py191_1	Charkadio cave, Paros isl., Greece
	Py192_1	Aneratza, Paros isl., Greece
	Py193_1	Megalos Votsos, Fournoi isl., Greece
	Py194_1	Syrna isl., Greece
	Py196_1	Chrysostomos, Ikaria isl., Greece
	Py197_1	Valaxa isl., Greece
	Py201_1	Anydros isl., Greece
	Py202_1	Levitha isl., Greece
	Py203_1	Korakas, Amorgos isl., Greece
	Py204_1	Malta, Sikinos isl., Greece
	Py206_1	Syringas, Syros isl., Greece
	Py207_1	Agia Paraskevi, Syros isl., Greece
	Py211_1	Lastos plateau, Karpathos isl., Greece
	Py225_1	Chodros, Crete, Greece
	Py230_1	Apostolos, Sifos isl., Greece
	Py239	Tilos island, Dodekanissa, Greece
	Py241	Anidro isl., Greece
	Py247	Syros isl., Greece
	Py248	Ikaria isl., Greece
	Py253	Andros isl., Greece
	Py259	Naxos isl., Greece
	Py260	Astypalaia isl., Greece
	Py261	Sifnos isl., Greece
	Py269	Lentas, Crete, Greece
Pyramidula jaenensis	Py019_1	Valle de Abdalajis, Málaga, Spain
	Py043_1	Anguiano, La Rioja, Spain
	Py056_1	Estoi, Faro, Portugal
	Py057_1	Torcal de Antequera, Málaga, Spain
	Py061_1	Itoiz, Navarra, Spain
	Py066_1	Los Alazores, Jaén, Spain
	Py069_1	Jódar, Jaén, Spain
	Py120_1	Capo Figari, Sardinia, Italy
	Py170_1	Near Arbaoun, Sétif, Algeria
	Py234_1	Nabeur, Tunisia

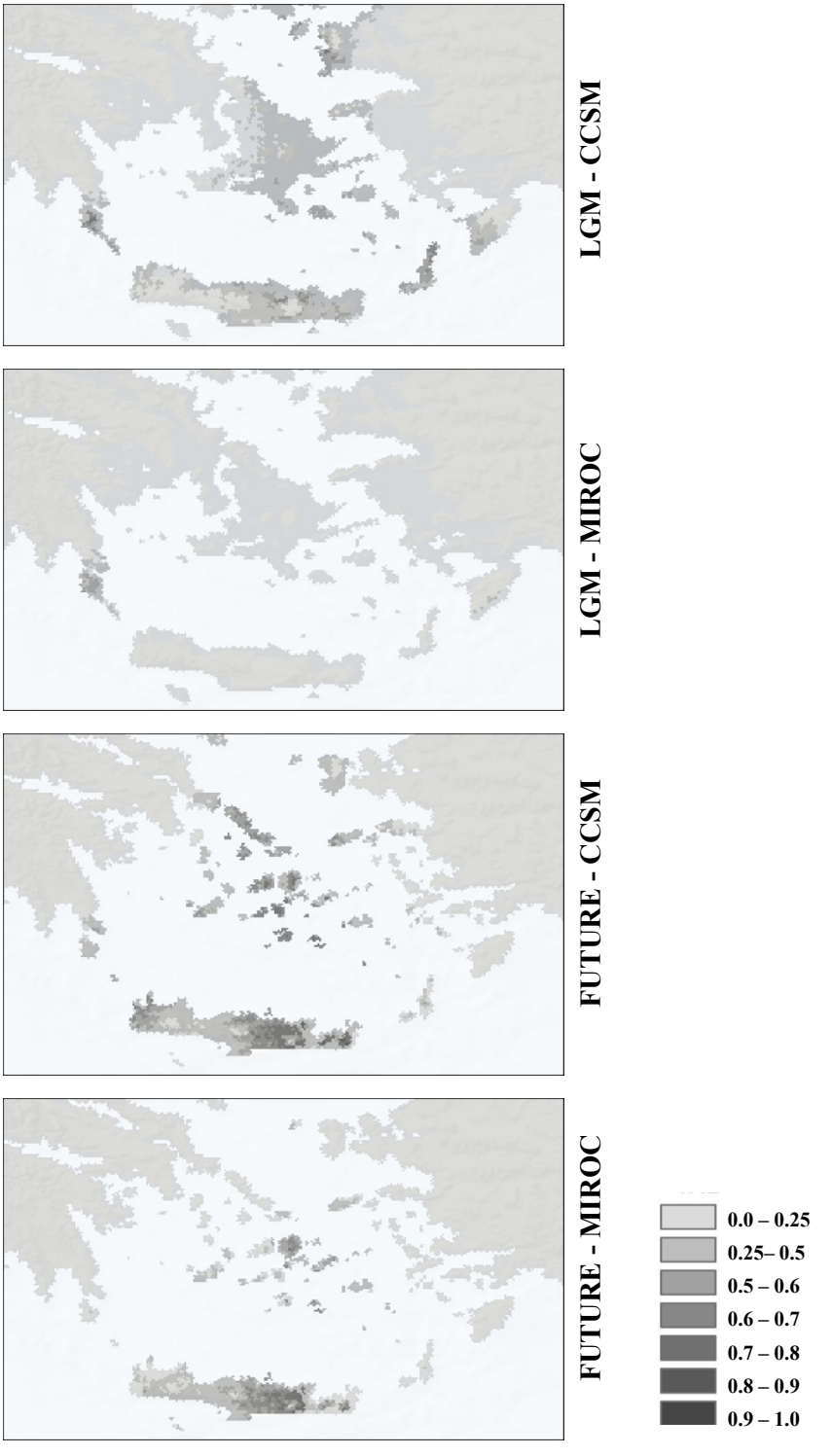
Supplementary material 3: Lengths of the fragments sequenced (minimum and maximum) before and after alignment and number of polymorphic sites.

Gene	Minimum lenght	Maximum lenght	Aligned length	Polymorphic sites
COI	621	621	621	194
16S	324	336	370	73
5.8S-ITS2	560	587	644	51
28S	831	840	843	9
Total	2348	2382	2493	327

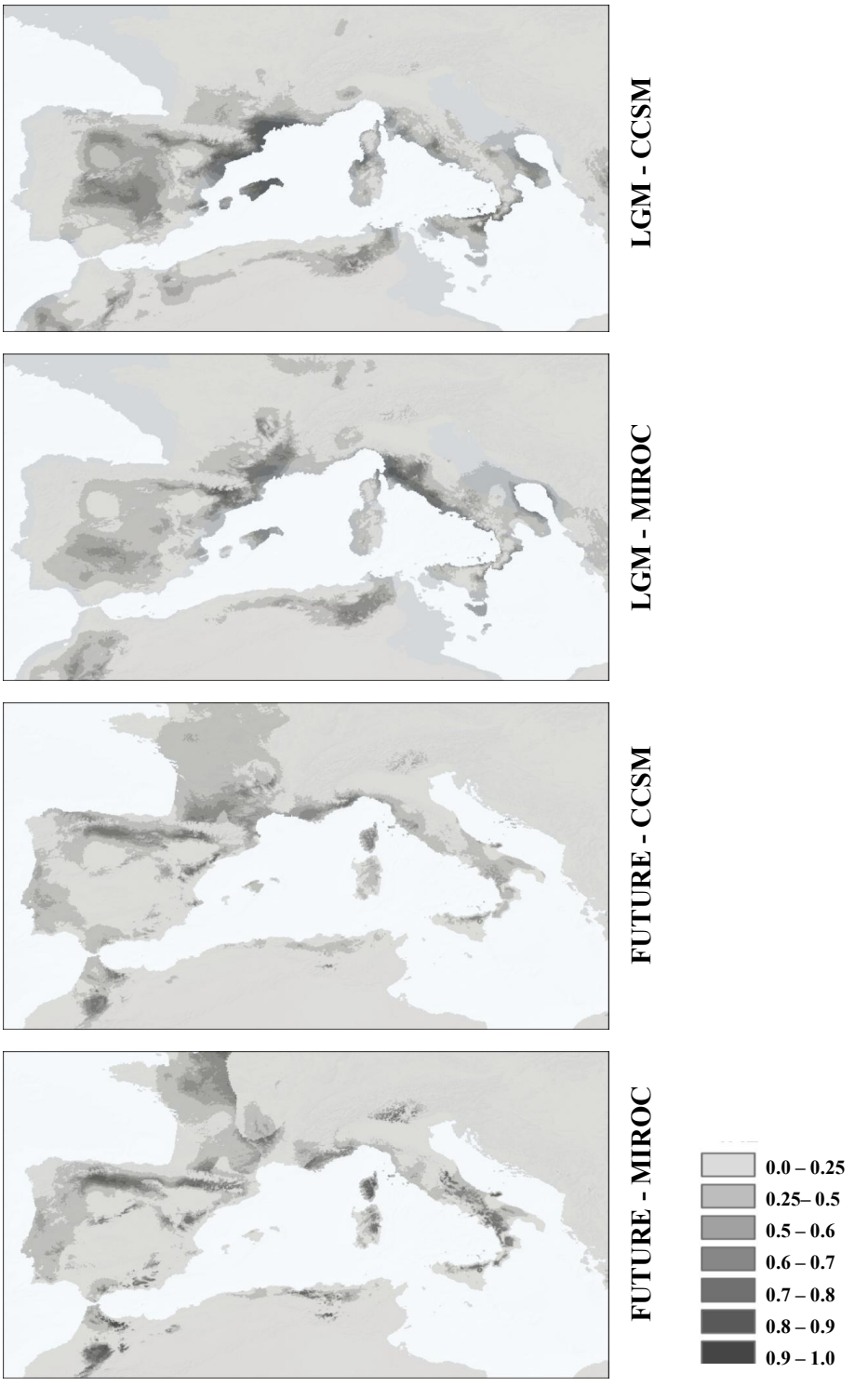
Supplementary material 4a: Ecological Niche Models for *P. cephalonica* for the Last Glacial Maximum and future (RCP 6.0) based on MIROC and CCSM models. Darker colours indicate better-predicted conditions.



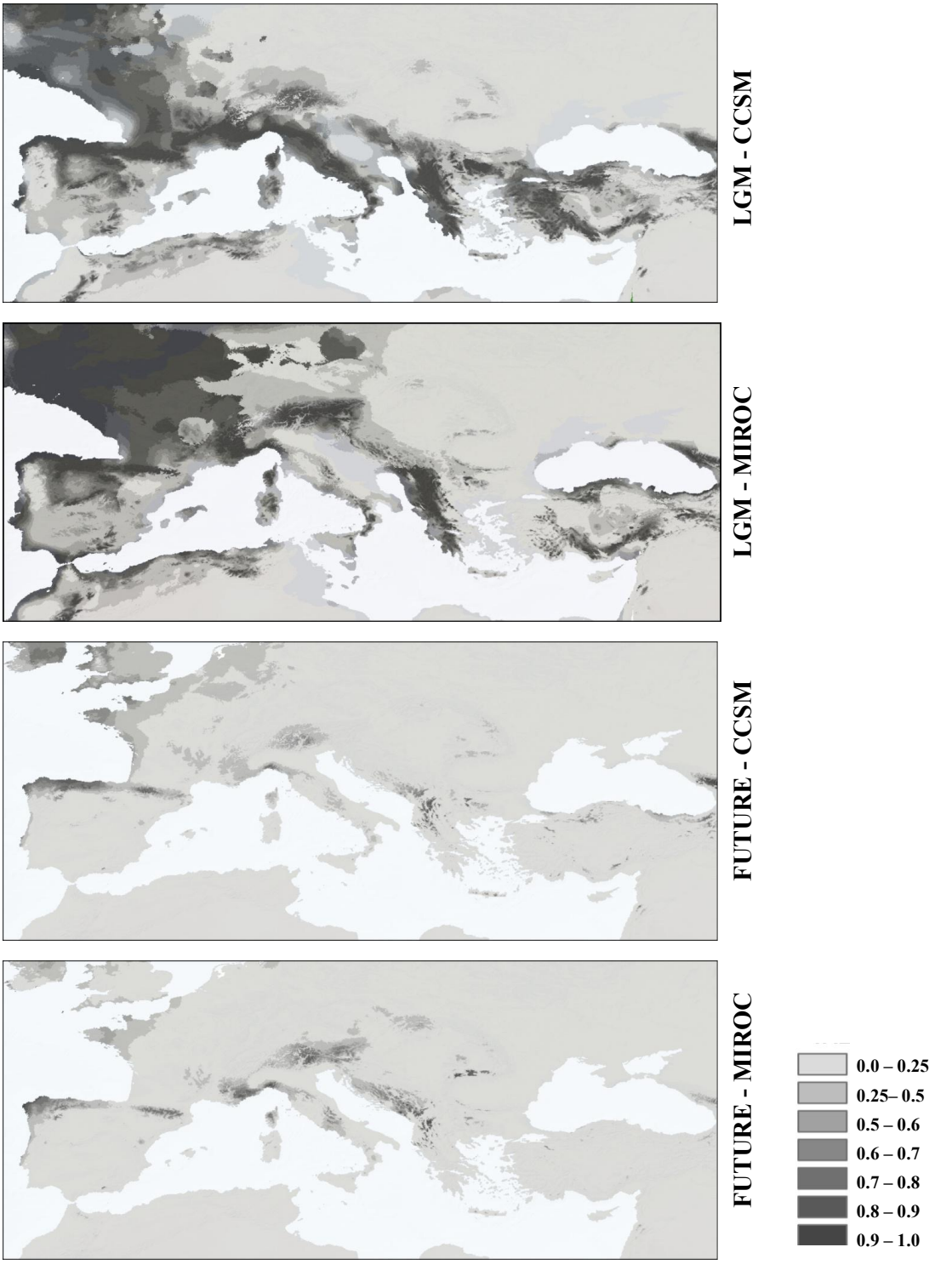
Supplementary material 4b: Ecological Niche Models for *P. chorismenostoma* for the Last Glacial Maximum and future (RCP 6.0) based on MIROC and CCSM models. Darker colours indicate better-predicted conditions.



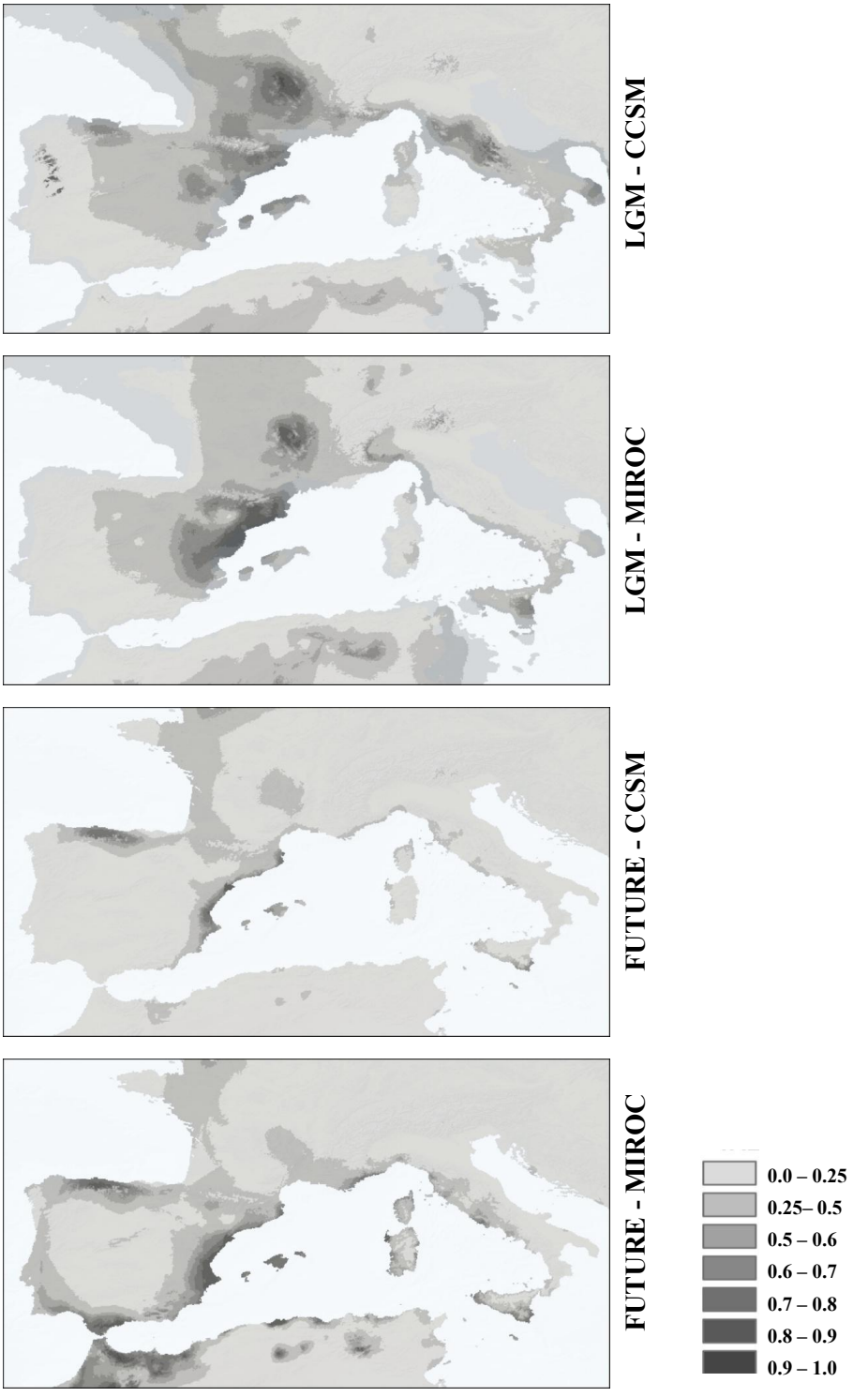
Supplementary material 4c: Ecological Niche Models for *P. jaenensis* for the Last Glacial Maximum and future (RCP 6.0) based on MIROC and CCSM models. Darker colours indicate better-predicted conditions.



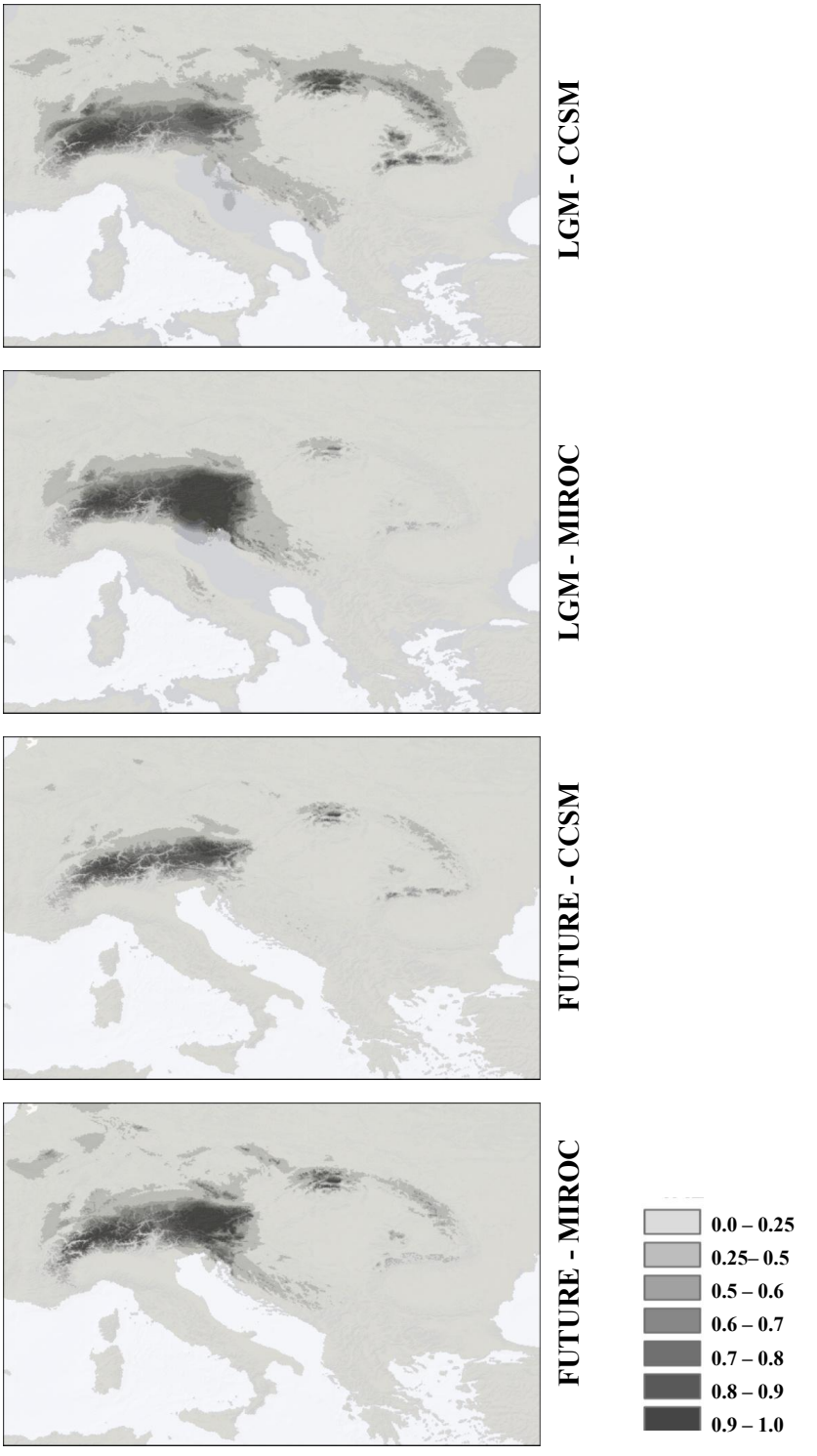
Supplementary material 4d: Ecological Niche Models for *P. pusilla* for the Last Glacial Maximum and future (RCP 6.0) based on MIROC and CCSM models. Darker colours indicate better-predicted conditions.



Supplementary material 4e: Ecological Niche Models for *P. rupestris* for the Last Glacial Maximum and future (RCP 6.0) based on MIROC and CCSM models. Darker colours indicate better-predicted conditions.



Supplementary material 4f: Ecological Niche Models for *P. saxatilis* for the Last Glacial Maximum and future (RCP 6.0) based on MIROC and CCSM models. Darker colours indicate better-predicted conditions.



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CHAPTER 5

CONCLUDING REMARKS

Next, the main conclusions that can be drawn from the studies performed in this PhD thesis are exposed:

1. The molecular phylogeny of the Helicoidea based on mitochondrial and nuclear genes was mainly focused on the families that exist in the western Palaearctic region and yielded new insights into their relationships. The family Hygromiidae s.l. was divided into three clades which were given familial rank: Canariellidae, Geomitridae and Hygromiidae s.str.. The family Cochlicellidae was given tribe rank belonging to the Geomitridae. Three subfamilies were recognized within Helicidae: Ariantinae, Helicinae and Murellinae. Some of the subfamilies recognized in current classifications were not recovered as monophyletic groups. A new rearrangements in tribes is proposed to Geomitridae and Helicidae.

2. The origin of the Helicoidea was estimated at the end of the Early Cretaceous and its families as Late-Cretaceous to Paleogene. Western Palaearctic Helicoidea belongs to two different lineages that diverged around 86 Ma ago, both starting their diversification at the end of the Cretaceous (around 73-76 Ma).

3. The integrative species delimitation approach, including multilocus and ecological data, proposed the existence of 9 species in the cryptic *Pyramidula* species complex inhabiting the western Palaearctic region. Seven of these species could be defined and nominated by means of morphological and geographical data and the inclusion of topotypes. However, further samples from eastern regions would be needed to fully resolve the taxonomy of *Pyramidula* genus in Asia.

4. Although shell shape in *Pyramidula* could discriminate between some of the delimited species, it is not valid as unique taxonomic criterium. Moreover, the shell shape in this genus is influenced by environmental factors. Ecological niche modelling procedure predicted a suitable habitat characterized by a higher altitude, more rainfall and lower temperatures for specimens with lower shells while opposite habitat conditions offered by a Mediterranean climate were predicted as more suitable for specimens with higher shells.

5. RADseq technique successfully resolved taxonomic and phylogenetic issues of *Pyramidula*, demonstrating its utility for phylogenetic studies of closely related taxa in non-model organisms. The increment in the number of markers of the RADseq data helped to fully resolve phylogenetic relationships between species in *Pyramidula*. Besides, the analyses of unlinked markers obtained from RADseq data found the species delimitation scenario of 9 species to be the most plausible one. The test on interspecific hybridization provided weak or null evidence of ancestral gene flow between species.

6. Ancestral area reconstructions suggested different migration patterns for the diversification of the species in *Pyramidula*. Some of the species could have had a Mediterranean origin and posterior diversification with a westward directionality (*P. cephalonica*, *P. chorismenostoma*, *P. jaenensis* and *Pyramidula* sp2); while other species originated and diversified mainly across Central Europe (*P. rupestris*, *P. saxatilis* and *P. pusilla*). The Eastern Mediterranean region was found to be the ancestral area for several *Pyramidula* species.

7. Ecological niche modeling analyses demonstrated that each *Pyramidula* species responded differently to climate fluctuations: temperate species living in semiarid areas, like *P. cephalonica*, *P. chorismenostoma*, *P. jaenensis* and *P. rupestris*, suffered contractions in their post-glacial range dynamics, while cold-adapted species living in areas with high rainfall, like *P. pusilla* and *P. saxatilis*, were expanded. Projections to future climate conditions predicted potential contractions in several species' distributions.

8. Analyses on shell shape evolution suggested that the current variability on shell shape and the crypticity of the species in *Pyramidula* may have been the results of several evolutionary processes, including adaptive radiation, parallel evolution, niche conservatism and constrained evolution.