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DNA BARCODING

A new approach to an old conundrum—DNA barcoding sheds new light on phenotypic plasticity and morphological stasis in microsnails (Gastropoda, Pulmonata, Carychiidae)

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Abstract

The identification of microsnail taxa based on morphological characters is often a time-consuming and inconclusive process. Aspects such as morphological stasis and phenotypic plasticity further complicate their taxonomic designation. In this study, we demonstrate that the application of DNA barcoding can alleviate these problems within the Carychiidae (Gastropoda, Pulmonata). These microsnails are a taxon of the pulmonate lineage and most likely migrated onto land independently of the Stylommatophora clade. Their taxonomical classification is currently based on conchological and anatomical characters only. Despite much confusion about historic species assignments, the Carychiidae can be unambiguously subdivided into two taxa: (i) *Zospeum* species, which are restricted to karst caves, and (ii) *Carychium* species, which occur in a broad range of environmental conditions. The implementation of discrete molecular data (COI marker) enabled us to correctly designate 90% of the carychiid microsnails. The remaining cases were probably cryptic *Zospeum* and *Carychium* taxa and incipient species, which require further investigation into their species status. Because conventional reliance upon mostly continuous (i.e. nondiscrete) conchological characters is subject to fallibility for many gastropod species assignments, we highly recommend the use of DNA barcoding as a taxonomic, cutting-edge method for delimiting microsnail taxa.

Keywords: Carychiidae, DNA barcoding, Gastropoda, microsnails, morphological stasis, phenotypic plasticity

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Introduction

The taxon Carychiidae Jeffreys, 1830 (Gastropoda, Pulmonata, Ellobioidea) is a group of terrestrial microsnails (approx. 1–2 mm shell height) of holarctic distribution (Pilsbry 1948, Morton 1955). It encompasses at least 40 nominal species comprising the two taxa *Carychium* and *Zospeum* (Morton 1955), which inhabit distinct habitats. *Carychium* lives in mesic environments constituting riparian zones, talus slopes, meadows, swamps and the interstitial layers of forest leaf litter. *Zospeum* is exclusively troglobitic and restricted to karstic hotspots in the Cantabrian Mountains, the Pyrenees, the southern European Alps and the Dinaric Alps (Watson & Verdcourt 1953; Gittenberger 1980; Doll 1982; Slapnik & Ozimec 2004) albeit a record from Korea has been reported recently (Prozorova *et al.* 2010). The Carychiidae probably represent one of a few gastropod lineages which migrated onto land (Martins 1996; Vermeij & Dudley 2000; Klussmann-Kolb *et al.* 2008) independently of the Stylommatophora and thereby, performed one of the major niche shifts from a marine to a terrestrial habitat.

The systematic position of the Carychiidae is largely based on conchological characters of the mature shell (Bourguignat 1856; Pilsbry 1948; Bank & Gittenberger 1985; Harry 1998; Egorov 2007). In general, shell measurements (e.g. mean ratio shell height/shell width and number of suture whorls), degree of surface striation and the shape of the aperture as well as the sinuosity of the folds along the columellar apparatus are used to describe carychiid species and subspecies (Winslow 1922; Zimmermann 1925; Burch & Van Devender 1980; Bank & Gittenberger 1985; Harry 1998; Stworzewicz 1999). Members of the taxa *Carychium* and *Zospeum* can be

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morphologically and anatomically well differentiated by the structure of the radula, the nervous system and the reproductive system (Martins 1996). However, the shell morphology of *Carychium* species as in other mollusks varies under diverse environmental conditions (Pintér 1967; Giusti & Manganelli 1992; Harry 1998; Pfenninger & Magnin 2001; Nekola & Barthel 2002) while the aphotic and stable subterranean conditions for members of *Zospeum* are believed to favour morphological stasis (Lefébure *et al.* 2006; Culver & Pipan 2009). In addition, many other anatomical characters underwent phenotypic reductions and are thereby not suitable for species designation (Morton 1955; Martins 1996, 2007). As a result, specific taxonomic assignments in this gastropod group differ greatly (Burch & Van Devender 1980; Harry 1998; Nekola & Barthel 2002), and the identification of described species is notoriously difficult.

DNA barcodes provide a complementary tool to species identification. They represent a promising and expedient new tool for accurately identifying and linking the varied ontogenetic stages of single species by using the ubiquitous and homologous COI mitochondrial gene sequence (Hebert *et al.* 2003). Indeed, DNA barcoding has demonstrated its success in several animal groups, e.g. in birds (Hebert *et al.* 2004b; Kerr *et al.* 2009), fishes (Ward *et al.* 2005; Hubert *et al.* 2008; Steinke *et al.* 2009), insects (Hebert *et al.* 2004a; Hajibabaei *et al.* 2006; Pfenninger *et al.* 2007; Foottit *et al.* 2008; Smith *et al.* 2008; Lukhtanov *et al.* 2009), crustaceans (Bucklin *et al.* 2007; Costa *et al.* 2007; Radulovici *et al.* 2009) and molluscs (Remigio & Hebert 2003; Kelly *et al.* 2007; Campbell *et al.* 2008; Johnson *et al.* 2008; Feng *et al.* 2010). Although gastropods represent the most speciose group within the Mollusca, very few barcoding studies have been conducted on them (Remigio & Hebert 2003; Meyer & Paulay 2005). Land snails, in particular, are one group for which there is a complete lack of baseline data for barcoding with the exception of one study that utilized exclusively GenBank data (Davison *et al.* 2009).

This study examines the patterns of sequence divergence at COI in predominantly European and North American carychiid microsnails. Our investigation not only provides a further qualitative exploration of COI barcodes for species identifications, but explores the application of DNA barcodes in flagging overlooked species as well as discusses the potential limitations to the system.

Material and methods

Sampling

We collected 113 specimens from 41 populations representing 21 nominal taxa of Carychiidae mainly from Europe and North America during the years 2006–2010 (Table 1, Fig. 1). The identification of morphospecies was based on a combination of external and internal shell characters, locality as well as habitat (ecological) information. Moreover and if available, the extreme and thus unambiguous morphotypes of *Carychium* species have been analysed to set up DNA barcodes. In the case of *Zospeum* taxa, mostly only one species has been described in each cave system. For that reason also juveniles or pre-adults from a cave system have been investigated.

DNA extraction and COI amplification

Freshly collected individuals were immediately preserved in 70–99% ethanol until DNA extraction. Prior to DNA extraction, shell and gut material were removed to minimize contamination risk. DNA extraction was performed following the DNeasy Blood and Tissue protocol (Qiagen, Hilden, Germany). All DNA samples are stored as voucher material at the Goethe-University of Frankfurt am Main, Germany. The barcoding region of COI was amplified by polymerase chain reaction (PCR) using the standard invertebrate primer pair LCO1490—5′GGTCA ACAAATCATAAAGATATTGG3′ and HCO2198—5′TA AACTTCAGGGTGACCAAAAAATCA3′ (Folmer *et al.* 1994). Each 25 μL PCR mixture included 1 μL (10 pmol) of each primer, 2.5 μL 10× PCR buffer, 2 μL (100 mM) $MgCl_2$, 0.3 μL (20 mM) dNTPs, 0.3 μL *Taq*-polymerase, 0.25 μL (0.5 M) tetramethylammonium chloride, 1.5 μL (10 mg/mL) bovine serum albumin, 11.15 μL ddH_2O and 5 μL template DNA. PCR cycles were run at the following conditions: 1 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 52 °C and 30 s at 72 °C, and finally, 3 min at 72 °C. Single PCR products were visualized on a 1.4% agarose gel and cleaned with the GeneJET PCR Purification Kit (Fermentas, St. Leon-Rot, Germany). We used the QIAquick Gel Extraction protocol (Qiagen) in case multiple PCR products were detected.

PCR products were bidirectionally sequenced using the PCR primer pair (Folmer *et al.* 1994) and the BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) on an ABI 3730 xl capillary sequencer following the manufacturer's instructions.

Sequence analyses

Sequence data were assembled and edited using the BioEdit 7.0.9.0 software (Hall 1999) and subsequently submitted to the Barcode of Life Data system (BOLD, http://www.barcodinglife.org, see Ratnasingham & Hebert 2007) and to GenBank (Table 1). Specimen and collection data, sequences, specimen images and trace files are provided in the project 'Barcoding Carychiidae' in BOLD. Sequences were aligned using the ClustalW

Table 1 Table shows detailed locality information, latitude and longitude data for each taxon; BOLD identifier and NCBI accession numbers are provided

Taxon	Locality	Latitude	Longitude	BOLD identifier	Accession no.
Carychium exile cf. *mexicanum* I (Pilsbry, 1891)	USA, GA, Adairsville	34.311233	−84.9866	BARCA015-10	HQ171518
				BARCA016-10	HQ171517
				BARCA017-10	HQ171516
				BARCA018-10	HQ171515
Carychium exile cf. *mexicanum* II (Pilsbry, 1891)	USA, GA, Adairsville	34.311233	−84.9866	BARCA014-10	HQ171519
Carychium exile cf. *mexicanum* III (Pilsbry, 1891)	USA, AL, Little River Canyon National Preserve	34.312233	−85.685733	BARCA019-10	HQ171514
				BARCA020-10	HQ171513
Carychium stygium I (Call, 1897)	USA, KY, Horse Cave (Hidden River Cave)	37.174167	−85.903833	BARCA021-10	HQ171569
				BARCA022-10	HQ171568
Carychium stygium II (Call, 1897)	USA, TN (Slit Cave, cave entrance)	36.456833	−85.375067	BARCA023-10	HQ171567
				BARCA024-10	HQ171566
				BARCA025-10	HQ171565
				BARCA026-10	HQ171564
Carychium clappi (Hubricht, 1959)	USA, TN, La Follette	36.332	−83.998	BARCA027-10	HQ171501
				BARCA028-10	HQ171500
				BARCA029-10	HQ171499
				BARCA030-10	HQ171498
				BARCA031-10	HQ171497
Carychium floridanum (Clapp, 1918)	USA, FL, Wakulla Springs	30.23548	−84.303087	BARCA032-10	HQ171525
				BARCA033-10	HQ171524
				BARCA034-10	HQ171523
				BARCA035-10	HQ171522
				BARCA036-10	HQ171521
				BARCA037-10	HQ171520
Carychium mexicanum (Pilsbry, 1891)	USA, Georgia, Flovilla	33.242367	−83.92035	BARCA038-10	HQ171531
				BARCA039-10	HQ171530
				BARCA040-10	HQ171529
Carychium exiguum (Say, 1822)	USA, NY, Naples	42.61591	−77.41355	BARCA041-10	HQ171504
				BARCA042-10	HQ171503
				BARCA043-10	HQ171502
Carychium exile (H. C. Lea, 1842)	USA, NY, Watkins Glen	42.375828	−76.871115	BARCA044-10	HQ171512
				BARCA045-10	HQ171511
				BARCA046-10	HQ171510
	USA, IN, Lawrence County	38.7555	−86.59105	BARCA049-10	HQ171507
				BARCA050-10	HQ171506
				BARCA051-10	HQ171505
	USA, NY, Portageville	42.57909	−78.04945	BARCA047-10	HQ171509
				BARCA048-10	HQ171508

Table 1 Continued

Taxon	Locality	Latitude	Longitude	BOLD identifier	Accession no.
Carychium occidentale (Pilsbry, 1891)	USA, WA, Mason County	47.3038	−123.0918	BARCA052-10	HQ171553
				BARCA053-10	HQ171552
				BARCA054-10	HQ171551
				BARCA055-10	HQ171550
				BARCA056-10	HQ171549
				BARCA057-10	HQ171548
				BARCA058-10	HQ171547
				BARCA059-10	HQ171546
				BARCA060-10	HQ171545
				BARCA061-10	HQ171544
Carychium minimum (O. F. Müller, 1774)	Switzerland, Wallis, Hérémence	46.176	7.396	BARCA062-10	HQ171541
				BARCA063-10	HQ171540
	Spain, Asturias, Gijón	43.5204	−5.6164	BARCA064-10	HQ171539
	Italy, Upper Adige, near Auer	46.3385	11.3503	BARCA065-10	HQ171538
	Scotland, Whitekirk	56.0433	−2.6375	BARCA066-10	HQ171537
	UK, Hampshire, Hartley Wintney	51.31	−0.89	BARCA067-10	HQ171536
	Bulgaria, Upper Thracian Lowland, Stara Zagora	42.4428	25.6386	BARCA068-10	HQ171535
	Poland, Słubice	52.3467	14.5806	BARCA069-10	HQ171534
	Germany, Bavaria, Dießen am Ammersee	47.94	11.11	BARCA070-10	HQ171533
	Italy, Trentino, near Tuenno	46.3261	11.0114	BARCA071-10	HQ171532
Carychium ibazoricum (Bank & Gittenberger, 1985)	Portugal, Azores, San Miguel, Furnas	37.770383	−25.306583	BARCA072-10	HQ171528
				BARCA073-10	HQ171527
	Portugal, Azores, San Miguel, Sete Cidades	37.847033	−25.780217	BARCA074-10	HQ171526
Carychium tridentatum (Risso, 1826)	Germany, Hesse, Baunatal	51.2574	9.3581	BARCA075-10	HQ171578
	Switzerland, Wallis, Hérémence	46.176	7.396	BARCA076-10	HQ171577
	Germany, Hesse, Kelkheim-Eppenhain	50.17	8.39	BARCA077-10	HQ171576
	France, Bretagne, near Lopreden	48.596667	−3.97	BARCA078-10	HQ171575
				BARCA079-10	HQ171574
	Italy, Upper Adige, Parcines	46.690083	11.06065	BARCA080-10	HQ171573
	Italy, Upper Adige, near Auer	46.3385	11.3503	BARCA081-10	HQ171572
	Germany, Hesse, Frankfurt am Main	50.1293	8.6583	BARCA082-10	HQ171571
	Spain, Asturias, Collia	43.409333	−5.199133	BARCA083-10	HQ171570
Carychium sp. nov.	Bulgaria, Rhodopi Mnt., Mostovo (Gargina Dupka Cave, cave entrance)	41.850667	24.926183	BARCA084-10	HQ171563
				BARCA085-10	HQ171562
				BARCA086-10	HQ171561
				BARCA087-10	HQ171560
				BARCA088-10	HQ171559
				BARCA089-10	HQ171558
				BARCA090-10	HQ171557
				BARCA091-10	HQ171556
				BARCA092-10	HQ171555
				BARCA093-10	HQ171554

Table 1 Continued

Taxon	Locality	Latitude	Longitude	BOLD identifier	Accession no.
Carychium cf. *pessimum* (Pilsbry, 1902)	Russia, Primorsky Kray, Vladivostok	43.193417	132.051100	BARCA094-10	HQ171496
				BARCA095-10	HQ171495
				BARCA096-10	HQ171494
				BARCA097-10	HQ171493
				BARCA098-10	HQ171492
Carychium nannodes (Clapp, 1905)	USA, TN (Slit Cave, cave entrance)	36.456833	−85.375067	BARCA099-10	HQ171543
	USA, TN, near Unaka Springs	36.0986	−82.4466	BARCA100-10	HQ171542
Zospeum spelaeum I (Rossmässler, 1839)	Slovenia, Mengeš, Loka Pri Mengsu (Cave 1 near Jablje)	46.1426	14.5533	BARCA101-10	HQ171601
				BARCA102-10	HQ171600
				BARCA103-10	HQ171599
				BARCA104-10	HQ171598
				BARCA105-10	HQ171597
Zospeum spelaeum II (Rossmässler, 1839)	Slovenia, Veliki Otok (Betalov Spodmol Cave)	45.7922	14.1877	BARCA106-10	HQ171596
Zospeum frauenfeldi (Freyer, 1855)	Slovenia, Dobrepolje, Podpeč (Podpeč Cave)	45.8393	14.6863	BARCA107-10	HQ171590
				BARCA108-10	HQ171589
				BARCA109-10	HQ171588
				BARCA110-10	HQ171587
				BARCA111-10	HQ171586
Zospeum subobesum (Bole, 1974)	Croatia, Ogulin, Tounj (Tounjčica Cave)	45.24385	15.3253	BARCA112-10	HQ171604
				BARCA113-10	HQ171603
				BARCA114-10	HQ171602
Zospeum exiguum (Kuščer, 1932)	Slovenia, Cerknica, Lož (Križna Cave)	45.7452	14.4673	BARCA115-10	HQ171585
				BARCA116-10	HQ171584
				BARCA117-10	HQ171583
				BARCA118-10	HQ171582
				BARCA119-10	HQ171581
Zospeum pretneri (Bole, 1960)	Croatia, Gračac, Kesići (Lower Cerovačka Cave)	44.2701	15.8855	BARCA120-10	HQ171595
Zospeum isselianum I (Pollonera, 1887)	Slovenia, Kobarid, Robič (Turjeva Cave)	46.2435	13.5046	BARCA121-10	HQ171594
Zospeum isselianum II (Pollonera, 1887)	Slovenia, Kamnik, Kamniška Bistrica (Jama pod Farjevim plazom)	46.3093	14.5832	BARCA122-10	HQ171593
Zospeum isselianum III (Pollonera, 1887)	Slovenia, Kamnik-Savinja Alps, Šmihel nad Mozirjem (Konečka zijalka Cave)	46.4024	14.9393	BARCA123-10	HQ171592
				BARCA124-10	HQ171591
Zospeum alpestre (Freyer, 1855)	Slovenia, Kamnik-Savinja Alps, Solčava (Ložekarjeva zijalka Cave)	46.4268	14.6240	BARCA125-10	HQ171580
				BARCA126-10	HQ171579

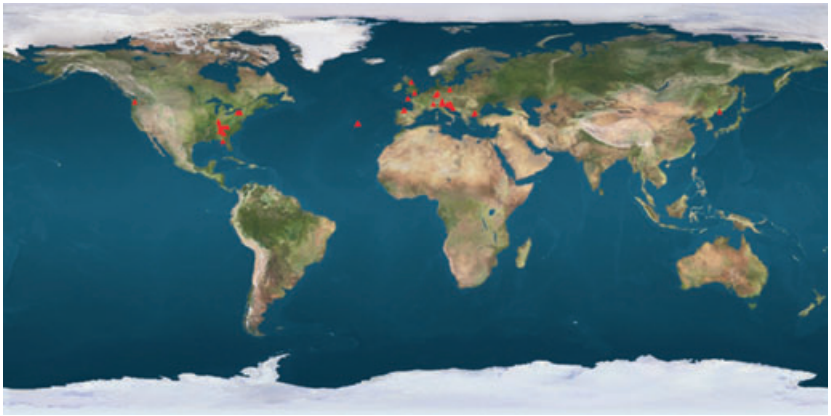

Fig. 1 Distribution map of Carychiidae. Red triangles indicate localities of the 21 *Carychium* and *Zospeum* taxa analysed in this study.

(Thompson *et al*. 1994) module in BioEdit 7.0.9.0. Inter- and intraspecific pairwise genetic distances were calculated with the Kimura 2-parameter (K2P) model in MEGA version 4 (Tamura *et al*. 2007) under the pairwise deletion option. Neighbour-joining (NJ) analyses were also executed in MEGA using bootstrap analysis with 2000 replicates.

Results

COI amplicons were recovered from all 113 individuals, and no indels or stop codons were encountered. Sequence length averaged 632 bp (range = 592–655 bp), and more than 97% of the records were above 600 bp. Overall nucleotide frequencies were as follows: C (15.0%), T (40.7%), A (26.4%) and G (17.9%).

A NJ tree of COI sequence divergences (K2P) indicated that most species (19) formed cohesive units (Fig. 2). Mean K2P sequence distance between congeneric species (5.7%) was approximately 8-fold higher than that within species (0.7%). This division between intra- and interspecific sequence variation is further illustrated through the histogram in Fig. 3. A threshold value of 3.2% COI sequence variation (i.e. barcoding gap) can be detected, which separates intra- and interspecific sequence pairs in most of the cases.

Among the 20 species in which two or more specimens were examined, two displayed intraspecific divergences greater than 3.2%. One (*Zospeum spelaeum*) formed two distinct clusters while another (*Zospeum isselianum*) included three groups with a maximum of intraspecific sequence variation of 5.4% and 7.0%, respectively.

Sequence divergences between most congeneric taxa were high, averaging 5.7%, but there were exceptions. *Carychium stygium* and *Carychium exile* cf. *mexicanum* demonstrated sequence sharing although the COI sequences were not tightly clustered, differing on average (range: 0.8–2.8%) by 1.7% divergence.

Discussion

The present study represents the first molecular survey of the Carychiidae (Gastropoda, Pulmonata, Ellobioidea). We examined 113 individuals representing 21 described taxa from 41 populations mainly from Europe and North America (Table 1, Fig. 1). Hence, this data set covers approximately half of the described *Carychium* microsnail species. Until now, no valid genetic distinctions or agreement could be reached regarding species assignments in European or American *Carychium*. Currently, inconsistencies abound in a number of factors including the paucity of material, improper analytical techniques, different evaluations of the respective results, subjectivity relative to historical context and any number of reasons respective to the literature consulted (Zimmermann 1925; Pilsbry 1948, Burch & Van Devender 1980; Azuma 1982; Bank & Gittenberger 1985; Harry 1998; Egorov 2007; Kantor *et al*. 2009).

In the case of *Zospeum*, the total number of described species is 17 (R. Slapnik, pers. comm.) of which we successfully collected and barcoded seven species from ten different caves in Croatia and Slovenia.

Our results show that 18 taxa (90%) possess COI sequences that designate their separation from any other taxon included in this study. Sequence divergence between congeneric taxa was typically high, averaging 5.7%. Conversely, within-species variation for most taxa was rather low (0.7%), matching lower levels of conspecific variation reported in prior barcoding studies on gastropods (Meyer & Paulay 2005; Johnson *et al*. 2008). There were a few exceptions to these general patterns. Two species showed markedly deeper COI variation, ranging from 5.4–7.0%. Conversely, one case was encountered where barcode divergence was very limited between recognized species (*C. stygium/C. exile* cf. *mexicanum*).

The high intraspecific variation within both *Z. spelaeum* and *Z. isselianum* can be attributed to allopatric

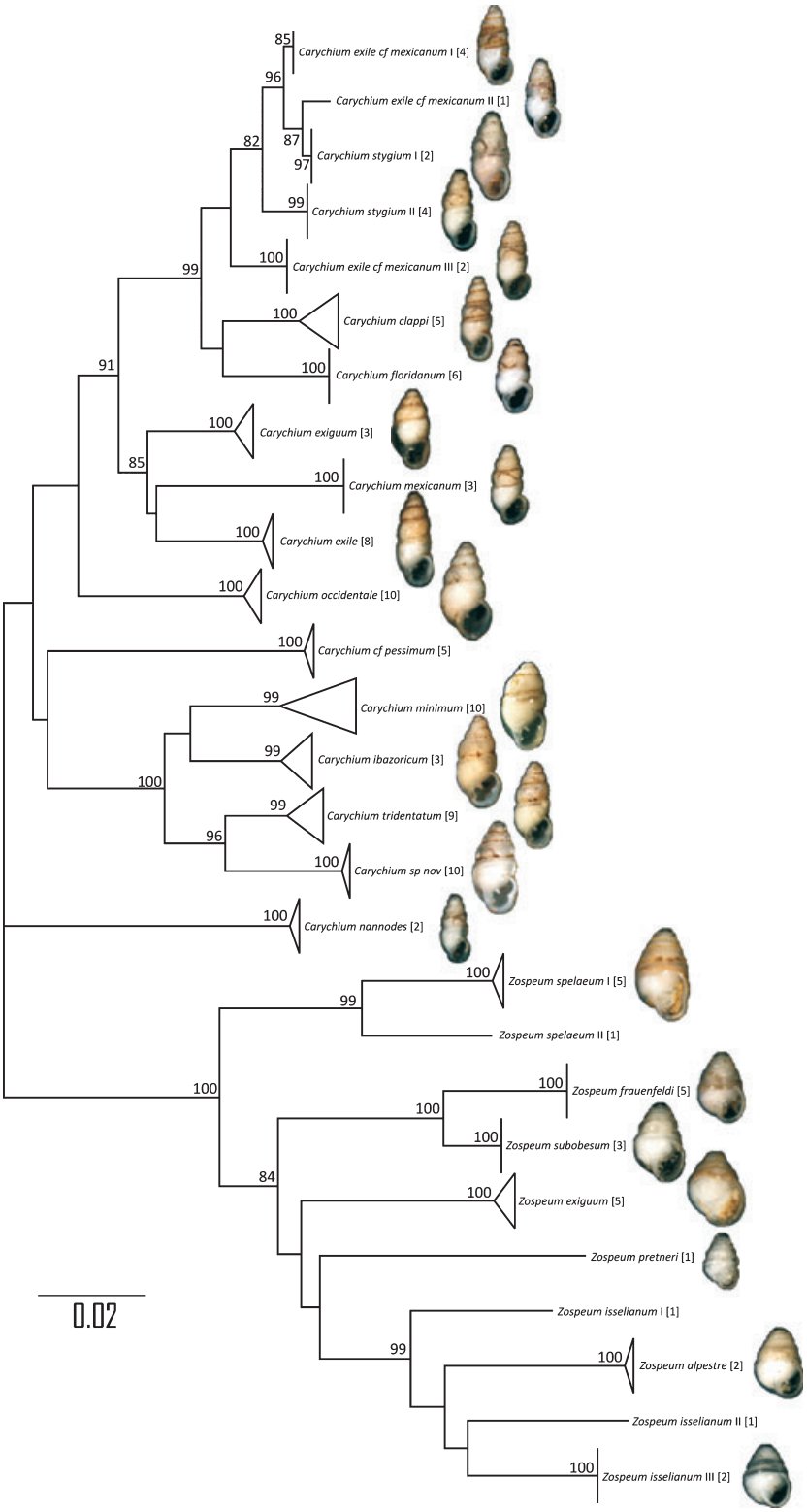

Fig. 2 NJ tree of the Carychiidae. Arabic numbers on the branches and in brackets represent bootstrap support and number of specimens analysed, respectively. Depth of the triangles indicates intraspecific COI sequence variation.

diversification, because it only occurs when different localities—caves in this case—were integrated in the analysis. Generally, within-cave intraspecific variability of all *Zospeum* clades ranged from 0% to 0.7%. *Z. spelaeum* is thought to be a widespread species throughout Slovenia, Croatia and Italy with relatively variable shell morphology (Bole 1974; Manganelli *et al*. 1995). This is contradicted by the fact that relatively stable

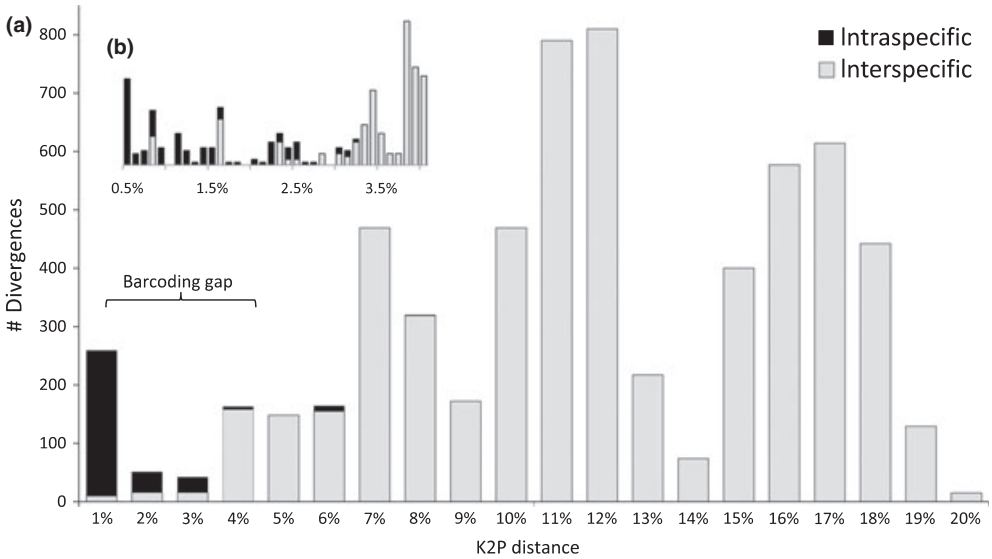

Fig. 3 DNA barcoding gap for Carychiidae. Histogram illustrates division of intraspecific (black) and interspecific (grey) COI sequence variation in Carychiidae (a). A barcoding gap can be set to a value of 3.2%, with most intraspecific variation below and interspecific variation above this threshold (b).

environmental conditions such as those encountered in subterranean habitats often favour existing morphotypes (morphological stasis) and thus, through time, cryptic species can evolve (Lefébure *et al.* 2006; Bickford *et al.* 2007; Pfenninger & Schwenk 2007). Hence, because of their small size and troglobitic lifestyle, *Zospeum* species are prone to morphological stasis. We found a high intraspecific variability of 5.1% between two clades of *Z. spelaeum* collected at two different localities in Slovenia. Given the rather atypical variable shell morphology (Bole 1974; Manganelli *et al.* 1995), this probably indicates cryptic species.

An even higher intraspecific variability of 7.0% can be observed for *Z. isselianum*. Specimens were sampled at three different locations in Slovenia and the groups referred to as *Z. isselianum* clade I, II and III in Fig. 2 again represent cave-specific clusters. Furthermore, representatives of *Z. alpestre* and *Z. isselianum* form a well-supported group (Fig. 2, bootstrap 99).

Despite cryptic allopatric speciation, a deep COI variation could alternatively be explained by the process of incomplete lineage sorting, where no reciprocal monophyly has yet been reached (McGuire *et al.* 2007). A cryptic allopatric diversification seems most likely because the high intraspecific variation for *Zospeum* taxa was only observed in our data set when different caves are considered. In this light, DNA barcoding enables first insights into the cryptic diversity of *Zospeum* taxa, which was not revealed by morphological approaches alone.

A surprising result is the positioning of the single troglobitic *Carychium* taxon, *Carychium stygium*, because DNA barcoding was not able to delineate it from another taxon, *Carychium exile* cf. *mexicanum*. The two populations of *C. stygium* were collected in caves of Kentucky (Hidden River Cave) and Tennessee (entrance to Slit Cave), whereas all *C. exile* cf. *mexicanum* specimens originate from epigean habitats in eastern Alabama and Georgia. Nevertheless, these two taxa form well-supported clades (Fig. 2, bootstrap 96 and 82) with an averaged intraspecific variability of 1.7% (range: 0.8–2.8%). In reference to the barcoding gap (Fig. 3), this value is typical for within-species divergences in Carychiidae and other taxa (Hebert *et al.* 2003; Barrett & Hebert 2005). An explanation for the relatively low interspecific variability and nonreciprocal monophyly for this clade could be incomplete lineage sorting, i.e. the presence of ancestral haplotypes in both groups (McGuire *et al.* 2007). Alternatively, DNA barcoding flags, and thus treats, the *C. stygium/C. exile* cf. *mexicanum* clade as a single taxon and neither the species status of *C. stygium* nor the subspecies rank of *C. exile* cf. *mexicanum* is supported.

Because other troglobiontic or trogloxenic *Carychium* populations exist, e.g. in Hubricht (1964) and Peck (1989) referring to *C. exile* and Lewis *et al.* (2003) who refer to *C. mexicanum*, cave dwelling per se as the most signature qualification for species assignment of *C. stygium* cannot be considered sufficient (Hubricht 1960). Moreover, our own histological observations of the 'somewhat reduced eyes' for *C. stygium* individuals as stated in Hubricht (1960) very well suggest either a relatively young species formation, incipient speciation or a broad range of suitable habitats for a *C. stygium* + *C. exile* cf. *mexicanum* taxon.

In Europe, DNA barcoding was able to delineate four taxa. Three species (*C. minimum*, *C. tridentatum* and *C. ibazoricum*) are well-known entities of the European gastropod fauna. A fourth taxon (*Carychium* sp. nov.) has been revealed through our approach and most likely should be described *de novo*. With a minimal interspecific variability of 3.8% to *C. tridentatum* and a maximal intraspecific variability of 0.2%, the population found in Bulgaria (entrance to Gargina Dupka Cave) meets our DNA barcoding criteria enough to delineate it from other carychiid species (intraspecific variability < 3.2% < interspecific variability).

Earlier taxonomic concepts and investigations of the Carychiidae have relied primarily on conchological characters (Pilsbry 1948, Burch & Van Devender 1980; Bank & Gittenberger 1985; Harry 1998). Critical consideration of varying environmental conditions respective to superficial subterranean and cave habitats as well as their influence on species morphology, i.e. phenotypic plasticity must be emphasized (West-Eberhard 1989; Pfenninger & Magnin 2001; Nekola & Barthel 2002). In this light, the gastropod shell alone does not always serve as an informative character for taxonomic purposes (Giusti & Manganelli 1992; Pfenninger *et al.* 2006) because it exhibits mostly nondiscrete (i.e. continuous) character states (DeSalle 2006). In addition, because of their minute size, microgastropods such as the Carychiidae have presented a very complicated, often subjective, taxonomic history of species assignment based on conchological characters (Burch & Van Devender 1980; Harry 1998). In this study, molecular (i.e. discrete) characters clearly designate *Carychium* microsnails in 86% (12/14) of all cases (Figs 2 and 3) while those remaining represent yet undescribed candidate species or failure in morphological taxonomy rather than failure of the barcoding method. This is indeed a taxonomic breakthrough because the tediousness and difficulty of overlapping shell morphology—as known for many *Carychium* taxa (Pintér 1967; Burch & Van Devender 1980; Harry 1998; Nekola & Barthel 2002)—can be overcome by nonoverlapping, taxon-specific DNA barcodes (Hajibabaei *et al.* 2007), which resolves the majority of these taxonomic discrepancies.

Generally, DNA barcoding demonstrates its applicability for species designation as well as underscores its potential to flag cryptic morphologically static or phenotypically plastic taxa. In the case of the Carychiidae, it is evident that a species revision is needed. Moreover, a first focus on the troglomorphic carychiid microsnails is paramount.

Conclusion

This study effectively demonstrates the applicability of DNA barcoding for the identification of carychiid microsnails. Despite their minute size, the shell is prone to phenotypic plasticity (*Carychium* taxa) or morphological stasis (*Zospeum* taxa). The taxonomical reliance upon continuous conchological characters is questionable particularly because it represents an inadequate, outdated system for phenotypically plastic and morphologically static taxa. The taxonomic value of DNA barcoding in this taxon and probably for many other gastropods using discrete molecular data is high. We recommend the application of this method for further species investigations of microsnail taxa. Additionally, DNA barcoding enables a first insight into the cryptic diversity common in subterranean habitats while presenting a promising approach to the dynamics of subterranean studies in general.

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