Org Divers Evol (2012) 12:377–386
DOI 10.1007/s13127-011-0070-2

ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

Outdated but established?! Conchologically driven species delineations in microgastropods (Carychiidae, *Carychium*)

Alexander M. Weigand · Marie-Carolin Götze · Adrienne Jochum

Received: 31 January 2011 / Accepted: 13 December 2011 / Published online: 6 January 2012

Abstract Valid taxonomic descriptions are paramount in evolutionary biology. Many date back centuries and are based on ambiguous morphological data. Microgastropods, in particular the taxon Carychiidae (Eupulmonata, Ellobioidea), demonstrate a paucity of informative conchological features. However, as exemplified by *Carychium mariae* Paulucci, 1878, their taxonomic classification is based almost entirely on these few features. Here we investigated the questionable taxonomic status of *Carychium mariae* combining DNA barcoding, field-emission scanning electron microscopy and conchological data. This taxon occurs in the Southern Alps, where it shows a sympatric distribution with two widely distributed members of *Carychium*—*C. minimum* Müller, 1774 and *C. tridentatum* (Risso, 1826). Our analyses do not support the species status of *C. mariae*. In contrast, DNA barcoding reveals a monophyletic grouping of *C. minimum* and *C. mariae* specimens with averaged intraspecific variability less than 3.2% (barcoding gap for Carychiidae). Hence, *C. mariae* is treated and should be regarded as a synonym of *C. minimum*, just representing a different morphotype. The differentiation and monophyletic status of *C. tridentatum* can be validated by showing an averaged interspecific variability of 5.9% to *C. minimum*. In general, we are critical of the sole use of conchological characters for microgastropod taxonomy and strongly recommend the implementation of molecular data (e.g., DNA barcoding) to reevaluate established species designations.

Keywords Microgastropod taxonomy · Species descriptions · Ellobioidea · *Carychium mariae*

A. M. Weigand (✉) · M.-C. Götze · A. Jochum
Institute for Ecology, Evolution and Diversity, Goethe University, Max-von-Laue-Str. 13,
60438 Frankfurt am Main, Germany
e-mail: a.weigand@bio.uni-frankfurt.de

Introduction

Understanding biodiversity is a crucial factor in evolutionary biology. Misinterpretation of biological patterns (e.g., as a result of taxonomic misidentifications) can lead to incorrect conclusions and consequently to the proposition of false hypotheses. The incorporation of ethological, molecular and morphological characters when describing and identifying species ("integrative taxonomy") has led to a plethora of new taxonomic descriptions within the last few years (e.g., Martens et al. 2008; Pauls et al. 2010; Bucklin et al. 2011). However, since they represent testable scientific species hypotheses, still many longer established species descriptions are in dire need of a taxonomic re-examination (Dayrat, 2011; Haszprunar 2011).

In this study, we use DNA barcoding (Hajibabaei et al. 2007) and scanning electron microscopy of conchological data to taxonomically revise the species status of a member of the microgastropod taxon, Carychiidae (Gastropoda, Eupulmonata, Ellobioidea) (Morton 1955a; de Frias Martins 1996, 2007). Gastropods are subject to various evolutionary processes, complicating species identifications. For example, historic taxonomic emphasis has been placed on shell morphology or on variable color patterns, characters now known to be often subject to phenotypic plasticity, therefore frequently leading to a number of erroneous taxonomic descriptions (species splitting). By contrast, complexes of morphologically static or cryptic species are treated as a single taxon (species lumping) (Pinceel et al. 2004; Pfenninger et al. 2006; Bickford et al. 2007; Jordaens et al. 2010; Weigand et al. 2011). In particular, carychiid microgastropods belong to a systematic group in which a taxonomic revision is absolutely necessary (Giusti and Manganelli 1992; Weigand et al. 2011). Due to their minute size (a few millimeters) and lack of sufficient, distinguishing

conchological and anatomical characters, they are particularly prone to misidentification (Watson and Verdcourt 1953).

The taxon *Carychium* inhabits permanently moist superficial subterranean habitats (e.g., leaf litter, crevices) (Watson and Verdcourt 1953; Harry 1998; Culver and Pipan 2009). According to the most recent summary of European *Carychium* by Bank and Gittenberger (1985), eight morphospecies are described. Recently, they have been partially supported by a DNA barcoding study (Weigand et al. 2011). However, the species status of *Carychium mariae* Paulucci, 1878 is still very questionable. This taxon is known from Northern Italy, France and the northwestern corner of the former Yugoslavia (Zimmermann 1925; Maassen 1987; Slapnik 1991; Cossignani and Cossignani 1995) (Fig. 1a), where it possesses a sympatric distribution with *Carychium minimum* Müller, 1774 and *Carychium tridentatum* (Risso, 1826) (Bank and Gittenberger 1985; Slapnik 1991; Cossignani and Cossignani 1995; Simon et al. 2010). Up to now, *C. mariae* has been described and distinguished from these two taxa based on conchological characters alone (Zimmermann 1925; Bank and Gittenberger 1985).

Material and methods

Sampling

Our data set comprised ten populations of the morphospecies *C. mariae*, *C. minimum* and *C. tridentatum* collected throughout the core distribution area of *C. mariae* in Northern Italy, Croatia and Slovenia during the years 2009 and 2010 (Table 1, Fig. 1a). Molecular analyses were conducted on all 83 individuals (a-j, maximal ten per population and morphospecies). Additional COI barcode sequences representing the ingroup taxa *C. minimum* and *C. tridentatum* as well as the outgroup taxa *Carychium ibazoricum* Bank and Gittenberger, 1985, *Zospeum subobesum* Bole, 1974 and *Zospeum frauenfeldi* (Freyer, 1855) (Table 2) were retrieved from Weigand et al. (2011). E-voucher information (e.g., georeference data, images, COI sequences) for all specimens can be obtained from the project 'PHYCA' stored in the Barcode of Life Data System (BOLD; Ratnasingham and Hebert 2007). Physical vouchers for all but one population (population 10) are deposited in the Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt am Main, Germany (SMF 336668–SMF 336679). Morphospecies identification was based upon historically determined conchological characters for Carychiidae (Table 3). All individuals have been investigated for striation and measured for shell dimensions (shell height, shell width, ratio shell height/shell width) by counting pixels in the images (Table 1 and BOLD project 'PHYCA'). Since striation was very prominent in specimens of all three taxa and other conventionally used characters for species delimitation of *C. mariae*, *C. minimum* and *C. tridentatum* can be inconclusive (see Discussion), we performed a conservative strategy: Based on the morphometric measurements, the degree of sinuosity of the parietal lamellae, the level of striation and the number of whorls, we identified the eight most characteristic specimens of each of the three *Carychium* morphospecies (Table 4) out of the pool for continuous conchological variability (Fig. 1b).

DNA isolation and marker amplification

Freshly collected individuals were immediately preserved in 70-99% ethanol. Individuals were photographed prior to DNA extraction. Shell and visceral materials were removed to minimize contamination risk. DNA extraction was performed following the DNeasy Blood and Tissue protocol (Qiagen, Hilden, Germany).

The COI Folmer fragment was amplified by polymerase chain reaction (PCR) using the standard invertebrate primer pair LCO1490-5'GGTCAACAAATCATAAAGATATTGG3' and HCO2198-5'TAAACTTCAGGGTGACCAAAAAATCA3' (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. PCR products were visualized on a 1.4% agarose gel and cleaned with the GeneJET PCR Purification Kit (Fermentas, St. Leon-Rot, Germany). 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 3730xl capillary sequencer following the manufacturer's instructions.

Molecular and morphological analyses

Sequence data were assembled, edited and aligned using Geneious 5.0.3 software (Biomatters, Ltd.). Neighbor-joining (NJ) analyses were executed in MEGA5 (Tamura et al. 2011) with bootstrap analysis of 2,000 replicates under the Kimura 2-parameter model (K2P) and pairwise-deletion option. Genetic distances were calculated using MEGA5 and the K2P pairwise-deletion option.

Well-preserved empty shells of each morphospecies were cleaned and prepared for field emission scanning

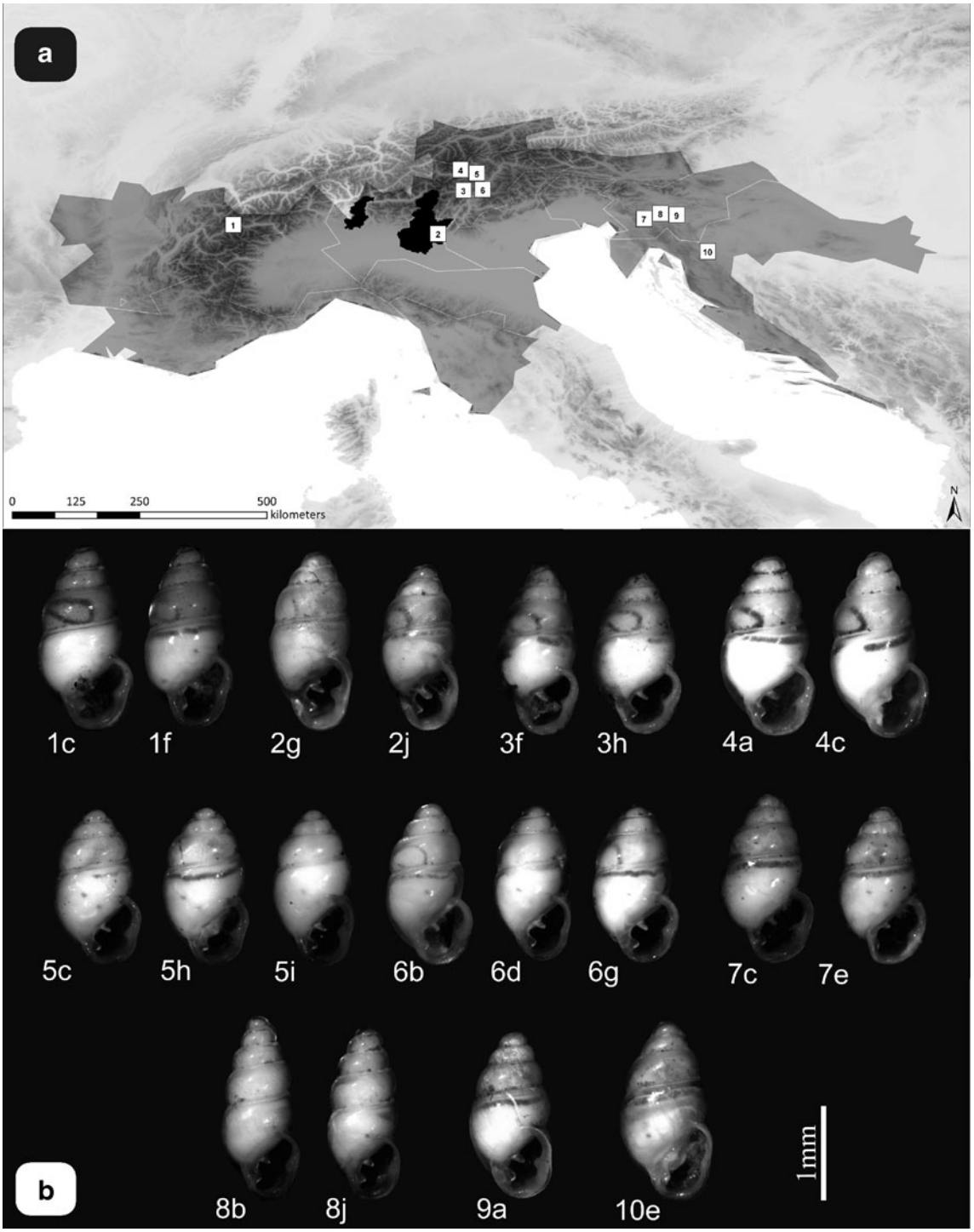

Fig. 1 **a** Geographic map with core distribution area of *C. mariae* shown in *grey* and sampling sites of *C. mariae* specimens from the first description by Paulucci (1878) given in *black*. *Numbers 1-10* indicate sampling localities of the current study (see Table 1). **b** Selected individuals (*a-j*) of analyzed populations 1-10

electron microscopy (FE-SEM) using a Hitachi S 4500 apparatus. FE-SEM samples were gold or gold-palladium sputtered for 4 min with an agar sputter coater and recorded with a Digital Image Processing System 2.6 (Point Electronic, Halle, Germany) or with a CamScan CS 24 apparatus.

Table 1 Georeference and morphometric data for populations under investigation. Sampling for population 3 has been conducted at two nearby locations (3-1 for *C. minimum* /*C.mariae* and 3-2 for *C. tridentatum* morphospecies). Shown are individual morphometrics (a-j) for shell height x shell width (in cm) and the ratio height/width (given in brackets). N = number of specimens for genetic analyses. * = only for FE-SEM data. / = juvenile specimen

Pop.	N	latitude	longitude	locality	a	b	c	d	e	f	g	h	i	j
1	9	45.7170	6.9445	Italy (ITA), Aosta Valley, La Thuile	2.02x0.91 (2.22)	2.00x0.88 (2.27)	1.92x0.89 (2.16)	2.05x0.88 (2.33)	1.93x0.86 (2.24)	1.91x0.84 (2.27)	1.78x0.87 (2.05)	1.90x0.82 (2.32)		1.89x0.89 (2.12)
2	10	45.5588	10.5617	Italy (ITA), Lombardy, Manerba del Garda	1.79x0.86 (2.08)	1.73x0.81 (2.14)	1.81x0.86 (2.10)	1.77x0.85 (2.08)	1.80x0.79 (2.28)	1.71x0.85 (2.01)	1.87x0.86 (2.17)	1.65x0.77 (2.14)	1.71x0.83 (2.06)	1.74x0.80 (2.18)
3-1	10	46.3261	11.0114	Italy (ITA), Trentino, Tuenno	1.78x0.87 (2.05)	1.71x0.84 (2.04)	1.73x0.88 (1.97)	1.76x0.86 (2.05)	1.79x0.85 (2.11)	1.79x0.85 (2.11)	1.80x0.89 (2.02)	1.69x0.88 (1.92)	1.78x0.87 (2.05)	1.70x0.86 (1.98)
3-2	*	46.3142	11.0200	Italy (ITA), Trentino, Tuenno										
4	3	46.6901	11.0607	Italy (ITA), Upper Adige, Partschins	1.90x0.94 (2.02)	1.83x0.87 (2.10)	1.95x0.93 (2.10)							
5	9	46.4889	11.2464	Italy (ITA), Upper Adige, Eppan	1.63x0.82 (1.99)	1.63x0.83 (1.96)	1.65x0.84 (1.96)	1.69x0.86 (1.97)		1.67x0.89 (1.88)	1.57x0.83 (1.89)	1.64x0.85 (1.93)	1.66x0.85 (1.95)	1.67x0.83 (2.01)
6	9	46.3385	11.3503	Italy (ITA), Upper Adige, Auer	/	1.69x0.82 (2.06)	1.68x0.84 (2.00)	1.63x0.81 (2.01)	1.76x0.84 (2.10)		1.66x0.83 (2.00)	1.73x0.86 (2.01)	1.61x0.80 (2.01)	/
7	10	45.8250	14.2480	Slovenia (SLV), Planinsko Polje, Planina	1.78x0.88 (2.02)	1.73x0.85 (2.04)	1.77x0.85 (2.08)	1.80x0.92 (1.96)	1.64x0.84 (1.95)	1.66x0.82 (2.02)	1.68x0.79 (2.13)	1.83x0.90 (2.03)	1.69x0.79 (2.14)	1.77x0.86 (2.06)
8	10	45.9189	14.4934	Slovenia (SLV), Krim Region, Gornji Ig	1.79x0.79 (2.27)	1.92x0.77 (2.49)	1.88x0.74 (2.54)	1.88x0.75 (2.51)	1.99x0.76 (2.62)	1.76x0.74 (2.38)	1.88x0.79 (2.38)	1.95x0.77 (2.53)	1.81x0.74 (2.45)	1.91x0.74 (2.58)
9	6	45.8897	14.7756	Slovenia (SLV), Gradiček, near Krka	1.73x0.85 (2.04)	1.65x0.85 (1.94)	/	/	/	/				
10	6	45.2439	15.3253	Croatia (CRO), Karlovac, Tounj	1.97x0.95 (2.07)	1.93x0.92 (2.10)	1.89x0.92 (2.05)	2.02x0.95 (2.13)	1.88x0.93 (2.02)	1.98x0.93 (2.13)				

Table 2 DNA barcodes and corresponding GenBank accession numbers

taxon	Barcode ID	GenBank Accession
Carychium minimum Müller, 1774	BARCA065-10	HQ171538
	BARCA066-10	HQ171537
	BARCA068-10	HQ171535
	BARCA071-10	HQ171532
Carychium tridentatum (Risso, 1826)	BARCA075-10	HQ171578
	BARCA078-10	HQ171575
	BARCA080-10	HQ171573
	BARCA082-10	HQ171571
Carychium ibazoricum Bank & Gittenberger, 1985	BARCA072-10	HQ171528
	BARCA073-10	HQ171527
	BARCA074-10	HQ171526
Zospeum frauenfeldi (Freyer, 1855)	BARCA107-10	HQ171590
	BARCA110-10	HQ171587
	BARCA111-10	HQ171586
Zospeum subobesum Bole, 1974	BARCA112-10	HQ171604
	BARCA113-10	HQ171603
	BARCA114-10	HQ171602

Results

Shell morphology

Morphospecies Carychium tridentatum (Risso, 1826)

The white translucent shell ranges from 1.7–2.1 mm in height and measures 0.7–0.9 mm in width. The shell is cylindrical to spindle shaped, the diameter being narrower in relation to height than in the sympatric species *C. minimum*. Although some individuals (e.g., FE-SEM material for columellar view, Fig. 2) have 4½ whorls; other representatives in our material possess 5¼ whorls. This variation in whorl number has been described by previous authors (Zimmermann 1925; Watson and Verdcourt 1953; Lozek 1957; Pintér 1967; Kerney et al. 1979; Bank and Gittenberger 1985). The surface sculpture of *C. tridentatum* consists of finely threaded, closely spaced transverse striae. The whorls are positioned more horizontally (dorsal and ventral views) relative to the central axis of the shell in contrast to those of *C. minimum* and *C. mariae*. The increased degree of undulation of the upper parietal lamella is

Table 3 Literature summary of conchological characters. Information is given for conventionally used conchological characters for the delimitation of the three morphospecies *Carychium minimum*, *C. mariae* and *C. tridentatum*. Data retrieved from Müller 1774, Risso 1826, Paulucci 1878, Zimmermann 1925, Watson and Verdcourt 1953, Lozek 1957, Strauch 1977, Kerney et al. 1979, Bank and Gittenberger 1985 and Schütt 2010

Carychium minimum, Müller 1774	*Carychium mariae* Paulucci, 1878	*Carychium tridentatum* (Risso, 1826)
Ovate-conic; well-rounded, lightly convex, slightly flattened whorls and indented suture; faint, rounded, closely-spaced transverse striae; penultimate and ultimate whorls more convex; shell transparent when fresh	Ovate-conic; "more obese form" (Paulucci 1878); well rounded whorls and indented suture; spire less slender than *C. tridentatum*; conspicuous transverse riblets; prominent strong striae easily differentiate this species from *C. minimum*; shell transparent when fresh	Proportionately more slender than *C. minimum*; cylindrical to spindle-shaped; well-rounded convex whorls and indented suture; fine, raised, closely-spaced transverse striae; penultimate whorl larger than *C. minimum;* shell transparent when fresh
Height: 1.6 - 1.9 mm (Ø 1.8 mm); Width: 0.8 - 1.0 mm; Dimensions: twice as long as broad (Ø H/W ~ 2)	Height: max. 1.5 - 2.0 mm (Ø 1.7 mm); Width: 0.85 - 1.0 mm; Dimensions: less than twice as long as broad (Ø H/W < 2)	Height: 1.7 - 2.1 mm (Ø 1.95 mm); Width: 0.8 - 1.0 mm; Dimensions: more than twice as long as broad (Ø H/W > 2)
Number of whorls: 4 ½ - 4 ¾	Number of whorls: 4.0 - 4 ½	Number of whorls: 4 ½ - 5 ¼
Ultimate whorl expands somewhat quicker and is thus more convex above the aperture as the penultimate whorl	Initial whorls increasing more quickly in height than *C. tridentatum*	Attenuate growth of whorls; evenly convex; ultimate and penultimate whorls less convex than *C. minimum* not extending over the aperture
Aperture oblique oval, 2/5 or more of shell height; outer lip thick and slightly turned back towards the base; single central outer lip denticle; side profile of lip sinuous Narrow, thin parietal lamella demonstrates simple, s-shaped profile; less elaborate than that of *C. tridentatum*	Columellar lamella broadly extended, directly under outer wall of shell; thickened on the edges; columellar lamella almost as wide as parietal lamella; Columellar lamella almost as wide as parietal lamella; parietal lamella characterized by adapical tongue-like, non-sinuous lateral wedge positioned in an upward direction; edges of lamellae contorted, comparable to *C. tridentatum* and quite different from *C. minimum*	Height of aperture smaller than 2/5 the shell height; aperture oblique elliptic; wellthickened lip flares more than *C. minimum*; single central denticle adorns outer lip Columellar fold more prominent in *C. tridentatum* than *C. minimum*; parietal lamella characterized by irregular, doubly twisted ridge-like shelf thickening as it bends upward and then abruptly vertically downward
Ecology hygrophilic; alluvial plains, fens, marshes and riparian zones	Ecology sympatric with *C. tridentatum* south of the Alps; wide range in Northern Italy, France and northwestern corner of former Yugoslavia	Ecology petrophilic ; shaded moist deciduous woodlands

Table 4 Morphometric data for the eight most characteristic specimens of each morphospecies. Provided are values for the minimum, maximum and the average (in brackets) of the investigated dimensions

	C. mariae	*C. minimum*	*C. tridentatum*
shell height [cm]	1.57 - 1.69 (1.64)	1.77 - 1.83 (1.79)	1.88 - 2.05 (1.95)
shell width [cm]	0.82 - 0.89 (0.85)	0.85 - 0.92 (0.88)	0.74 - 0.88 (0.79)
width/height	1.88 - 1.99 (1.93)	1.96 - 2.10 (2.05)	2.27 - 2.62 (2.48)
number of whorls	4 - 4 ¼ (4)	4 ¼ - 4 ¾ (4 ½)	5 (5)
striation	prominent	less prominent	less prominent

characteristic for this morphospecies. Our FE-SEM ventral and side observations and image data of *C. tridentatum* reveal neither new dimensions nor considerable differences to all previous descriptions of this species (Fig. 2).

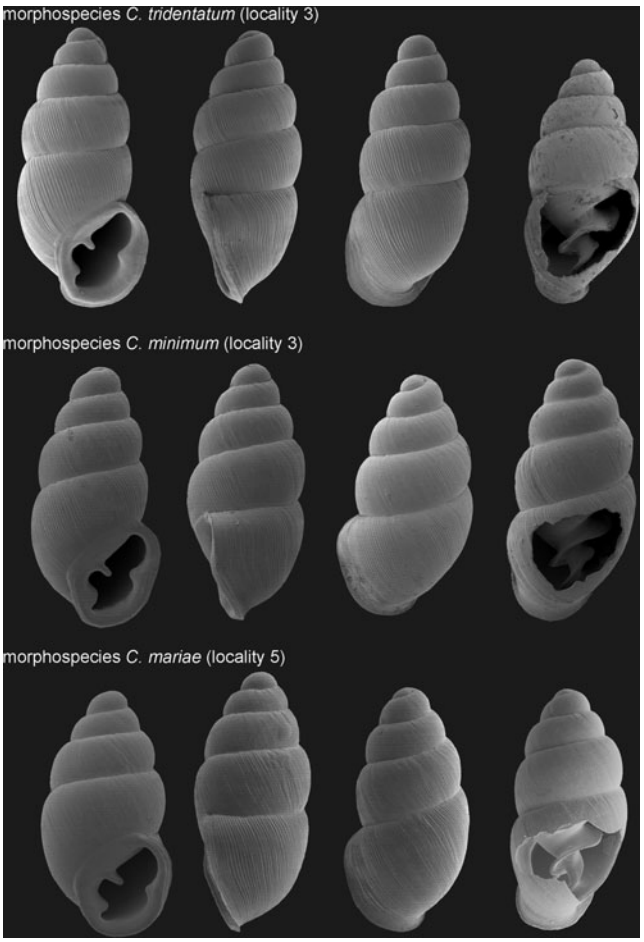

Fig. 2 FE-SEM results of conchological details of *Carychium tridentatum*, *C. minimum* and *C. mariae* morphospecies. Shells are arranged in the following order (*left* to *right*): ventral view, side view, dorsal view, columellar view. Locality information can be retrieved from Table 1

Morphospecies Carychium minimum Müller, 1774

The white translucent shell is ovate-conic and ranges from 1.6–2.0 mm in height. The shell is less slender than that of *C. tridentatum*, and the diameter is broader in relation to height, ranging from 0.8–1.0 mm. The surface sculpture consists of fine, thread-like, closely spaced transverse striae. The 4½–5 whorls are separated by more oblique sutures compared with *C. tridentatum*. The upper parietal lamella reveals the characteristic, weak sinuous flexion as described by previous authors (Zimmermann 1925; Watson and Verdcourt 1953; Lozek 1957; Pintér 1967; Kerney et al. 1979; Bank and Gittenberger 1985). Our FE-SEM ventral and side observations and image data of *C. minimum* reveal neither new dimensions nor substantial differences in contrast to all previous descriptions of this species (Fig. 2).

Morphospecies Carychium mariae Paulucci, 1878

The translucent, thin, uniform whitish shell "differs from all others by its more obese form" (Paulucci 1878), which is ovate-conic and ranges from 1.6–1.8 mm in height and 0.8–0.9 mm in width. The surface sculpture consists of prominent, fine, thread-like closely spaced, transverse striae coursing the 4–4 ½ convex whorls. The spire is more convex and less cylindrical than in *C. tridentatum*. The characteristic, sinuous parietal lamella resembles that of *C. tridentatum* in the slightly increased degree of undulation as well as its orientation in relation to the aperture opening. Our FE-SEM frontal, ventral and side observations and image data reveal neither new dimensions nor any distinguishable differences (Zimmermann 1925; Watson and Verdcourt 1953; Lozek 1957; Pintér 1967; Kerney et al. 1979; Bank and Gittenberger 1985) (Figs. 2, 3). Finally, the eight most characteristic specimens identified as *C. mariae* within our study resemble those of investigated *C. mariae* syntypes (Fig. 4).

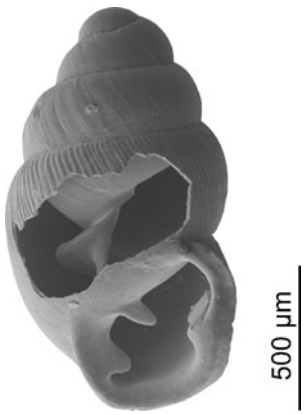

Fig. 3 FE-SEM image of typical morphospecies *Carychium mariae* Paulucci, 1878 (frontal view) from locality 5

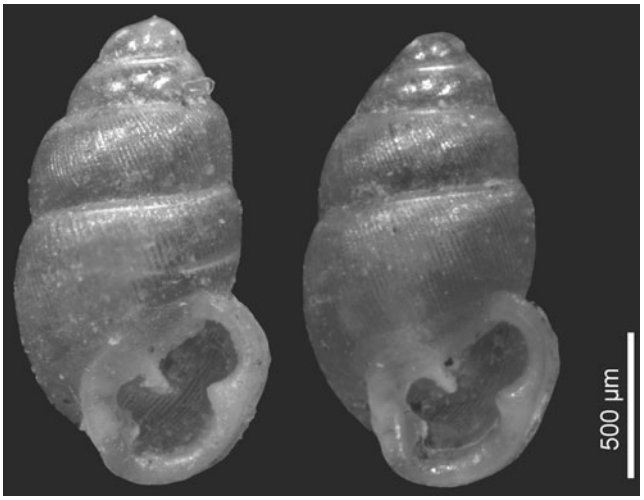

Fig. 4 Two syntype specimens of *Carychium mariae* Paulucci, 1878. Depicted are syntypes stored in the Museo di Storia Naturale dell'Università di Firenze, Sezione di Zoologia de "La Specola" with collection number MZUF GC/11971; 6 sp.; Italy, Lombardy region (MN), Castel Goffredo; M. Paulucci. Additional syntypes (not shown) are MZUF GC/11985 (1 sp.), MZUF GC/13599 (32 sp.) and MZUF GC/13600 (202 sp.)

Our conchological observations of the three *Carychium* morphospecies are in congruence with Bank and Gittenberger's (1985) conchological analyses of *C. mariae* (their figs. 9-14), *C. tridentatum* (their figs. 15-28) and *C. minimum* (their figs. 29-30) (Table 3). These observations also underscore fossil analyses performed by Strauch (1977) in which he describes the degree of characteristic sinuosity of the parietal lamella in association to its placement on the columellar apparatus while viewing the shell ventrally. Although the three morphospecies in this study depict varying degrees of sinuosity from the posterior view, the concept is the same: flexure of the S-sinuate lamella varies with each individual shell and individual species.

DNA barcoding

The alignment of 96 COI sequences of *Carychium mariae*, *C. minimum* and *C. tridentatum* morphospecies and barcodes of the in- and outgroup taxa (Table 2) has a total length of 655 base pairs. The resulting neighbor-joining tree shows a significant split between an evolutionary significant unit (ESU) including the eight most characteristic *C. tridentatum* specimens (ESU1) and another ESU including the eight most characteristic specimens of *C. mariae* and *C. minimum* (ESU2) (bootstrap support 100, Fig. 5a, b). The averaged between-group genetic variability (ESU1/ESU2) is 5.9% with averaged within-group variabilities of 0.6% and 1.7% for ESU1 and ESU2, respectively. DNA barcoding of the ESUs using in- and outgroup barcode IDs (Table 2) clearly identifies the specimens of ESU1 as *C. tridentatum* and all members of ESU2 as *C. minimum* with maximal genetic variability lower than 3.2% (barcoding gap for Carychiidae, Weigand et al. 2011) within both groups.

Discussion

We tested whether the three microgastropod morphospecies of *Carychium mariae*, *C. minimum* and *C. tridentatum* represent distinct and monophyletic units. Scanning electron microscopy of conchological features and molecular data were used to test our hypothesis. In all previous studies, only conchological characters (striation, shell dimensions and folds along the columellar apparatus) were consulted for identification of the three species in their sympatric habitats (e.g., Zimmermann 1925; Bank and Gittenberger 1985; Cossignani and Cossignani 1995) (Table 3). However, these characters must be considered with caution and are discussed in the following.

Striation as one of the most prominent features distinguishing *C. mariae* and *C. minimum* has to be regarded as an insufficient character. Despite critical evaluation of shell dimensions, we were hardly able to identify three morphospecies (Figs. 2, 5a). Moreover, our FE-SEM results indicate that even *C. minimum* and *C. tridentatum* individuals from Northern Italy show a remarkable degree of striation (Fig. 2). Hence, we strongly support the opinion proposed in previous studies on *Carychium* species (Watson and Verdcourt 1953; Morton 1955b; Nekola and Barthel 2002) of striation being a phenotypically plastic character varying with changing environment. One explanation for an environment-dependent variation of striation could be the amount of moisture available in the habitat (Watson and Verdcourt 1953). In dry habitats, the soft tissue, particularly the glandular margin of the mantle secreting the shell, will be less swollen with moisture. As a result, this will lead to a narrower shell with whorls tending to expand more rapidly and to the striae being more prominent and distant from each other.

Nevertheless, shell dimensions such as shell height or width, the number of whorls and proportions of the shell (shell width/shell height) play a significant role in carychiid species assignments (Pilsbry 1948; Burch and Van Devender 1980; Bank and Gittenberger 1985), and thus also for *C. mariae*. This taxon is slightly smaller than *C. minimum* and possesses an average shell ratio of approximately 2 (Zimmermann 1925) (Table 3). In his study and other studies referring to him, Zimmermann (1925) proposed a scenario with *C. mariae* replacing the widespread *C. minimum* in the Southern European Alps. Nekola and Barthel (2002) conducted a morphometric study analyzing shell dimensions of two North American carychiid microgastropods, *C. exiguum* and *C. exile*. In a similar manner, they found larger individuals of the same species present in the

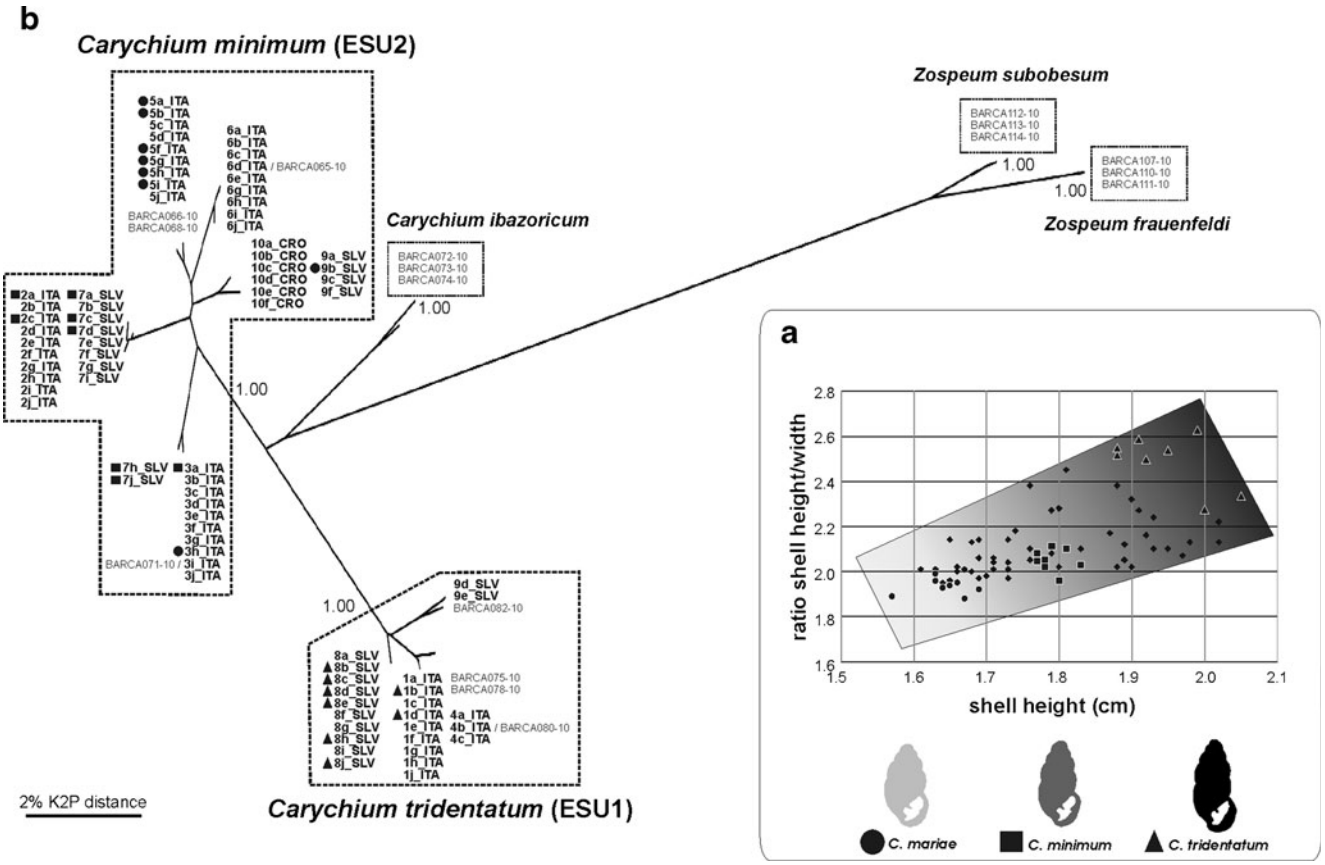

Fig. 5 **a** Range of conchological variability for the parameters shell height (*x-axis*) and the ratio shell height/width (*y-axis*). The eight most characteristic specimens per morphospecies are indicated by a *circle* (*Carychium mariae*), *square* (*C. minimum*) and *triangle* (*C. tridentatum*), respectively. *Dark grey*, more typical *C. tridentatum* specimens; *light grey*, more typical *C. mariae* specimens; typical *C. minimum* individuals are in between those two extremes. **b** Neighbor-joining tree of carychiid COI sequences. The same eight most characteristic specimens per analyzed morphospecies are marked with a *circle* (*C. mariae*), *square* (*C. minimum*) and *triangle* (*C. tridentatum*), respectively. Barcode sequences are indicated with their barcode ID from the BOLD project 'BARCA'. *Numbers* provided at the branches represent bootstrap support. *CRO*=Croatia, *ITA*=Italy, *SLV*=Slovenia

north and proposed a clinal variation of shell dimensions. In light of our results, we observe an identical phenomenon for the designated '*C. mariae* morphotype' of the widely distributed species, *C. minimum*.

The most frequently used characters for *Carychium* species identification are structure and form of the folds (parietal lamellae) encompassing the columellar apparatus (Winslow 1922; Pilsbry 1948; Burch and Van Devender 1980; Bank and Gittenberger 1985). However, Bulman (1990) showed the characteristics of the columellar apparatus of *C. tridentatum* to be a continuous trait and to vary as a function of the number of whorls. Morphospecies can be identified as different (sub-) species just by viewing the columellar apparatus from a different angle. In their review of European *Carychium* species, Bank and Gittenberger (1985) state that there is considerable structural variation in the shape of the internal plate comprising the parietal lamella in *C. mariae* when investigated in frontal view (as is often the case).

Due to these tenuous conchological characters, we included molecular data for our analyses of carychiid microgastropod taxa – in particular for *Carychium mariae*. DNA barcoding has proven suitable for the complementary investigation and delimitation of established taxa (Hajibabaei et al. 2006, 2007; Smith et al. 2008; Radulovici et al. 2009; Steinke et al. 2009). Moreover, DNA barcodes successfully identify cryptic taxa and link varying ontogenetic stages of single species (Hebert et al. 2004; Pfenninger et al. 2007; Johnson et al. 2008; Weigand et al. 2011). These results can be ascribed to the fact that DNA barcoding uses discrete characters instead of relying on mostly continuous traits such as color and size. Molecular identification of the ESUs was able to undoubtedly assign *C. tridentatum* DNA barcodes to all sequences of ESU1 and *C. minimum* DNA barcodes to all members of ESU2. COI sequences of assigned *C. mariae* specimens did not form a monophyletic group (Fig. 5b) and remained in ESU2 within the maximal intraspecific variability of 3.2%, characteristic for species members of the Carychiidae (Weigand et al. 2011). Hence, molecular data only reveal a significant distinction into two evolutionary significant units, *C. minimum* and *C. tridentatum*, with an averaged interspecific variability of 5.9% (bootstrap

support 100, Fig. 5b). Because individuals resembling all three morphospecies were analyzed (Figs. 2, 5a, Table 4) and DNA barcoding only reveals two ESUs (Fig. 5b), the species status of *C. mariae* cannot be supported by our molecular data, further contradicting the validity of the use of established conchological characters (e.g., striation and shell dimensions) for this taxon. Although *C. mariae* is widely distributed in Northern Italy (Zimmermann 1925; Bank and Gittenberger 1985), we have to acknowledge the fact of not sampling the species. In this case, the conventionally used diagnostic characters for the delimitation of this taxon (shell dimensions, degree of striation and sinuous flexion of parietal lamellae) have to be regarded as inconclusive. A survey of these traits is absolutely needed as they reveal a continuous pool of variable character states and are used more by intuition than with confidence. However, even though we only would have analyzed one single *C. mariae* specimen and this morphospecies indeed embodies a species and not a morphotype, this should be clearly visible in the molecular data.

Consequently, we propose demoting *Carychium mariae* Paulucci, 1878 from species status and prefer to consider it not only as a synonym, but rather a morphotype of *Carychium minimum* Müller, 1774. Moreover, a morphospecies concept alone is no longer applicable for microgastropod designations and in particular for carychiid taxonomy. Hence, we strongly suggest the incorporation of molecular data to re-investigate established but outdated taxonomic first descriptions.

Acknowledgements We thank the Malacological Society of London and the BiK-F Biodiversity and Climate Research Center of the research-funding programme 'LOEWE—Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz' of Hesse's Ministry of Higher Education, Research, and the Arts for their financial support. We also thank Jana Valentinčič, Frank Hardie and Rajko Slapnik for their collecting efforts as well as Manfred Ruppel and Yaron Malkowsky for their support in preparing the FE-SEM photographs. We are grateful to Annette Klussmann-Kolb, Hannah Schweyen and Eugenia Zarza, who provided valuable insights on the conceptual design of this study. Special gratitude goes to Ronald Janssen (Senckenberg Forschungsinstitut und Naturmuseum) and Bruno Dell'Angello for their insights and help in locating the type specimens of *C. mariae*. We especially wish to thank Simone Cianfanelli (Museo di Storia Naturale dell'Università di Firenze, Sezione di Zoologia de "La Specola") for kindly photographing the syntypes as well as for constructive help in accessing the collection of Marianna Paulucci. We also thank the anonymous reviewers and editors, who provided valuable input on an earlier version of the manuscript.

References

Bank, R. A., & Gittenberger, E. (1985). Notes on Azorean and European *Carychium* species (Gastropoda Basommatophora: Ellobiidae). *Basteria, 49*, 85–100.

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K. L., Meier, R., Winker, K., et al. (2007). Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution, 22*, 148–155.

Bole, J. (1974). Rod *Zospeum* Bourguignat 1856 (Gastropoda, Ellobiidae) v Jugoslaviji. *Razprave/Slovenska akademija znanosti in umetnosti Razred za naravoslovne vede, 17*, 249–291.

Bucklin, A., Steinke, D., & Blanco-Bercial, L. (2011). DNA Barcoding of Marine Metazoa. *Annual Review of Marine Science, 3*, 471–508.

Bulman, K. (1990). Shell variability in *Carychium tridentatum* (Risso, 1826) and its importance for infraspecific taxonomy (Gastropoda, Pulmonata: Ellobiidae). *Malakologische Abhandlungen Staatliches Museum für Tierkunde Dresden, 15*, 37–50.

Burch, J. B., & Van Devender, A. S. (1980). Identification of eastern North American land snails. The Prosobranchia, Opisthobranchia and Pulmonata (Actophila). *Walkerana, 1*, 60–80.

Cossignani, T., & Cossignani, V. (1995). *Atlante delle conchiglie terrestri e dulciacquicole italiane, first ed. L´Informatore Piceno, Mostra Mondiale Malacologia*. Ancona: Cupra Marittima (AP).

Culver, C. D., & Pipan, T. (2009). *The Biology of Caves and Other Subterranean Habitats*. Oxford University Press.

Dayrat, B. (2011). A warning for ecologists and conservation biologists using species checklists: How the European marine fauna 'lost' all of its 16 Discodoris species (Mollusca: Gastropoda). *Organisms Diversity and Evolution, 11*, 75–82.

de Frias Martins, A. M. (1996). Relationships within the Ellobiidae. In J. Taylor (Ed.), *Origin and Evolutionary Radiation of the Mollusca* (pp. 285–294). London: Oxford University Press, The Malacological Society of London.

de Frias Martins, A. M. (2007). Morphological and anatomical diversity within the Ellobiidae (Gastropoda, Pulmonata, Archaeopulmonata). *Vita Malacologica, 4*, 1–28.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology, 3*, 294–299.

Freyer, C. (1855). Über neu entdeckte Conchylien aus den Geschlechtern *Carychium* und *Pterocera*. *Sitzungsberichte der Akademie der Wissenschaften in Wien, Mathematisch-Naturwissenschaftliche Klasse*, 15, 18–23.

Giusti, F., & Manganelli, G. (1992). The problem of the species in malacology after clear evidence of the limits of morphological systematic, in: Gittenberger, E., Goud, J. (Eds.), *Proceedings of the 9th International Malacological Congress*. Edinburgh, pp. 153–172.

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. N. (2006). DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Sciences of the United States of America, 103*, 968–971.

Hajibabaei, M., Singer, G. A. C., Hebert, P. D. N., & Hickey, D. A. (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics, 23*, 167–172.

Harry, H. W. (1998). *Carychium exiguum* (Say) of Lower Michigan; morphology, ecology, variation and life history (Gastropoda, Pulmonata). *Walkerana, 9*, 1–104.

Haszprunar, G. (2011). Species delimitations – not 'only descriptive'. *Organisms Diversity and Evolution, 11*, 249–252.

Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 14812–14817.

Johnson, S. B., Waren, A., & Vrijenhoek, R. (2008). DNA Barcoding of *Lepetodrilus* limpets reveals cryptic species. *The Journal of Shellfish Research, 27*, 43–51.

Jordaens, K., Pinceel, J., Van Houtte, N., Breugelmans, K., & Backeljau, T. (2010). *Arion transsylvanus* (Mollusca, Pulmonata, Arionidae): rediscovery of a cryptic species. *Zoologica Scripta, 39*, 343–362.

Kerney, M. P., Cameron, R. A. D., & Jungbluth, J. H. (1979). *Die Landschnecken Nord- und Mitteleuropas*. Hamburg und Berlin: Paul Parey Verlag.

Lozek, V. (1957). Die Tschechoslowakischen Arten der Gattung *Carychium* (Mollusca, Basommatophora). *Acta Societatis Zoologicae Bohemoslovenicae, 21*, 225–232.

Maassen, W. J. M. (1987). Carychium mariae Paulucci in Jugoslawien. *Basteria, 51*, 2.

Martens, J., Sun, Y.-H., Wei, L., & Päckert, M. (2008). Intraspecific differentiation of Sino-Himalayan bush-dwelling *Phylloscopus* leaf warblers, with description of two new taxa (*P. fuscatus*, *P. fuligiventer*, *P. affinis*, *P. armandii*, *P. subaffinis*). *Vertebrate Zoology, 58*, 233–266.

Morton, J. E. (1955a). The evolution of the Ellobiidae with a discussion on the origin of the Pulmonata. *Proceedings of the Zoological Society of London, 125*, 127–168.

Morton, J. E. (1955b). Notes on the ecology and annual cycle of *Carychium tridentatum* at Box Hill. *Proceedings of the Malacological Society London, 31*, 31.

Müller, O. F. (1774). *Vermivm terrestrium et fluviatilium, seu animalium infusoriorum, helminthicorum, et testaceorum, non marinorum, succincta historia*. Volumen alterum. Havniæ & Lipsiæ. (Heineck & Faber).

Nekola, J. C., & Barthel, M. (2002). Morphometric analysis of the genus *Carychium* in the Great Lakes region. *Journal of Conchology, 37*, 515–531.

Pauls, S. U., Blahnik, J. R., Zhou, X., Wardwell, C. T., & Holzenthal, R. W. (2010). DNA barcode data confirm new species and reveal cryptic diversity in Chilean Smicridea (Smicridea) (Trichoptera: Hydropsychidae). *Journal of the North American Benthological Society, 29*, 1058–1074.

Paulucci, M. (1878). *Matériaux pour servir à l'étude de la faune malacologique terrestre et fluviatile de l'Italie et de ses îles*. Paris. (Savy).

Pfenninger, M., Cordellier, M., & Streit, B. (2006). Comparing the efficacy of morphologic and DNA-based taxonomy in the freshwater gastropod genus *Radix* (Basommatophora, Pulmonata). *BMC Evolutionary Biology, 6*, 1–14.

Pfenninger, M., Steinke, D., Nowak, C., Kley, C., & Streit, B. (2007). Utility of DNA-taxonomy and barcoding for the inference of larval community structure in morphologically cryptic *Chironomus* (Diptera) species. *Molecular Ecology, 16*, 1957–1968.

Pilsbry, H. A. (1939–1948). *Land Mollusca of North America (North of Mexico)*. Academy of Natural Sciences of Philadelphia, Monographs Number 3.

Pinceel, J., Jordaens, K., Van Houtte, N., De Winter, A. J., & Backeljau, T. (2004). Molecular and morphological data reveal cryptic taxonomic diversity in the terrestrial slug complex *Arion subfuscus/fuscus* (Mollusca, Pulmonata, Arionidae) in continental northwest Europe. *Biological Journal of the Linnean Society, 83*, 23–38.

Pintér, L. (1967). A revision of the genus *Carychium* O. F. Müller, 1774, in Hungary (Mollusca, Basommatophora). *Annales historico-naturales Musei nationalis hungarici, 59*, 399–407.

Radulovici, A. E., Sainte-Marie, B., & Dufresne, F. (2009). DNA barcoding of marine crustaceans from the Estuary and Gulf of St Lawrence: a regional-scale approach. *Molecular Ecology Resources, 9*, 181–187.

Ratnasingham, S., Hebert, P. D. N., 2007. BOLD: the barcode of life data system (http://www.barcodinglife.org). *Molecular Ecology Notes*, 7, 355–364.

Risso, A. (1826). *Histoire naturelle des principales productions de l'Europe méridionale et particulièrement de celles des environs de Nice et des Alpes Maritimes*. Paris: Tome quatrième. Levrault.

Schütt, H. (2010). *Turkish land snails*. Solingen: Verlag Natur & Wissenschaft.

Simon, A. T. C., Weigand, A. M., & Klussmann-Kolb, A. (2010). Comparative phylogeography of two (micro-) sympatric *Carychium* species in Europe (Gastropoda, Pulmonata, Ellobioidea). *Tropical Natural History*, Suppl. 3, 291.

Slapnik, R. (1991). Localities of Carychium tridentatum (Risso 1826) and C. minimum O. F. Müller 1774 in Slovenia and the distribution of C. mariae Paulucci, 1878 (Gastropoda: Carychiidae) in Yugoslavia. *Biološki vestnik, 39*, 45–62.

Smith, M., Rodriguez, J., Whitfield, J., Deans, A. R., Janzen, D. H., Hallwachs, W., et al. (2008). Extreme diversity of tropical parasitoid wasps exposed by iterative integration of natural history, DNA barcoding, morphology, and collections. *Proceedings of the National Academy of Sciences of the United States of America, 105*, 12359–12364.

Steinke, D., Zemlak, T. S., & Hebert, P. D. N. (2009). Barcoding Nemo: DNA-based identifications for the ornamental fish trade. *PLoS ONE, 4*, e6300.

Strauch, F. (1977). Die Entwicklung der europäischen Vertreter der Gattung *Carychium* O. F. Müller seit dem Miozän. *Archiv für Molluskenkunde, 107*, 149–193.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*. doi:10.1093/molbev/msr121.

Watson, H., & Verdcourt, B. J. (1953). The two British species of *Carychium*. *Journal of Conchology, 23*, 306–324.

Weigand, A. M., Jochum, A., Pfenninger, M., Steinke, D., & Klussmann-Kolb, A. (2011). A new approach to an old conundrum—DNA barcoding sheds new light on phenotypic plasticity and morphological stasis in microsnails (Gastropoda, Pulmonata, Carychiidae). *Molecular Ecology Resources, 11*, 255–265.

Winslow, M. L. (1922). Notes on the internal lamellae of *Carychium*. *Occasional papers of the Museum of Zoology University of Michigan, 128*, 1–17.

Zimmermann, F. (1925). Untersuchungen über die Variabilität einiger Arten des Genus *Carychium* Müller. *Molecular and General Genetics, 37*, 291–342.