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Description of the reproductive system of *Helicodonta angigyra* (Rossmässler, 1834) and differential diagnosis with *Helicodonta obvoluta* (O.F. Müller, 1774) (Gastropoda: Helicodontidae)

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Abstract. This study investigates whether the conchological differences observed between *Helicodonta obvoluta* (O.F. Müller, 1774) and *Helicodonta angigyra* (Rossmässler, 1834) are also reflected in their reproductive anatomy and genetic divergence, thereby corroborating their status as distinct species. To this end, we present the first detailed anatomical description of both species, complemented with a DNA barcoding approach. Our results demonstrate consistent and stable differences in the arrangement and morphology of pilasters and spicules in the penis of the two species. DNA barcoding based on the mitochondrial COI gene further supports the taxonomic distinctness of *H. angigyra*, which forms a well-supported monophyletic lineage sister to *H. obvoluta*. Within *H. obvoluta*, we identified two haplogroups separated by approximately 7% genetic divergence, which exceeds the commonly recognised species-level threshold. This suggests either a complex evolutionary history of the mitochondrial genome across time and space, or a more intricate taxonomy within this nominal species. At the family level, our molecular results challenge the current infrafamilial classification of Helicodontidae, as well as some of the traditional anatomical characters used to define it. They also call into question the monophyly of the family. While more comprehensive multilocus datasets are still required, this study provides new diagnostic features that allow *H. angigyra* to be unambiguously distinguished from *H. obvoluta*.

Key words. Auxiliary copulatory organs, reproductive system, comparative anatomy, COI barcoding, species delimitation

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Introduction

Within the Helicoidea, the Helicodontidae Kobelt, 1904 constitute a relatively species-poor family of land snails, comprising six Recent and three fossil genera (*Atenia* E. Gittenberger, 1968; *Darderia* Altaba, 2007†; *Drepanostoma* Porro, 1836; *Falkneria* H. Nordsieck, 1989; *Helicodonta* A. Férussac, 1821; *Lindholmiola* P. Hesse, 1931; *Protodrepanostoma* Germain, 1929†; *Prosoosia* H. Nordsieck, 1986†, *Soosia* P. Hesse, 1918) and 17 extant species (Bank *et al.* 2001; Schileyko 2006; Subai & Neubert 2014; MolluscaBase Eds 2021). The group's distribution spans from eastern Spain to western Turkey (Schütt 2010; Talaván Serna & Talaván Gómez 2012; Welter-Schultes 2012), with a centre of diversity in the region between the southern slopes of the Central Alps and the Balkans but including the Aegean Islands and

Crete. While most species are endemic to small geographical areas, *Helicodonta obvoluta* (O.F. Müller, 1774) is the only species with a broader distribution across much of Western Europe. Its range extends from northern Spain to southern England, covering almost all of mainland France. Northwards, it reaches as far as northern Germany and Poland, while southwards it spreads into the Italian Apennine Peninsula. Its easternmost limit includes Slovakia, Hungary and Serbia (Maltz 2007; IUCN 2011a).

The shells of helicodontid species are typically strongly depressed to nearly flat, with an umbilicus open to varying degrees. The whorls are narrow, spiral striations are absent, and the periostracum often bears hairs. The margins of the aperture are oblique, not aligned in a single plane, and reflexed, occasionally featuring one or two tooth-like bulges on the peristome (Schileyko 2006).

Beyond these general shell characteristics, no recent study has tested the monophyly of the group or its subfamilial entities using molecular tools. Although several phylogenetic studies have included members of the family (Manganelli *et al.* 2005; Gómez-Moliner *et al.* 2012; Calcutt *et al.* 2020; Zhang *et al.* 2024), sampling of the taxon remains incomplete, as some of the currently recognised genera are unrepresented among available gene sequences. In addition, the results may vary depending on the genetic marker used, as reported by Gómez-Moliner *et al.* (2012), who found that monophyly was supported by mitochondrial but not by nuclear data.

Consequently, there is currently no widely accepted diagnosis of the family, particularly one based on morpho-anatomical characters that could support its taxonomic delimitation. According to Schileyko (2006) and Subai & Neubert (2014), the helicodontid genera share a reproductive system with a reduced (in *Falkneria*) or absent flagellum, an epiphallus of variable length, and an elongated, often bulbous penis. Although absent in *Soosia*, the mucous gland is present in other genera, in which it consists of a single branch in *Atenia* and *Lindholmiola* (Martìnez-Ortì 2005) or of two branches in *Drepanostoma*, *Falkneria* and *Helicodonta*. In the absence of a well-resolved phylogenetic framework, the character states and their value as diagnostic traits within the group remain uncertain (Gómez-Moliner *et al.* 2012). In addition, comprehensive descriptions of the reproductive system are lacking for most species, except for those of the genus *Lindholmiola* (Subai & Neubert 2014), even though the internal structure of the penis can provide particularly robust diagnostic characters at the species level (e.g. Giusti & Manganelli 2002; Pizá & Cazzaniga 2010; Giusti *et al.* 2011; Pholyotha *et al.* 2021).

This is the case for the genus *Helicodonta*, which includes four species: *H. obvoluta*, *H. angigyra* (Rossmässler, 1834), *H. langhofferi* A.J. Wagner, 1912 and *H. wilhelminae* Maassen, 1991 (Wagner 1912; Maassen 1991; Bank & Neubert 2017).

Helicodonta langhofferi was described from empty shells found in marine debris near Dubrovnik and the island of Lokrum (Croatia). No additional shells or live individuals have been recorded since (IUCN 2020). *Helicodonta wilhelminae* was originally described by Maassen (1991) as a subspecies of *Helicodonta gyria* (Roth, 1839). However, Subai & Neubert (2014) argued that, despite the shell similarity between the nominal species *Helix gyria* and *H. obvoluta*, differences in the structure of the genital apparatus support assigning the former to the genus *Lindholmiola*. This view is strongly supported by Mylonas *et al.* (2024), who re-examined the morphology of the shell and reproductive system of *H. wilhelminae* and subsequently transferred the species to *Lindholmiola*. This Cretan endemic species is recognised as Endangered by the IUCN (2011b).

The situation for *H. angigyra* is markedly different. Endemic to the southern slopes of the Central Alps with numerous occurrences, this species is present only in the Canton of Ticino, southern Switzerland (Turner *et al.* 1988; Boschi 2011) and in Lombardy and Trentino-Alto Adige, in northern Italy; however, it has been introduced in the Italian Piedmont (Bodon *et al.* 2021). Furthermore, the species was reported in the early 20th century from the western Alps, particularly in Haute-Savoie (France) and north-western Italy near the French border (Germain 1929, 1931). Shell material housed in the MNHN collections corroborates these historical records. However, no reliable evidence confirms its current presence in France, and the few shell-based citations from the last two decades remain doubtful and unverified (see the French species' distribution data on INPN, https://inpn.mnhn.fr).

This species is distinguishable from *H. obvoluta* by its shell morphology (Girod 1968; Manganelli *et al.* 1995; Ghezzi 2012), and its generic assignment has been supported by anatomical characters described in earlier literature (Saint Simon 1856; Vogel 1933). However, the historical descriptions of the reproductive system are now outdated and fail to provide a reliable basis for distinguishing *H. angigyra* from *H. obvoluta*. To establish robust diagnostic criteria for distinguishing these two species, a detailed and standardised description of the internal anatomy of the reproductive system is required. Such data could provide the basis for a critical reassessment of the genus *Helicodonta* as a whole.

In addition, although the distinctness of *H. angigyra* and *H. obvoluta* has long been accepted in the literature, the actual robustness of this separation has never been reassessed within an integrative framework. In particular, no study has simultaneously tested the congruence of conchological, anatomical and mitochondrial signals, leaving open the question of whether the traditionally recognised species boundaries are taxonomically justified.

For these reasons, this article aims to investigate the interspecific boundaries between *H. obvoluta* and *H. angigyra* through a dual approach combining (i) DNA barcoding and (ii) anatomical analysis, with particular emphasis on the internal structure of the penis. This integrative framework is intended to support a critical re-evaluation of both anatomical and conchological traits underlying species delimitation.

Materials and Methods

All specimens included in our anatomical and/or molecular studies were collected opportunistically without a formal sampling plan (for further details, refer to the material examined provided below). Identification of the species follows the diagnostic characters proposed by Kerney & Cameron (1999). Living animals were first drowned in water for about 12–24 h and then preserved in a 75% ethanol solution. For anatomical studies, the shells were broken to extract the animal's soft body parts. The tissues were immediately and thoroughly washed with water to eliminate any residual shell fragments. All dissections were performed under a stereomicroscope in a solution of 60% water, 30% ethanol, and 10% glycerine using thin, pointed forceps (0.06 mm). The reproductive systems were dissected out and photographed. Line drawings were realised by hand from these photographs. The nomenclature used here to describe the different parts of the reproductive system follows Gómez (2001). In this study, anatomical orientation follows a standard convention whereby the terms *proximal* and *distal* are defined with reference to the location of the gonad: *proximal* indicating a position closer to the gonad, and *distal* referring to a position further away along the reproductive tract.

DNA extraction was performed on four individuals: one *H. obvoluta* (sample B4) and three *H. angigyra* (samples F3, G3 and H3) from Switzerland (45.8955°N, 009.0394°E; elevation 635 m; 17.IV.2024; leg. Damien Combrisson). Prior to extraction, each specimen was rinsed with 95% ethanol to avoid contamination, and a 4 mm^2 fragment of the tail was collected using a sterile blade. Total genomic DNA was isolated according to the DNeasy® Blood & Tissue Kit protocol (Qiagen, France), with final elution in 50 µL of buffer.

The standard DNA barcoding region (Hebert *et al.* 2003) was amplified by PCR. This region consists of a 655-bp fragment located at the 5′ end of the mitochondrial COI gene. PCR reactions were performed in a final volume of 25 µL containing 5 µL of 1× colourless GoTaq® Buffer Master Mix (Promega, France), 0.5 µL of 0.2 µM of each primer, 1.25 µL of 0.5 mM dNTPs, 0.125 µL of 0.025 U GoTaq® G2 DNA polymerase (Promega, France), 2 µL of about 20 ng/µL extracted DNA, and 15.625 µL of ultrapure water. Forward and reverse primers were LCO1490-GGTCAACAAATCATAAAGAT TGG and HCO2198-TAAACTTCAGGGTGACCAAAAA ATCA (Folmer *et al.* 1994). An initial denaturation step at 94 °C for 3 min was followed by 35 cycles at 94 °C for 30 s, an annealing step at 50 °C for 45 s and an extension step at 72 °C for 1 min, and a final extension step at 72 °C for 10 min (T100 thermal Cycler Bio-Rad, France).

The PCR products were sent to Eurofins Genomics (Cologne, Germany) for Sanger sequencing. Sequences were manually corrected using Chromas v. 2.6.6 (https://technelysium.com.au), aligned using the Muscle program in SeaView (Gouy *et al.* 2010), and submitted to GenBank (PX418023, PX418024, PX418025, PX418026).

Thirty-four sequences retrieved from the GenBank and BOLD databases were added to the alignment: 19 sequences of *H. obvoluta*, each with its country of origin, and 15 sequences of other Helicodontidae species (2 from the genus *Atenia*, 1 from *Drepanostoma*, 11 from *Lindholmiola*, and 1 from *Soosia*). Maximum likelihood (ML) and Bayesian inference (BI) were used for phylogeny reconstruction. Maximum-likelihood analyses were run with PhyML (Guidon *et al.* 2005) with 1000 bootstrap replicates and the model of nucleotide substitution selected by Smart Model Selection with the Akaike Information Criterion (SMS, Lefort *et al.* 2017). Bayesian inference was run using MrBayes v. 3.2.7a (Ronquist *et al.* 2012). Two independent Markov Chain Monte Carlo (MCMC) analyses were performed, each comprising four chains and running for 10 million generations, with sampling occurring every 100 generation. The first 25% of the sampled generations were discarded as burn-in to construct a majority-rule consensus tree and estimate posterior probabilities. GenBank sequences from *Arianta arbustorum* (Linnaeus, 1758) (Helicidae), *Hygromia limbata* (Draparnaud, 1805) (Hygromiidae), and *Caracollina lenticula* (Michaud, 1831) (Trissexodontidae) were chosen as the presumed outgroups, based on the phylogenetic tree obtained by Gómez-Moliner *et al.* (2012) using mitochondrial sequences. Interspecific genetic distances were calculated according to the Kimura 2-parameter (K2P) model using MEGA X (Kumar *et al.* 2018).

A haplotype list was generated in DnaSP v. 6 (Rozas *et al.* 2017) using sequences from the genus *Helicodonta* (*N* = 23 sequences) as an input file. The representative haplotype sequences were exported in Nexus format with trait blocks added to represent geographical regions (i.e. countries). A Median-joining haplotype network was produced using the default parameters in PopART v. 1.7 (available at http://popart.otago.ac.nz; accessed on 01/08/2025).

Concerning species delimitations, we followed the Hennigian inter-nodal species concept formalized by Samadi & Barberousse (2006). In this framework, species are considered sets of organisms that have genealogic relationships and form isolated, irreversible evolutionary lineages. Consequently, species-level taxa were delineated based on (i) the cohesiveness of haplotype networks and (ii) the monophyly criterion derived from the Phylogenetic Species Concept, in which species constitute the smallest diagnosable monophyletic groups.

Abbreviations

Institutions and collection

GNC	Gianbattista Nardi collection, Brescia (Italy)
MHNEC	Musée d'Histoire naturelle et d'Ethnographie de Colmar (France)
MHNNice	Musée d'Histoire naturelle de Nice (France)
MNHN	Museum national d'Histoire naturelle, Paris (France)
SMF	Senckenberg Research Institute Frankfurt am Main (Germany)

Anatomical description

ag	Albumin gland
at	Atrium
bc	Bursa copulatrix
cp	Central pilaster
dbc	Duct of the bursa copulatrix
dp	Distal part of the penis
epp	Proximal part of the epiphallus (between Retractor penis muscle insertion and vas deferens)
ep	Epiphallus
epd	Distal part of the epiphallus (between retractor penis muscle insertion and penis)
g	Gonad
gu	Gutter-like structure formed by V-shaped folds
hd	Hermaphodite duct
lp	Longitudinal pilaster
mg1	Longest branch of the mucous gland
mg2	Shorter branch of the mucous gland
ov	Oviduct
pa	Penial papillae
pc	Penial caecum
plp	Proximal part of the longitudinal pilaster
pp	Proximal part of the penis
ppo	Penial pouch
rpm	Retractor penis muscle
rs	Receptaculum seminis
sh	Sheath (very thin, transparent covering of epiphallus and penis)
sp	Secondary pilasters
spi	Conical spines (spicules)
so	Spermoviduct
vp	Valve pilaster (V-shaped folds in the penis)
vd	Vas deferens
vg	Vagina
wp	Penis wall

Systematics and Results

Class Gastropoda Cuvier, 1795

Order Stylommatophora Schmidt, 1855

Family Helicodontidae Kobelt, 1904

Genus *Helicodonta* A. Férussac, 1821

Helicodonta angigyra (Rossmässler, 1834)

Basionym. *Helix angigyra* Rossmässler, 1834.

Original description. "Dagegen steht unserer Art [*Helix obvoluta* Müller, 1774] die neue *H. angigyra* Z.[iegler] aus Oberitalien sehr nahe, unterscheidet sich jedoch durch ihre mindere größte und einen Umgang mehr. Man findet von unserer Art bei weitem die meisten Exemplare mit unbehaarter Oberfläche, da sich die Epidermis mit den haaren, selbst so lange das Thier noch lebt, sehr leicht abreibt."

English translation: In contrast, our species [*Helix obvoluta* Müller, 1774] is very similar to the new *H. angigyra* Z.[iegler] from northern Italy but differs in its smaller size and one additional whorl. By far the most specimens of our species are found with a hairless surface, as the epidermis with the hairs rubs off very easily, even while the animal is still alive.

Type locality. "… *aus Oberitalien* …"; English translation: from northern Italy.

Type material. Four syntypes preserved in the Senckenberg Research Institute Frankfurt am Main: one specimen labelled as "lectotype" under catalogue number SMF 285259 (Fig. 1A), and three specimens labelled as "paratypes" under catalogue number SMF 285260. The type

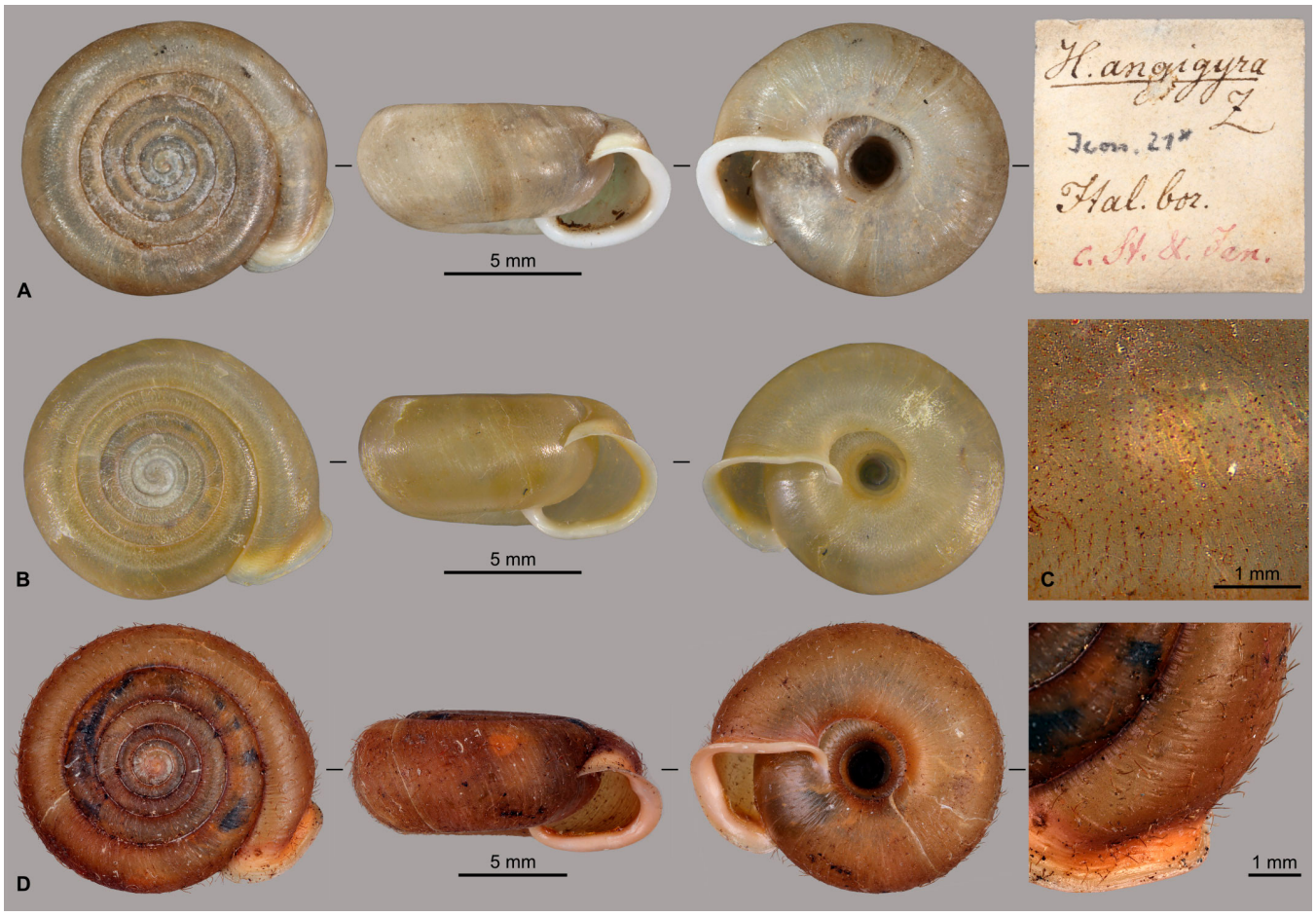

Figure 1. Shells and periostracal hair arrangement of *Helicodonta angigyra* (Rossmässler, 1834) and *Helicodonta obvoluta* (O.F. Müller, 1774). **A,** syntype of *H. angigyra* (SMF 285259), possibly the specimen illustrated in Rossmässler's Iconography (1835: 70, pl. 1, fig. 21*), and label (not to scale) (photograph provided by Sigrid Hof, Senckenberg Research Institute & Natural History Museum). **B,** *H. angigyra* from Lugano (Switzerland) (MOLL-0000445; 27/02/2024; collector Paul Godet; photograph provided by Estée Bochud, Natural History Museum Bern). **C,** *H. angigyra* from Val di Scalve (Italy) (MHNEC-Moll20250602-N2); detail of the periostracal hairs. **D,** *H. obvoluta* from Foncine-le-Bas, Jura (France) (MHNNice2019.0.218; 29/09/2018; Olivier Gerriet leg).

series originates from Rossmässler's collection, ex Stenz & Jan, with the locality given as "Oberitalien [Ital. bor.]". To our knowledge, no designation of a lectotype and/or paratypes has ever been published. The putative lectotype is probably the specimen illustrated by Rossmässler in his *Iconographie* (1835: 70, pl. 1, fig. 21).

Material examined. Switzerland • 3 specimens (shell and animal, soft parts housed at the MHNEC under the collection number MHNEC-Moll20250601-F3, -G3 and -H3) and 4 specimens (shell housed in the private collection of Damien Combrisson); Valle di Muggio, municipality of Cabbio, Canton of Ticino; 45.8955°N, 009.0394°E; elevation 635 m; 17.IV.2024; Damien Combrisson.

Italy • 4 specimens (shell and animal); Val di Scalve, municipality of Angolo Terme, Brescia Province, Lombardy; 45.9089°N, 10.1295°E; elevation 500 m; 24.IX.2005; Gianbattista Nardi & Antonio Braccia (2 specimens housed at the GNC under the collection number 3799; soft parts of 2 specimens housed at the MHNEC under the collection number MHNEC-Moll20250602-N1 and -N2; Fig. 1B).

France • 1 empty shell housed at the MNHN under the collection number IM-2010-13183; Faucigny area, department of Haute-Savoie; Hippolyte Blanc.

Global distribution and habitat

The species' range extends across the southern slopes of the Central Alps, encompassing parts of Switzerland and Italy. In Switzerland, it is restricted to the southern half of the canton of Ticino, from the Locarno region to Mendrisio, and also occurs in the Bernina region of the canton of

Graubünden (Rüetschi *et al.* 2012). In Italy, it is widespread throughout the Lombardian Mountains, ranging from the Lake Maggiore region in the west to the Lake Garda region in the east (Girod 1968, 1969; Ghezzi 2012; Welter-Schultes 2012; Nardi & Braccia 2023). The species' southernmost distribution reaches the plains of the Cremona region (Ghezzi 2012). In France, the species has only a marginal record dating from the early 20th century in the Haute-Savoie region (Faucigny area), supported by a specimen preserved in a collection (MNHN-IM-2010-13183) (Germain 1929, 1931). Germain (1929, 1931) also reported *H. angigyra* in north-western Italy near the French border, specifically in the Mont Cenis region. However, no reliable records subsequent to Germain's publications have ever confirmed the presence of the species in France. Previous records from the southern part of Lake Annecy, reported by the data provider himself (Alain Thomas pers. comm.) over the past two decades, were later shown to be erroneous. These data had not yet been corrected on the INPN website (https://inpn.mnhn.fr) at the time of writing. According to IUCN (2011c), the species occurs at elevations up to 1,700 m above sea level. Its habitat includes dense soil litter and rock fields within humid mountain forests at lower elevations, as well as cultivated lands and more anthropogenically influenced areas on the plains.

Molecular species delimitation

The final molecular dataset comprised 624 bp of aligned COI sequences from 41 individuals (four from this study and 37 from GenBank and BOLD), with 253 variable sites, and included 12 of the 17 species described in the family Helicodontidae. Phylogenetic reconstructions show that *H. angigyra* sequences are clearly distinct from those of *H. obvoluta*, and together these species form a monophyletic group (Fig. 2).

The three *H. angigyra* specimens from this study are represented by a single haplotype (Hap_8), whereas the *H. obvoluta* specimens are represented by seven haplotypes belonging to two haplogroups: Ho1 (Haplotypes Hap_3 and Hap_4) and Ho2 (Haplotypes Hap_1, Hap_2, Hap_5, Hap_6 and Hap_7) (Appendix Fig. A1). Haplogroup Ho1 comprises haplotypes from France, Germany, Slovakia, Spain and Switzerland, including the sequence from this study. Haplogroup Ho2 comprises haplotypes from Austria, Germany, the Netherlands, Slovakia, Switzerland, and the United Kingdom.

Considering all sequences except for outgroups, the mean Kimura 2-parameter (K2P) distance between species is 0.19. The mean K2P distances are 0.19 and 0.20 between *H. angigyra* and *H. obvoluta* Ho1, and *H. angigyra* and *H. obvoluta* Ho2, respectively, while the mean distance between the two *H. obvoluta* haplogroups Ho1 and Ho2 is 0.07.

Shell description

Nine adult individuals were examined, confirming in all key features the descriptions provided by Germain (1929, 1931), Kerney & Cameron (1999), and Welter-Schultes (2012). The shell of *Helicodonta angigyra* is planispiral, composed of 6–7 moderately convex, narrow whorls, and does not exceed 11 mm in maximum diameter or 5 mm in height (Fig. 1A, B). Morphometric data from 41 adult specimens with fully developed, thickened apertural lips (Ghezzi 2012) indicate an average diameter of 9.8 mm (range: 9.0–10.3 mm) and an average height of 4.6 mm (range: 4.3–4.9 mm). In comparison, shells of *H. obvoluta* can reach 9.9–14.2 mm in diameter and 4.4–6.9 mm in height (n = 668 measured specimens; Maltz 2007) (Fig. 1C). The last whorl often declines abruptly towards the aperture in lateral profile. The umbilicus is open and may be markedly eccentric relative to the shell axis on the final whorl. The periostracum bears evenly spaced, short hairs approximately 100 μm in length, arranged in a regular, staggered pattern (Fig. 1B); these are particularly conspicuous in juvenile to subadult specimens and may be worn or lost in older individuals.

Reproductive system

Five adult individuals were examined. Both the epiphallus (**ep**) and the penis are covered by a thin, transparent sheath (**sh**). The retractor penis muscle (**rpm**) originates from the columellar muscle and attaches to the epiphallus (Fig. 3A, B, C). The epiphallus gradually narrows, and the boundary between it and the vas deferens (**vd**) is not clearly defined (Fig. 3C). The vagina (**vg**) is long, with 4–7 prominent longitudinal internal folds (not illustrated here). The mucous gland is forked, with the longest branch (**mg1**) shorter than both the duct (**dbc**) and the bursa copulatrix (**bc**) (Fig. 3A). The shorter branch (**mg2**) folds back at the base of the longest mucous gland, forming a short, finger-like structure. The duct of the bursa copulatrix inserts at the base of the mucous gland, its length nearly identical to that of the bursa copulatrix.

The penial complex (comprising the penis and epiphallus) is either shorter than or slightly longer than the combined length of the vagina and the duct of the bursa copulatrix (Fig. 3A).

Once the penial complex sheath has been removed, the penis appears divided into two parts (Fig. 3B, C). The prox-

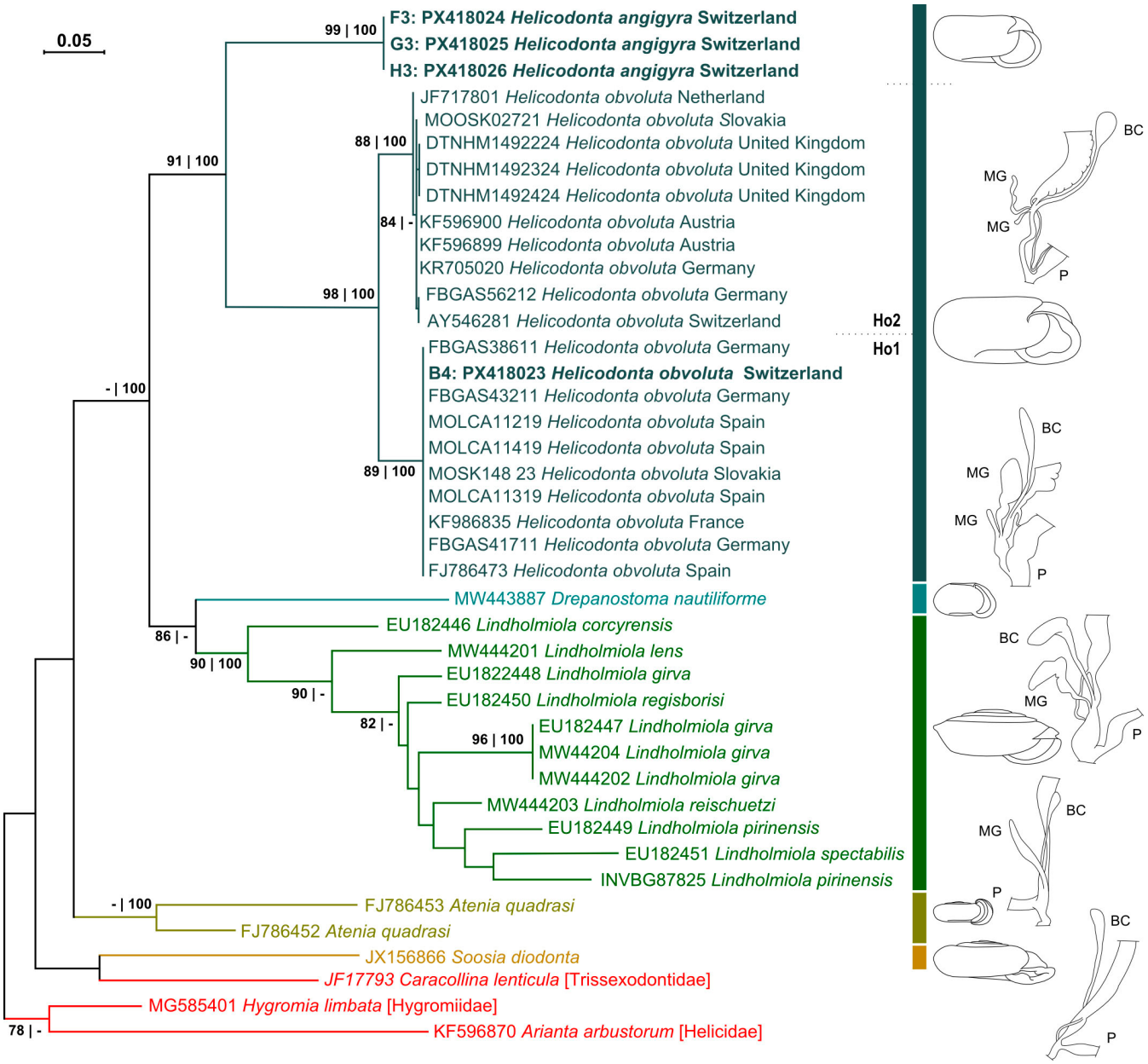

Figure 2. ML phylogenetic tree based on COI sequences and reconstructed with 1000 bootstrap replicates and the GTR evolutionary model. Sequences from this study are shown in bold. Numbers correspond to ML bootstrap values and BI posterior probabilities. Only nodes with bootstrap support or Bayesian posterior probabilities greater than 70% and 95%, respectively, were retained. The tree is rooted with *Arianta arbustorum* (Helicidae), *Hygromia limbata* (Hygromiidae) and *Caracollina lenticula* (Trissexodontidae). Each genus is represented by a distinct colour, with a shell profile and details of the auxiliary copulatory organs (*Helicodonta obvoluta* for *Helicodonta* and *Lindholmiola lens* for *Lindholmiola*). Auxiliary copulatory organs are drawn after Schileyko (2006). Outgroups are indicated in red.

imal part of the penis (**pp**) is irregularly shaped and bears bulges of varying prominence. These include two pouch-shaped dilations, referred to here as the penial caecum (**pc**) and the penial pouch (**ppo**). The latter contains two penial papillae (**pa**). The distal part of the penis (**dp**) has a more consistent diameter and varies in length.

The inner surface of the penis exhibits a large, flat thickening (here called the central pilaster, **cp**), which occupies up to two-thirds of the lumen of the proximal penis (Fig. 4A, B). The proximal tip of the central pilaster can be externally detected as a bulge at the base of the penial caecum (Fig. 3B, C), opposite to the insertion of the epiphallus. There are additional, less developed thickened areas and folds termed secondary pilasters (**sp**), which are densely covered with

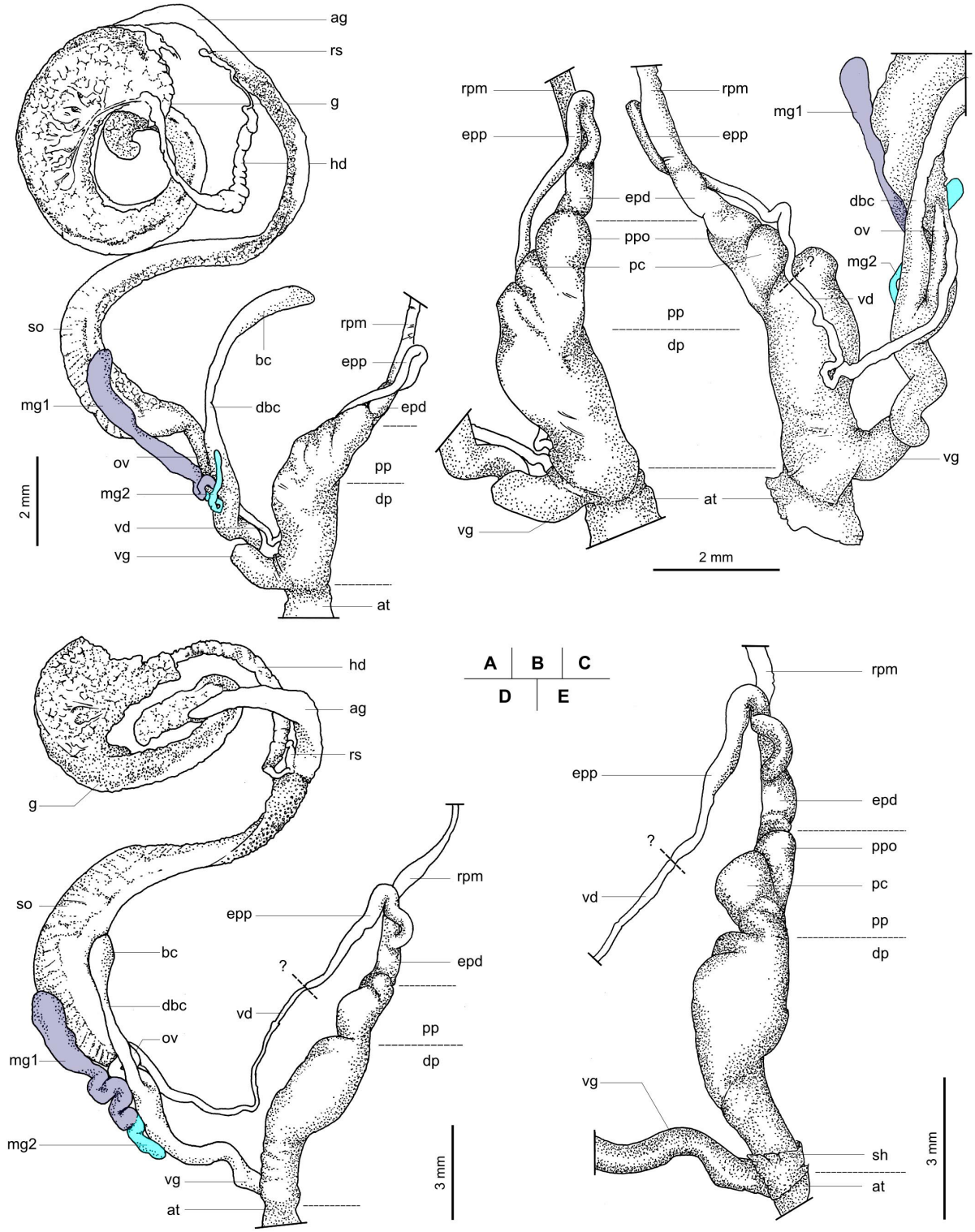

Figure 3. Reproductive systems of *Helicodonta angigyra* (Rossmässler, 1834) and *Helicodonta obvoluta* (O.F. Müller, 1774). **A**, specimen of *H. angigyra* from Val di Scalve, Italy, (MHNEC-Moll20250602-N2). **B**, dorsal view of the penial complex after removal of the sheath (MHNEC-Moll20250602-N2, same specimen as in Fig. 3A). **C**, ventral view of the penial complex after removal of the sheath (same specimen as in Fig. 3A). **D**, specimen of *H. obvoluta* from Hautes-Vosges, northeastern France, (MHNEC-Moll20250603-HV5). **E**, dorsal view of the penial complex after removal of the sheath (MHNEC-Moll20250603-HV5, same specimen as in Fig. 3D). The question mark and dashed line indicate the possible transition between the *vas deferens* (vd) and the proximal part of the epiphallus (epp).

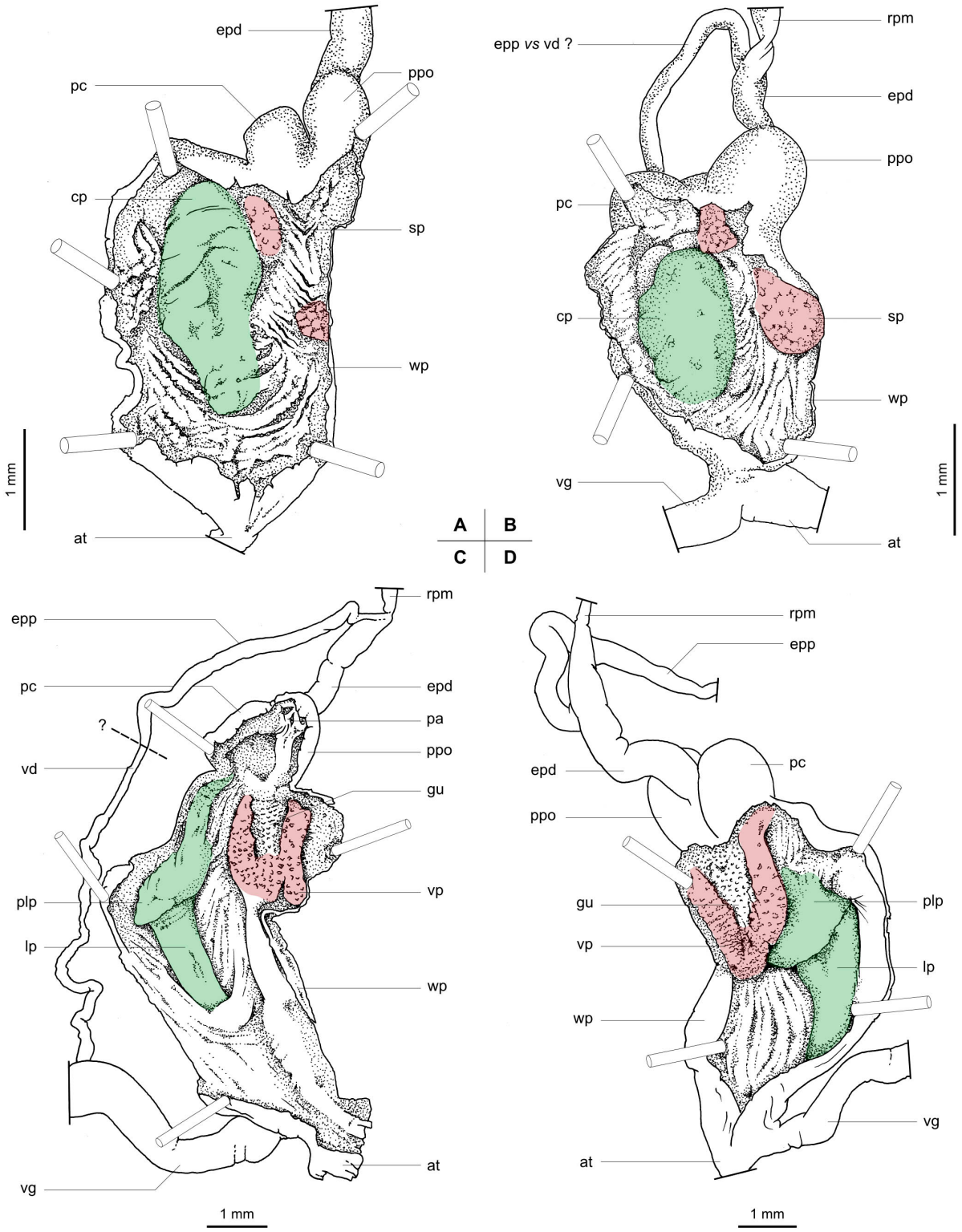

Figure 4. Internal structure of the penis in *Helicodonta angigyra* (Rossmässler, 1834) and *Helicodonta obvoluta* (O.F. Müller, 1774). **A**, *H. angigyra* from Val di Scalve, Italy (MHNEC-Moll20250602-N2, same specimen as in Fig. 3A). **B**, *H. angigyra* from Valle di Muggio, Switzerland (MHNEC-Moll20250601-G3). **C**, *H. obvoluta* from Hautes-Vosges, northeastern France (MHNEC-Moll20250603-HV4). **D**, *H. obvoluta* from Hautes-Vosges, northeastern France (MHNEC-Moll20250603-HV5, same specimen as in Fig. 3D). The question mark and dashed line indicate the possible transition between the vas deferens (vd) and the proximal part of the epiphallus (epp).

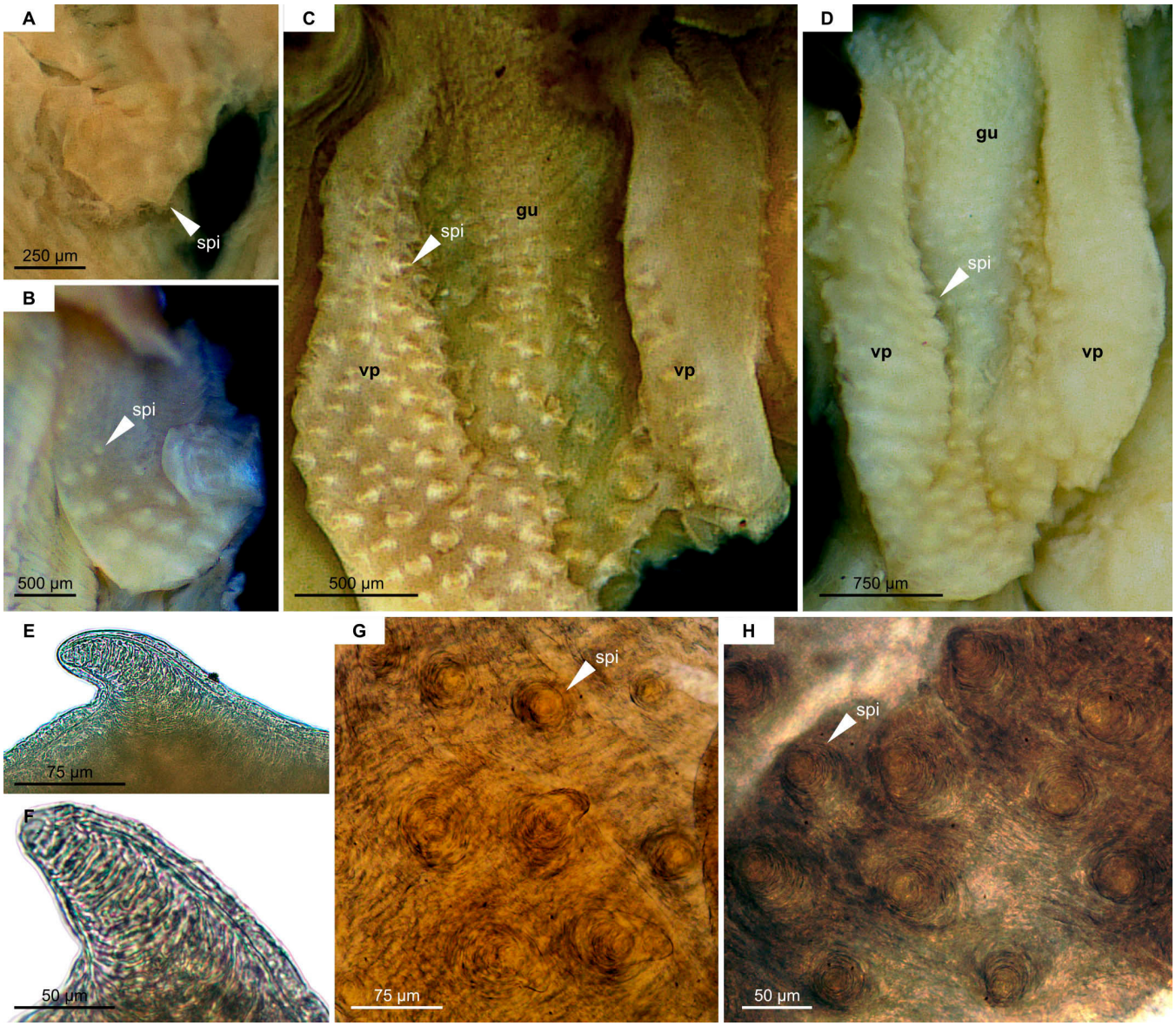

Figure 5. Structure of the pilasters and spicules in *Helicodonta angigyra* (Rossmässler, 1834) and *Helicodonta obvoluta* (O.F. Müller, 1774). **A**, secondary pilaster of *H. angigyra* from Val di Scalve, Italy (MHNEC-Moll20250602-N2, same specimen as in Figs 3A and 4A). **B**, secondary pilaster of *H. angigyra* from Valle di Muggio, Switzerland (MHNEC-Moll20250601-G3, same specimen as in Fig. 4B). **C**, valve pilaster of *H. obvoluta* from the Hautes-Vosges, northeastern France (MHNEC-Moll20250603-HV4, same specimen as in Fig. 4C). **D,** valve pilaster of *H. obvoluta* from the Hautes-Vosges, northeastern France (MHNEC-Moll20250603-HV5, same specimen as in Fig. 4D). **E**, **F**, detail of a spicule from the secondary pilaster of *H. angigyra* (MHNEC-Moll20250602-N2, same specimen as in Fig. 5A). **G**, detail of spicule implantation in the secondary pilaster of *H. angigyra* (MHNEC-Moll20250601-G3, specimen shown in Fig. 5B). **H**, detail of the spicule implantation in the valve pilaster of *H. obvoluta* (MHNEC-Moll20250603-HV5, same specimen as in Fig. 5D). White arrows indicate spicules (spi).

spicules (**spi**) (Fig. 5E, F, G). These two secondary pilasters are found on the penile wall opposite the central pilaster (Fig. 4A, B): one is located at the base of the penial caecum (Fig. 5A) and the other is situated next to the central pilaster (Fig. 5B). Together, these pilasters form a central canal whose surface has finer, spicule-free folds.

Reproductive system

Five adult individuals were examined, and their anatomical characteristics align with the descriptions provided by Nordsieck (1989), Schileyko (2006), and Maltz (2003a). Both the epiphallus and penis are covered by a very thin, transparent sheath (**sh**). The retractor penis muscle (**rpm**)

originates from the columellar muscle and attaches to the epiphallus (Fig. 3D, E). The transition between the epiphallus and the vas deferens (**vd**) is unclear. Schileyko (2006) and Maltz (2003a) suggested that the retractor penis muscle inserts at the top of the epiphallus, whereas Nordsieck (1989) proposed that it inserts in the middle. Consequently, Nordsieck (1989) distinguished between the short, wide, flared distal part of the epiphallus (the section between the retractor penis muscle insertion and the penis, **epd**) and the long proximal part of the epiphallus (the section between the retractor penis muscle insertion and the vas deferens, **epp**), which gradually narrows in diameter (Fig. 3D).

The vagina (**vg**) is long and thin, with smooth internal surfaces or vague longitudinal folds. The mucous gland is forked; its longest branch (**mg1**) is approximately as long as both the duct (**dbc**) and the bursa copulatrix (**bc**) (Fig. 3D). Although these two structures may be difficult to differentiate during dissection, their tissue compositions are distinct (Maltz 2003a). The shortest branch (**mg2**) folds back at the base of the longest mucous gland, forming a short, finger-like structure. This structure is referred to by several names, including "appendicula" (Nordsieck 1989), "short branch of the mucous gland" (Maltz 2003a; Schileyko 2006), "accessory sac" (Gómez-Moliner *et al.* 2012), or "tubular organ" (Subai & Neubert 2014).

The duct of the bursa copulatrix inserts at the base of the mucous gland. It is of moderate length and has no clear boundary with the bursa copulatrix itself.

The penial complex (penis and epiphallus) is always longer than the combined length of the vagina and the duct of the bursa copulatrix (Fig. 3D). Once the epiphallus and penial sheath have been removed, the penis appears to be divided into two parts (Fig. 3E). The proximal part (**pp**) has irregular bulges of varying prominence. The distal part (**dp**) maintains a consistent diameter and may feature a distinct longitudinal bulge along its entire length.

The inner surface of the penis exhibits several prominent longitudinal folds or pilasters (Fig. 4C, D). One of these, the longitudinal pilaster (**lp**), is wider and smoother, extending almost the entire length of the penis. The proximal part of the longitudinal pilaster (**plp**) may dilate to form a pad-like thickening, in an approximately transverse position. The longitudinal pilaster causes an external bulge, especially along the distal part of the penis.

The inner surface of the proximal part of the penis also features V-shaped folds (valve pilaster, **vp**), which form a depressed, gutter-like structure (**gu**) (Figs 4C, D, 5C, D, H). Both the folds and the gutter are covered with tiny conical spines (**spi**), as described by Schileyko (2006), who suggested that these structures (**vp**, **gu**, and **lp**) work together to create a conspicuous valve that isolates the distal part of the penis from the proximal part.

The epiphallus connects the proximal end of the penis to a pouch-like (penial pouch, **ppo**) structure located behind the valve (Fig. 4C, D). Two short, flattened penial papillae (**pa**) are located at the emergence of the epiphallus in the penial pouch (Fig. 4C). Separated by a strongly thickened wall on the lateral side of this pouch-like structure is located a dilation of the proximal part of the penis that resembles a penial caecum (**pc**) (Figs 3D, E, 4C, D). Both structures are clearly visible externally once the sheath covering the penis and epiphallus has been removed. While Nordsieck (1989) illustrated the penial caecum, it was not mentioned by Schileyko (2006).

Differential diagnosis: *H. obvoluta* vs *H. angigyra*

The following characters distinguish *H. angigyra* from *H. obvoluta* based on shell and internal anatomy.

Helicodonta angigyra differs from *H. obvoluta* by its smaller overall shell size, the more tightly coiled whorls resulting in an additional whorl in adult specimens, and the presence of short (100 μm vs 1 mm), densely set periostracal hairs arranged in a highly regular, staggered (quincuncial) pattern.

The penial complex (penis and epiphallus) is proportionally smaller in *H. angigyra*, its total length only slightly exceeding that of the duct of the bursa copulatrix. The two species differ markedly in the internal penial morphology. In *H. obvoluta*, the penial lumen contains (i) a thick, elongated longitudinal pilaster, smooth and devoid of spicules, which may proximally expand into a cushion-like structure, and (ii) a V-shaped arrangement of thickened folds forming a depressed, gutter-like region; both the V-shaped folds and the gutter are covered with spicules. In contrast, *H. angigyra* exhibits a large, flat thickening (the central pilaster) that appears to lack spicules and extends over two-thirds of the penile cavity. In addition, at least two smaller pilasters, densely covered with spicules, are present nearby.

Discussion

Molecular results

Our molecular analyses clearly confirm the taxonomic distinctness of *Helicodonta angigyra* from *H. obvoluta*. In the COI phylogeny, *H. angigyra* forms a well-supported, monophyletic lineage sister group to *H. obvoluta*. The mean Kimura 2-parameter (K2P) distance between *H. angigyra* and the two *H. obvoluta* haplogroups (Ho1 and Ho2) is approximately 19–20%, far exceeding the frequently

cited threshold of 2–5% for species-level divergence that was commonly applied during the early development of DNA barcoding (Hebert *et al.* 2003; Davison *et al.* 2009). Under the Hennigian inter-nodal species concept (Samadi & Barberousse 2006), this level of differentiation strongly supports the recognition of *H. angigyra* as an evolutionary lineage distinct from other species.

However, although all the available sequences attributed to *H. obvoluta* form a monophyletic group, this clade is split into two distinct haplogroups (Ho1 and Ho2). These haplogroups do not exhibit a clear geographical structure and are separated by an average K2P distance of 7%. Although this level of divergence is unusually high for intraspecific variation, similar or higher values have been reported for Stylommatophora (Davison *et al.* 2009) particularly for mountain-dwelling land snails such as *Faustina faustina* (Rossmässler, 1835) (Helicidae; 0.2-18.1%; Groenenberg *et al.* 2016; Zajac *et al.* 2020), *Orcula dolium (Draparnaud, 1801) (Orculidae)*, *Arianta arbustorum* (Linneaus, 1758) (Helicidae) (12.5–18.9%; Haase *et al.* 2013; Harl *et al.* 2014; Kruckenhauser *et al.* 2014), and *Cepaea nemoralis* (Linnaeus, 1758) (Helicidae) (up to 20% at the 16S rRNA locus; Davison 2000).

Several processes have been proposed to explain these patterns, including long-term isolation, restricted gene flow, and the persistence of cryptic lineages. Additional mechanisms such as mitochondrial introgression or incomplete lineage sorting may also play a part in maintaining divergent haplogroups within morphologically uniform species. Consequently, the ~7% divergence observed within *H. obvoluta, a broad ranging taxon,* should not be regarded as evidence for species-level separation in the absence of congruent nuclear or morphological data (Hausdorf & Sauer 2011).

Our results also indicate that COI sequences from some *Lindholmiola* species do not form clusters (Fig. 2: *L. girva* (Frivaldsky, 1835); *L. spectabilis* Urbański, 1960; *L. pirinensis* Jaeckel, 1954). As the COI data are derived from unpublished GenBank sequences lacking detailed specimen identification, future studies examining the monophyly of these species should primarily be based on newly generated data from reliably identified voucher material, and an integrative taxonomic perspective should be considered.

At the supraspecific level, the COI phylogeny obtained here does not fully support the monophyly or the current infrafamilial classification of the Helicodontidae (*MolluscaBase* Eds 2021). In our analyses, the genus *Soosia* emerges as sister group to *Caracolina lenticula* (Trissexodontidae) (Prieto *et al.* 1993). The monophyly of the Lindholmiolinae (genus *Lindholmiola*) is well supported, whereas the Helicodontinae (genera *Atenia*, *Drepanostoma*, *Falkneria*, and *Helicodonta*) appear polyphyletic. This topology is consistent with Schileyko's (2006) earlier proposal regarding the subfamily Drepanostomatinae, and it further supports the possible recognition of an additional subfamily, based on the genus *Atenia*.

The position of the genus *Falkneria* remains unresolved since it was not represented in our dataset.

However, our study is based on a single mitochondrial marker (COI), which is well suited for resolving species-level relationships but less reliable for inferring deeper phylogenetic nodes compared to nuclear markers. In addition, incomplete taxon sampling and the limited number of specimens per species may bias estimates of genetic distance and haplotype diversity. For these reasons, although our molecular results challenge the current family- and subfamily-level classification, they should be interpreted with caution.

Anatomical characters

The internal structure of the penis, including the pilasters, penial papillae, as well as the various folds of the penis wall, are often used to delineate species of Stylommatophora when their genital morphology remains homogeneous and does not provide obvious distinguishing features (Groenenberg *et al.* 2016). The penis and its internal structure are widely accepted to play a key role in species recognition during mating, particularly regarding the success of copulation (Gómez 2001). Our anatomical study of the penis's internal structure reveals significant differences between *H. angigyra* and *H. obvoluta*, particularly in the pilasters' structure and spatial arrangement. These structures appear to be highly consistent within both species, suggesting that they could be used as reliable, specific character states.

The function of the various pilasters during copulation, and their histological structure, as well as the spicules present on some of them, remains unknown. Schileyko (1978) suggested that the pilasters in *H. obvoluta* are arranged like a valve to facilitate the transfer of spermatophores. However, Matz (2003b) found no evidence of spermatophores in any of the 90 individuals of *H. obvoluta* studied and instead proposed that seminal fluid circulates in an amorphous "sperm mass". The putative absence of spermatophores may explain why there is no clear distinction between the epiphallus and the vas deferens (Nordsieck 1989). The latter is a narrow duct lined with ciliated cells and circularly oriented muscle fibres. Peristaltic movements and ciliary action enable seminal fluids to circulate throughout the penis. In contrast, the epiphallus is usually a highly muscular organ with a

wider, pleated lumen involved in forming spermatophores (Gómez 2001). Therefore, the presence of a "sperm mass" rather than a spermatophore could explain the presence of a regressed epiphallus that nevertheless remains variably distinct from the vas deferens. The appropriate terminology for this structure thus remains unresolved.

Another difficulty within the genus *Helicodonta* concerns the organisation of the auxiliary copulatory organs (ACO), particularly regarding the true identity of the structure referred to as the accessory sac (Gómez-Moliner *et al.* 2012), the appendicula (Nordsieck 1989) or the small branch of the mucous gland (Maltz 2003a). The morphology of the ACO is of prime importance in the taxonomy of the Helicoidea and particularly in the classification of subfamilies within the Helicodontidae (Gómez-Moliner *et al.* 2012; Groenenberg *et al.* 2016). Descriptions of ACO are based on the number of mucous glands, the presence of a dart sac (with or without a calcareous dart), as well as the number and position of the accessory sac and its opening into the vagina.

However, the presence of a single two-branched mucous gland, as opposed to a one-branched gland, accompanied by an accessory organ in Helicodontidae, has implications for the subfamily-level classification and for our understanding of the evolution of the ACO within Helicoidea. Based on histological approaches, Maltz (2003a) suggested that the mucous gland in *H. obvoluta* is composed of two branches of unequal size. Therefore, a mucous gland with two forked branches appears to be a derived state at the family level, since the basal groups have a single-branched mucous gland (*Atenia quadrasi*). The topology suggests that the accessory organ was lost in the ancestor of Helicodontidae, leaving a single gland in *Atenia*, which evolved into a forked gland in *Helicodonta*.

Shell characters and infraspecific taxonomy

In light of our molecular and comparative anatomical results, shell morphology indeed provides reliable characters for distinguishing *H. angigyra* from *H. obvoluta*. The former generally has a regular pattern of short, dense periostracal hairs and an additional spire whorl (Kerney & Cameron 1999; Welter-Schultes 2012). However, extreme variability in shell morphology has been observed between populations of *H. obvoluta* across its range (Maltz 2007), leading to the description of several subspecies (Molluscabase Eds 2021), some of which are sometimes treated at the species level (Manganelli *et al.* 1995). These subspecies are recognised based on overall shell size, aperture shape, and the presence or absence of a pronounced tooth on the peristome. These morphs have never been re-evaluated using an integrative taxonomy approach, and the high degree of morphological plasticity within *H. obvoluta* makes it difficult to define distinct evolutionary entities based on shell alone, even though some discrete, non-overlapping shell characters may occur. The deep split revealed by molecular analyses between the two *H. obvoluta* haplogroups cannot be interpreted as evidence for distinct evolutionary lineages in the absence of nuclear markers, but this split may instead reflect historical population-level processes.

Conclusion

Our integrative approach, combining DNA barcoding and comparative anatomical analyses, confirms that *Helicodonta angigyra* and *H. obvoluta* are two distinct evolutionary lineages identifiable by their penile internal structure and shell morphology. However, our molecular results based on the mitochondrial COI gene also challenge the currently accepted infraspecific boundaries and raise questions about the monophyly of the Helicodontidae. These results require confirmation through multilocus datasets incorporating both mitochondrial and nuclear markers, as well as more comprehensive taxonomic sampling across the family. In addition, the various organs of the genital apparatus within the family should be redefined, described and named using a standardised framework. Such integrative data will be essential for testing the robustness of the two *H. obvoluta* haplogroups, clarifying the position of *Falkneria camerani (Lessona, 1880) and Soosia diodonta* (A. Férussac, 1832), and ultimately providing a more stable phylogenetic framework for the family.

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Appendix

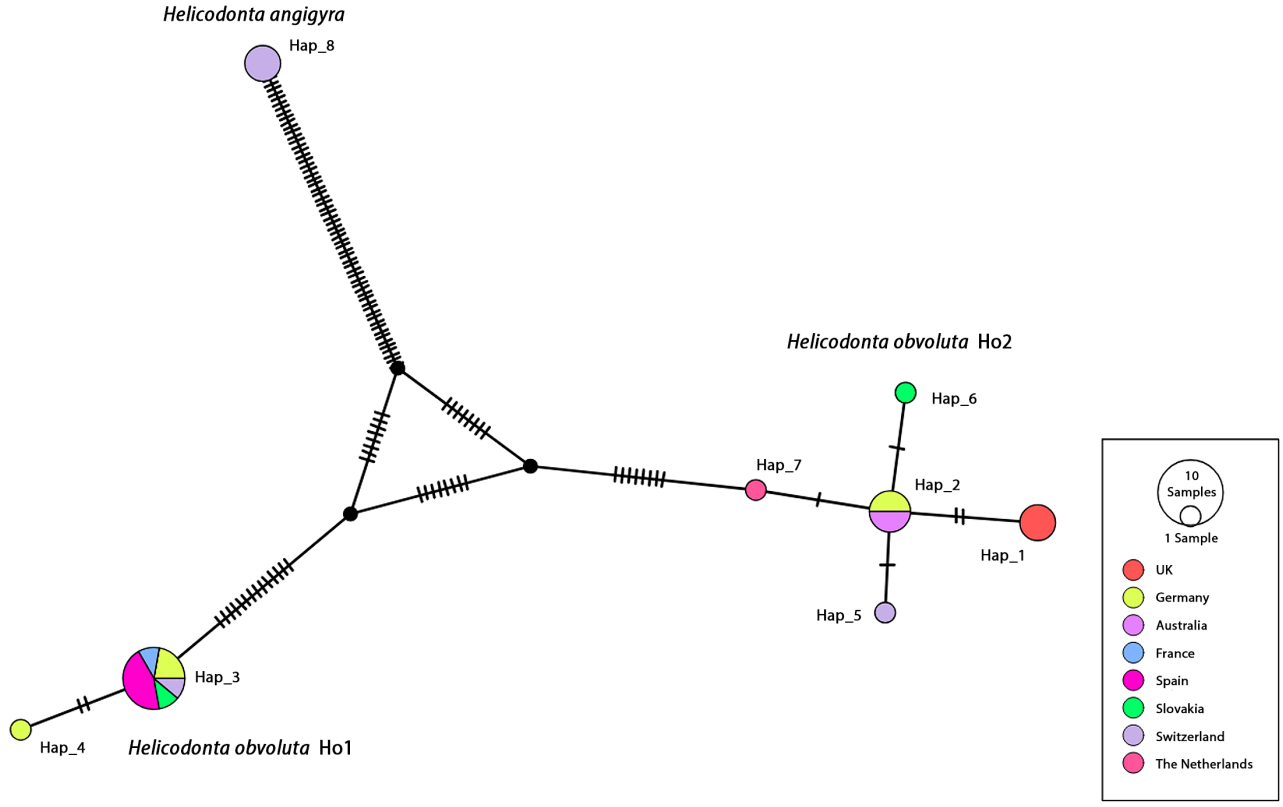

Figure A1. Median-joining haplotype network of *Helicodonta obvoluta* and *H. angigyra* based on the COI gene. Each circle represents an identified haplotype, and the circle's size is proportional to the number of individuals carrying it. The geographical origin of the sequences is indicated by a colour code (see the legend). Lines on the branches indicate the number of mutations between haplotypes.