Reconciling comparative anatomy and mitochondrial phylogenetics in revising species limits in the Australian semislug *Helicarion* Férussac, 1821 (Gastropoda: Stylommatophora)

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Helicarion Férussac, 1821 currently comprises four semislug species from south-eastern Australia (Tasmania to New South Wales). We comprehensively revise the taxonomy of this group based on comparative morphology and on the mitochondrial genes *COI* and 16S, provide a new generic diagnosis and revise species descriptions. Contrary to the previous classification, we found that *Helicarion* encompasses only two species, differentiated by anatomy and mitochondrial genetics. Both contain several divergent mitochondrial DNA lineages. Based on their rather inconsistent morphological differentiation and their allopatric to parapatric distribution, we suggest that these intraspecific lineages may be in a transient stage of speciation. The type species, *Helicarion cuvieri* (Férussac, 1821), is redefined to include populations from Tasmania, Victoria and southern New South Wales, as far north as Wyong. *Helicarion niger* (Quoy & Gaimard, 1832), *Helicarion leopardinus* Iredale, 1941 and *Helicarion mastersi callidus* Iredale, 1941 are now recognized as synonyms of *H. cuvieri*. *Helicarion mastersi* (Cox, 1868) is restricted to an area from north of Nowra to southern Sydney in New South Wales.

ADDITIONAL KEYWORDS: morphology – Pulmonata – reproductive biology – taxonomy.

INTRODUCTION

In this study, we seek to resolve species limits within the Australian endemic genus *Helicarion* Férussac, 1821 by means of comparative morphology and anatomy and by mitochondrial phylogenetics. This genus has been used as a dumping ground for poorly known semislugs from Australia and Southeast Asia (e.g. Schileyko, 2002). However, the genus had previously been delineated exclusively to contain semislugs that, in the reproductive system, lack a stimulator, epiphallic retractor caecum and elaborate atrial diverticulum, but possess a coiled flagellum that produces an elaborate spermatophore, and sometimes a small fleshy penial papilla (Kershaw, 1979, 1980, 1981). Based on comparative morphology, *Helicarion* has indeed been found to contain only five Australian species, ranging from Tasmania to northern New South Wales (NSW) (Kershaw, 1979, 1980, 1981; Hyman &

Ponder, 2010). One of these species, *Helicarion rubicundus* Dartnall & Kershaw, 1978, has recently been excluded from *Helicarion* and placed in the new genus *Attenborougharion* Hyman & Köhler, 2017 based on its morphological and mitochondrial distinctiveness (Hyman & Köhler, 2017). The remaining species are comparatively common throughout suitable habitats within their respective ranges in south-eastern Australia, which comprise a variety of forest and woodland types from rainforest to dry sclerophyll forest.

According to the current classification, *Helicarion* species differ considerably in their external morphology and are, for the most part, considered to be geographically isolated (i.e. allo- or parapatric). The type species of the genus, *Helicarion cuvieri* Férussac, 1821, is grey, greyish buff or white in colour and widespread throughout southern Tasmania (Kershaw, 1979). Dartnall & Kershaw (1978) stated that distinct morphs exist in the highlands of the west, central and eastern regions of Tasmania and the lower levels of the north and Tamar valley. However, the work underpinning this statement was never published, and the degree of

distinctiveness of these forms is therefore uncertain. The second species, *Helicarion niger* (Quoy & Gaimard, 1832), ranges in colour from pale to dark grey, rarely black, with paler shell lappets and a darker tail, and has been reported from Port Philip Bay to Wilson's Promontory in Victoria (Dartnall & Kershaw, 1978). Specimens from other parts of Victoria may represent potentially undescribed species (Dartnall & Kershaw, 1978); however, a comparative study of these forms has not yet been conducted. In contrast, the anatomy of both *H. cuvieri* and *H. niger* was studied rather extensively (Kershaw, 1979, 1980, 1981).

Two species, *Helicarion mastersi* (Cox, 1868) and *Helicarion leopardinus* Iredale, 1941, have been recorded from NSW. Hyman & Ponder (2010) described and figured the anatomy of *H. mastersi* based on the examination of material from the Royal National Park south of Sydney, whereas the anatomy of *H. leopardinus* has remained unknown. *Helicarion mastersi* has been recorded from Eden in southern NSW to Mt Coricudgy, northwest of Sydney, ranging in colour from a pale orange-brown to nearly black, often with a contrasting sole of white, pink or orange (Stanisic *et al.,* 2010). Very few morphological differences between *H. cuvieri* and *H. mastersi* were noted by Hyman & Ponder (2010), who also synonymized *H. mastersi callidus* Iredale, 1941, described from Twofold Bay near Eden (Iredale, 1941), with *H. mastersi*. *Helicarion leopardinus* was described from Ourimbah in mid-eastern NSW and is the least-known species. It is reported to be greyish white and both paler and smaller than *H. mastersi* (Stanisic *et al.,* 2010).

The neat demarcation of three of the four species along state boundaries appears suspicious in view of the otherwise near-continuous distribution of *Helicarion* semislugs in south-eastern Australia and the scarcity of apparent physical distribution barriers in this region. We scrutinize the presently accepted species-level taxonomy with molecular phylogenetic tools (i.e. analyses of partial sequences of the mitochondrial genes cytochrome *c* oxidase subunit I and 16S rRNA) and investigate the purported existence of potentially undescribed species. To this end, we have studied a comprehensive collection of material from throughout the entire range of this genus, including newly collected samples and samples from various museum collections.

MATERIAL AND METHODS

MATERIAL

This study is based on the examination of ethanol-preserved specimens and supplementary dry material from the Australian Museum (AM), the Queensland Museum (QM), Museum Victoria (NMV), the Tasmanian Museum and Art Gallery (TMAG) and the Queen Victoria Museum and Art Gallery (QVMAG), including freshly collected material from south-eastern NSW.

MOLECULAR STUDIES AND PHYLOGENETIC ANALYSES

DNA was extracted from small pieces of foot muscle by use of a QIAGEN DNA extraction kit for animal tissue (Qiagen, Hilden, Germany) following the standard procedure of the manual. A fragment of the 16S gene ~900 bp long was amplified by PCR using the primers 16S3F and 16S4Ra (Hyman, Ho & Jermiin, 2007). Whenever we failed to amplify the whole fragment owing to DNA fragmentation, as typically encountered in extracts from older museum specimens, we amplified two overlapping shorter fragments or even performed nested PCRs by using the internal primers 16S3R and 16S4F (Hyman *et al.,* 2007). In addition, a fragment of the *COI* gene 823 bp long was amplified by using the primers LCOH1940 (Folmer *et al.,* 1994) and COI-H865 (Hyman, Lamborena & Köhler, 2017). For samples with highly fragmented DNA, we performed a nested PCR using the primers LCOH1490 and HCOI2198 (Folmer *et al.,* 1994) to amplify a fragment 655 bp long. Reactions were performed using standard protocols with annealing temperatures/elongation times of 55 °C/90 s for 16S and 60 s 50 °C/60 s for *COI*, respectively. Both strands of PCR fragments were purified and cycle sequenced by use of the PCR primers. Electropherograms were corrected for misreads, and forward and reverse strands were merged into one sequence file using CodonCode Aligner v. 3.6.1 (CodonCode Corp., Dedham, MA, USA). Sequences of the previous helicarionid study (Hyman *et al.,* 2007, 2017) were retrieved from GenBank and included in our data set. All newly produced sequences have been deposited in GenBank under the accession numbers MH120212-81, MG823183-243.

The 16S sequences were aligned using the online version of MAFFT (version 7) available at http://mafft.cbrc.jp/alignment/server/ by using the iterative refinement method E-INS-i suitable for sequences with multiple conserved domains and long gaps (Katoh *et al.,* 2002). Uncorrected p-distances between sequences were calculated by using the phylogenetic software MEGA7 (Kumar, Stecher & Tamura, 2016) under the option 'pair-wise deletion of gaps'. The aligned 16S and *COI* sequences were concatenated into one partitioned data set. Four partitions were designated: the entire 16S fragment plus each of the three codon positions of the *COI* fragment. The best-fit model of nucleotide substitution was identified for each sequence partition separately by means of the corrected Akaike information criterion (AICc) using the software Partitionfinder version 2.1.1 (Lanfear *et al.,* 2012). Phylogenetic relationships were estimated by using a maximum likelihood (ML)-based method of tree reconstruction and

Bayesian inference (BI). Maximum likelihood phylogenies were reconstructed by using the program RAxML version 8.0.0 (Stamatakis, 2014), available on the Cipres Science Gateway (Miller, Pfeiffer & Schwartz, 2010). Nodal support of the best ML tree was estimated by performing ten independent runs, each with 200 thorough bootstrap replicates. A second ML analysis was performed using the software IQ-TREE version 1.6 (Nguyen *et al.,* 2015). This analysis was performed using the integrated modelfinder function (Kalyaanamoorthy *et al.,* 2017). Nodal support was estimated by performing 10 000 ultra-fast bootstrap repeats and by using Shimodaira-Hasegawa's (1999) approximate likelihood ratio test (SH-aLRT). Bayesian posterior probabilities of phylogenetic trees were estimated by running a 50 000 000 generations Metropolis-coupled Markov chain Monte Carlo (four runs, each with four chains, of which one was heated) as implemented by MrBayes 3.2.2 (Ronquist & Huelsenbeck, 2003). A data partition was applied that allowed all parameters to be estimated separately for each partition. The sampling rate of the trees was 1000 generations. Generations sampled before the chain reached stationarity were discarded as burn-in. Stationarity was reached when the average standard deviation of split frequencies was < 0.01 and the log-likelihood of sampled trees reached a stationary distribution (Ronquist & Huelsenbeck, 2003).

ANCESTRAL STATE RECONSTRUCTION

Phenotypic trait values for the proportion of penis to epiphallus length were estimated for ancestral nodes in the tree and visualized by using the ML-based ancestral character estimation implemented in the functions fastAnc and contMap in the R package phytools (Revell, 2012).

MORPHOLOGICAL STUDIES

Genital anatomy was examined through dissection of ethanol-preserved specimens using a Leica MZ8 stereo microscope with a drawing apparatus. Before dissection, the shell was removed, cleaned and mounted on carbon tabs for scanning electron microscopy (SEM). The reproductive system was examined *in situ* in order to retain the folding of the spermoviduct. Penial interiors were examined and the total number of penial lamellae was counted. Penis and epiphallus length were measured subsequently from drawings. The demarcation between penis and epiphallus was determined by an abrupt narrowing in diameter. The ratio of penis to epiphallus length was calculated and used in an ancestral character estimation and to inform species descriptions. Spermatophores were removed from the bursa copulatrix and prepared for drawing by rinsing and the

removal of extraneous tissue with fine forceps. To measure complexity, spermatophore spines were divided into four categories: spines containing more than one major branch; spines with multiple minor branches; spines with a bifurcation; and unbranched spines. The number of spines in each category was counted. Owing to the scarcity of recovered spermatophores, these data were not used for statistical testing but were used to inform species descriptions.

Shells were measured with callipers with a precision of 0.1 mm. We excluded specimens of fewer than three whorls, because dissection showed that specimens with a lower whorl count were often immature. Dimensions measured were height (SH = maximal dimension parallel to axis of coiling), width (SW = maximal dimension perpendicular to SH) and number of whorls (NW = whorl count using method shown by Köhler, 2011). Analyses of covariance (ANCOVAs) of morphometric parameters were performed using XLStatistics (Rodney Carr 1997–2011, Deakin University).

ABBREVIATIONS

Interim Biogeographic Regionalisation of Australia (IBRA) regions: AUA, Australian Alps; FUR, Furneaux; SCP, South East Coastal Plain; SEC, South East Corner; SEH, South Eastern Highlands; SYB, Sydney Basin; TAS, eight Tasmanian bioregions (King Island, Ben Lomond, Tasmanian Northern Slopes, Tasmanian Central Highlands, Tasmanian West, Tasmanian South East, Tasmanian Southern Ranges, Tasmanian Northern Midlands).

Geographical: NP, National Park; NR, Nature Reserve; NSW, New South Wales; Qld, Queensland; SF, State Forest; Tas, Tasmania; Vic, Victoria.

Institutional: AM, Australian Museum; MNHP, Muséum National d'Histoire Paris; NMV, Museums Victoria; QM, Queensland Museum; QVMAG, Queen Victoria Museum and Art Gallery; TMAG, Tasmanian Museum and Art Gallery.

Morphological: alb, albumen gland; bc, bursa copulatrix; ep, epiphallus; fl, flagellum; her, hermaphrodite duct; ov, ovotestis; p, penis; pl, penial lamellae; pr, prostate; prm, penis retractor muscle; pt, penial tunica; sc, spermatophore capsule; st, spermatophore tail-pipe; ut, uterus; v, vagina; vd, vas deferens.

RESULTS

MOLECULAR PHYLOGENETIC ANALYSES

The final concatenated sequence data set contained sequences of 78 *Helicarion* specimens and four outgroup representatives that were used to root the trees (Supporting Information, Table S1). The outgroup representatives were selected based on a comprehensive

phylogeny of the southeastern Australian Helicarionidae (Hyman *et al.,* 2017). Two *Helicarion* sequences were obtained from GenBank, whereas all other sequences were produced in the present study. Some samples were represented by only one sequence fragment, because three 16S and 12 *COI* sequences were missing from the data set. The final 16S alignment had a total length of 1160 aligned nucleotide positions, and the *COI* sequences had a length of 655 bp.

The best-fit models of sequence evolution for the different data partition were Hasegawa-Kishino-Yano model with gamma distributed rates and invariant sites (HKY+G+I) (Hasegawa *et al.,* 1985) for 16S, HKY+I for the first codon position of *COI*, and the general time reversible model with gamma distributed rates (GTR+G) (Tavaré 1986) for second and third codon positions of *COI*.

All phylogenetic analyses confirmed the monophyly of *Helicarion* with respect to the chosen outgroup. However, none of the species as currently delimited was monophyletic (Figs 1–3). The trees consistently revealed two principal clades, both well differentiated and well supported in terms of basal branch lengths. Uncorrected pairwise genetic distances between members of these principal clades ranged from 7 to 11% in *COI* and from 5 to 8.6% in 16S (Table 1). In contrast, the uncorrected distances between sequences within the same principal clade ranged from 0 to 9.6 % in *COI* and from 0 to 6.4% in 16S (Table 1, Fig. 4).

One of these principal clades included populations traditionally classified as *H. mastersi*, spanning a region that stretches from Kiama to southern Sydney, including specimens from the type locality of this species. Therefore, hereafter this principal clade is referred to as the *H. mastersi* complex. The second principal clade encompassed populations from Tasmania, through Victoria and southern NSW, to north of Sydney. The type localities of all remaining species and subspecies of *Helicarion* were included in the range of this clade, which is hereafter referred to as the *H. cuvieri* complex because this is the earliest introduced species name.

Both principal clades contained several subclades (labelled A–K; Fig. 1) that were well supported in terms of Bayesian posterior probabilities and ML bootstrap values (usually > 90%). The average genetic p-distances between different subclades ranged from 5.6 to 8.2% (on average 6.1%) in *COI* and from 3.4 to 5.4% (on average 4.6%) in 16S. The average within-group p-distances in these subclades ranged from 0.3 to 4.0% (on average 2.5%) in *COI* and from 0.5 to 2.5% in 16S (Tables 2, 3).

The topology of ML and BI trees was consistent with respect to the clustering into clades and subclades, with the single exception that the ML trees showed subclade H containing two sequences from the Brindabella Range in a sister-group relationship with all other clades (Fig. 2), whereas this subclade is shown in an unresolved position within the *H. cuvieri* complex in the BI phylogram (Fig. 1). The relative amount of lineage differentiation of all clades is visualized in the radiation tree shown in Figure 3.

COMPARATIVE MORPHOLOGY

Comparative examinations were conducted to investigate whether members of the mitochondrial clades and subclades could consistently be distinguished from each other by anatomical and/or morphological traits.

We found that all *Helicarion* specimens share a number of characteristics that distinguish them from other south-eastern Australian helicarionids. They have a glossy, reduced, ear-shaped shell of 3.0–3.7 whorls, a robust body with a well-developed caudal horn and slime grooves, and although their body colour varies considerably, the overall pattern is of a single body colour, becoming darker on the tail, with a contrasting, paler sole. The genital morphology is also highly consistent. The ovotestis is embedded in the digestive gland and generally consists of two to four lobes. The convoluted hermaphrodite duct leads to the talon and carrefour, embedded in the base of the triangular albumen gland. The spermoviduct is folded in a distinctive way unique to this genus: it descends from the albumen gland, curves 180° into the foot (towards the tail), then reverses direction towards the head, descending to the junction of the free oviduct and bursa copulatrix. The bursa copulatrix has a broad duct and a swollen, oval to tear-shaped sac; the free oviduct is moderately short with an indistinct capsular gland. The vagina is short. The penis is generally moderately long, slender and tubular, with no penial verge and with an internal anatomy of V-shaped rows of folded lamellae, sometimes becoming pustules proximally. The epiphallus is usually approximately one to four times the length of the penis, with no epiphallic caecum and with a short, spiralling flagellum containing internal cryptae. The ascending and descending arms of the epiphallus are often slightly twisted around one another. The spermatophore always consists of a soft-walled capsule with a short, spiralling, branched tail-pipe; the number, arrangement and branching pattern of spines vary considerably.

The division into two principal clades was well reflected in morphological differences in the penial interior. In the *H. mastersi* complex, the penial lamellae were fine, numerous and closely spaced, arranged in a shallow V shape, whereas in the *H. cuvieri* complex the penial lamellae were larger, less numerous, more widely spaced, and arranged in a deep V shape. In addition, semislugs of the *H. mastersi* complex had smooth shell lappets, lacking the distinctive pigmented warts seen in most (but not all) members of the *H. cuvieri* complex.
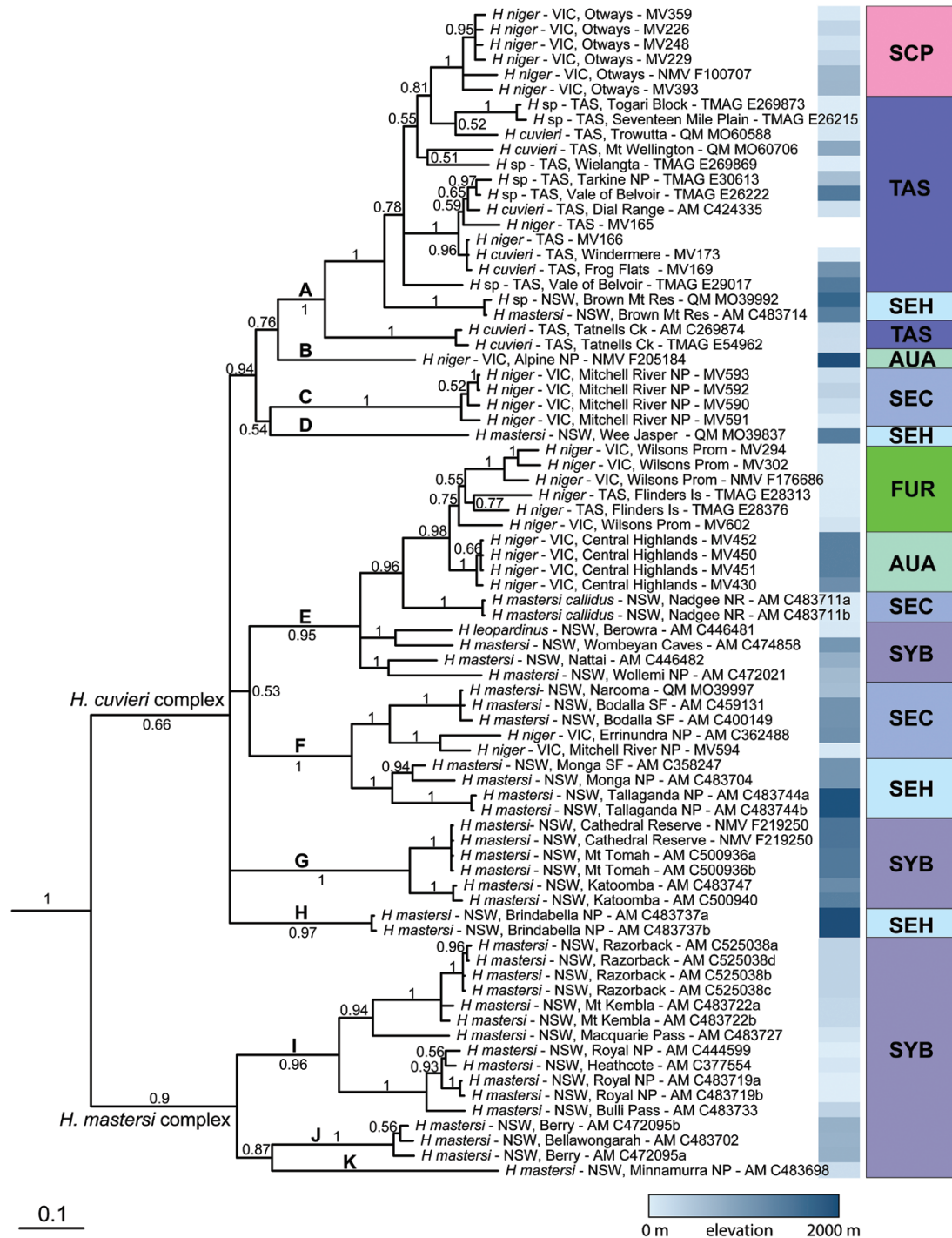
Figure 1. Majority-rule consensus tree based on Bayesian analysis of the concatenated data set of fragments of the mitochondrial genes 16S and *COI*. Ambiguous alignment sites in 16S were removed using MAFFT. Numbers on branches indicate posterior probabilities. Scale bar indicates 10% of modelled sequence divergence. Species identifications are according to previous taxonomy. Blue bars immediately to the right of the sample names indicate elevation; coloured bars on the far right refer to Interim Biogeographic Regionalisation of Australia (IBRA) geographical regions (for abbreviations see Material and Methods).
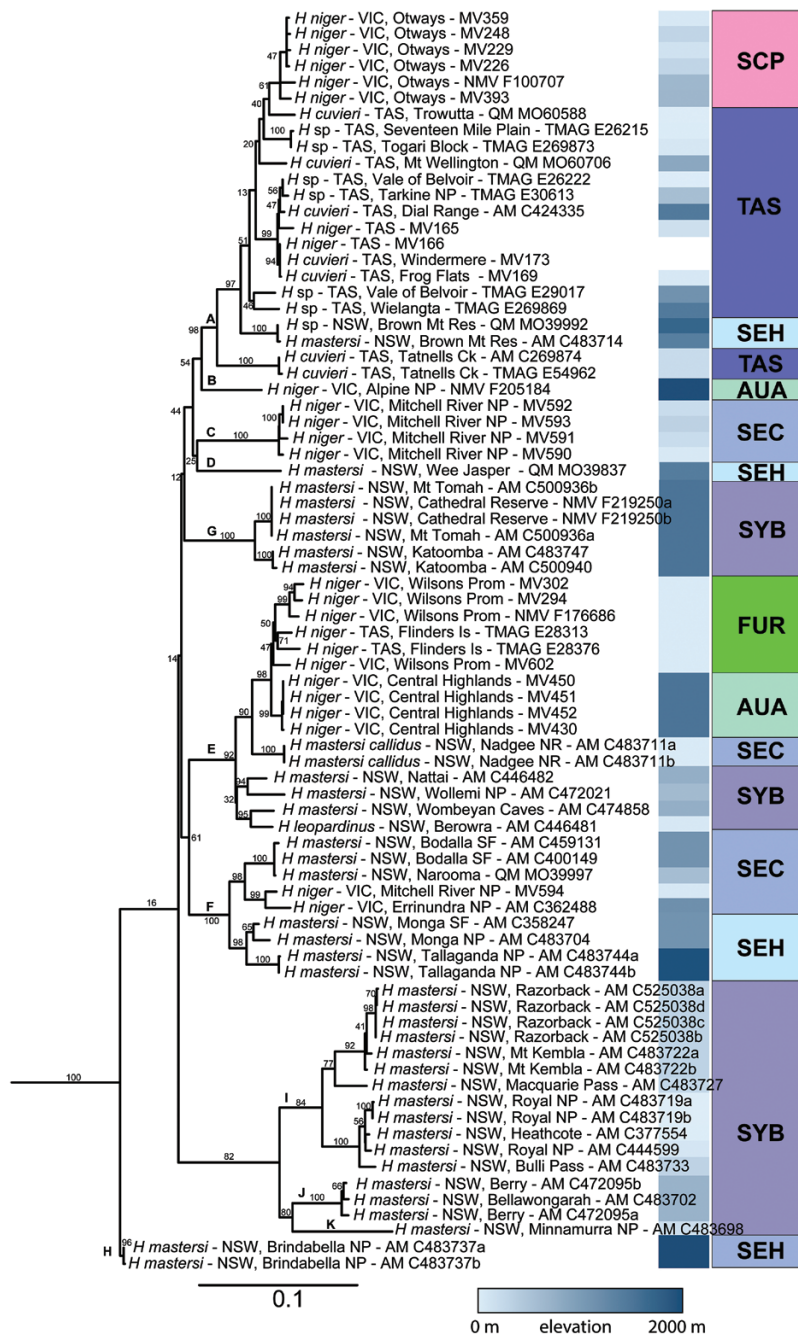
Figure 2. Best maximum likelihood tree based on analysis of the concatenated data set of fragments of the mitochondrial genes 16S and *COI*. Ambiguous alignment sites in 16S were removed using MAFFT. Numbers on branches indicate nodal support based on 200 thorough bootstrap replicates for a total of ten independent runs. Scale bar indicates 10% of modelled sequence divergence. Species identifications are according to previous taxonomy. Blue bars immediately to the right of the sample names indicate elevation; coloured bars on the far right refer to Interim Biogeographic Regionalisation of Australia (IBRA) geographical regions (for abbreviations see Material and Methods).

The two clades did not differ consistently in shell shape or external morphology and body colour, although members of *H. cuvieri* were generally darker in body colour, with dark eyestalks and a longer keel on the tail.

In the next two sections, we address the anatomical differentiation within each of the two principal clades (for detailed descriptions refer to taxonomy sections further below).
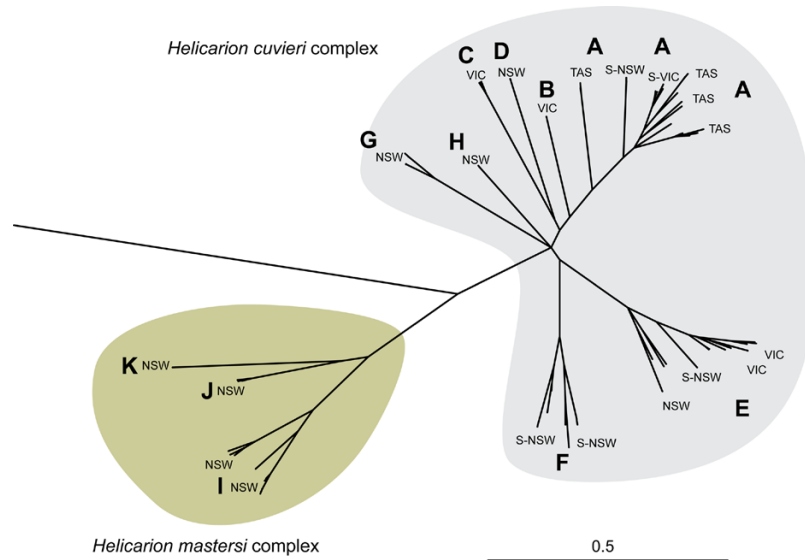
Figure 3. Bayesian phylogram depicted as a radiation tree, showing lineage differentiation between the two species complexes. Scale bar indicates 50% of modelled sequence divergence.

Table 1. Comparison of intra- and interspecific genetic p-distances for the two mitochondrial fragments analysed, both between principal clades (species) and between subclades

	Principal clades				Subclades			
	Within		Between		Within		Between	
	COI	16S	COI	16S	COI	16S	COI	16S
Minimum	0.000	0.000	0.070	0.050	0.000	0.000	0.049	0.029
Average	0.064	0.040	0.087	0.070	0.034	0.018	0.078	0.056
Maximum	0.096	0.064	0.110	0.086	0.067	0.038	0.110	0.086

Helicarion cuvieri *complex*

Covering a very broad geographical range, including all Tasmanian and Victorian populations and continuing through southern NSW to Wyong, north of Sydney, the *H. cuvieri* complex is subdivided into eight well-supported mitochondrial subclades (A–H; Fig. 2). This complex encompasses a relatively high degree of morphological variation overall, but this variation is largely not consistent with the clustering into different subclades.

The broad-ranging subclades A, E and F exhibit a very similar morphology. Each encompasses large semislugs, in most cases with three pigmented warts on the shell lappets (two on the right lappet, one on the left lappet). The primary distinguishing features are related to the penial complex; specimens belonging to subclades A and F had average penis-to-epiphallus ratios of 0.59 and 0.60, respectively (Table 4), whereas members of subclade E differed in having a generally shorter penis (penis-to-epiphallus ratio for most specimens of 0.18–0.50), with fewer internal lamellae.

Most specimens across all three subclades had a spermatophore with 13–16 evenly spaced branching spines.

However, within each subclade there was considerable variation. In subclade A, specimens from the Otway Ranges had only nine to ten spermatophore spines, and specimens from Brown Mountain had 20 spermatophore spines, contrasting with the 13–16 spines seen in Tasmanian specimens. In subclade E, body morphology and penis size were variable. Specimens from Victoria had three pigmented warts on their shell lappets and were significantly larger than specimens from NSW, which lacked the pigmented warts (SH, SD: *P* < 0.01). In addition, the considerable variation in the penis length relative to the epiphallus in subclade E gave rise to three penial morphotypes. A moderate-length penis was observed throughout Victoria and Flinders Island; a very short penis was present in disjunct populations from Nadgee NR, the southern Blue Mountains and Wollemi NP; and a long penis was seen in Wombeyan Caves (south of Sydney) and in Wyong (north of Sydney).
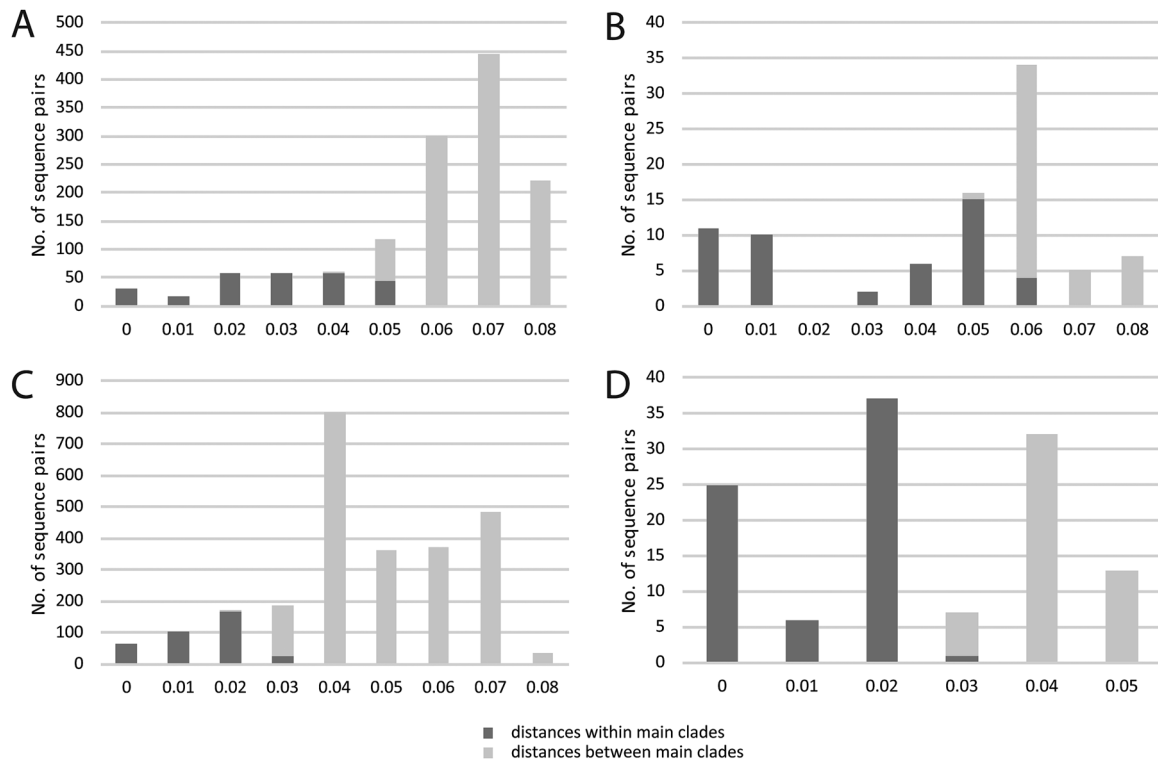
Figure 4. Comparison of intra- and intercladal genetic uncorrected p-distances for the two mitochondrial fragments analysed. A, B, frequency distributions of distances in *COI*. A, *Helicarion cuvieri* complex. B, *Helicarion mastersi* complex. C, D, frequency distributions of distances in 16S. C, *H. cuvieri* complex. D, *H. mastersi* complex.

Likewise, within subclade F there was considerable variation in the flagellum, spermatophore and penial complex. In particular, in populations from Mt Gulaga and Tallaganda NP the branching patterns of the spermatophore spines were very divergent, with differences significant enough to be reflected in the flagellum shape. Additionally, in a clade from Errinundra

NP and Fairy Dell (Vic) the penis was significantly shorter.

Subclades B, C, G and H could each be distinguished morphologically from the rest of the *H. cuvieri* complex. Subclade B from Alpine NP was represented by only a single specimen, which had a very dark body, three pigmented warts on the lappets, a moderately

Table 2. Average pairwise distances in *COI* within and between species under pairwise removal of gaps

		Helicarion cuvieri complex							*Helicarion mastersi* complex		
		A	B	C	E	F	G	H	I	J	K
Helicarion cuvieri complex	A	0.033									
	B	0.062	–								
	C	0.071	0.057	0.003							
	E	0.077	0.075	0.082	0.038						
	F	0.068	0.072	0.074	0.074	0.040					
	G	0.070	0.066	0.082	0.081	0.078	0.012				
	H	0.062	0.056	0.068	0.068	0.068	0.063	0.036			
Helicarion mastersi complex	I	0.085	0.082	0.086	0.086	0.087	0.094	0.084	0.035		
	J	0.087	0.075	0.077	0.081	0.087	0.085	0.075	0.066	0.004	
	K	0.093	0.093	0.093	0.097	0.103	0.104	0.085	0.078	0.082	–

Letters A–K refer to subclades of the phylogenetic analysis.

Table 3. Average pairwise distances in 16S within and between species under pairwise removal of gaps

		Helicarion cuvieri complex							*Helicarion mastersi* complex		
		A	B	C	E	F	G	H	I	J	K
Helicarion	A	0.020									
cuvieri	B	0.034	–								
complex	C	0.049	0.047	0.005							
	E	0.046	0.043	0.050	0.018						
	F	0.049	0.054	0.053	0.042	0.022					
	G	0.049	0.050	0.054	0.045	0.053	0.006				
	H	0.040	0.038	0.044	0.039	0.045	0.043	0.026			
Helicarion	I	0.073	0.074	0.076	0.069	0.074	0.073	0.067	0.016		
mastersi	J	0.064	0.062	0.063	0.059	0.069	0.061	0.055	0.047	0.005	
complex	K	0.066	0.067	0.077	0.068	0.074	0.073	0.064	0.052	0.036	–

Letters A–K refer to subclades of the phylogenetic analysis.

Table 4. Shell and penial complex measurements in *Helicarion*

Species	Shell					Penial complex	
	N	SH (mm)	SW (mm)	NW		*N*	P/E
H. cuvieri (A)	10	4.25–6.30 (5.295 ± 0.21)	10.71–15.89 (13.128 ± 0.49)	3.0–3.5 (3.355 ± 0.05)		6	0.41–0.84 (0.59 ± 0.072)
H. cuvieri Tatnells Creek	–	–	–	–		1	0.59
H. cuvieri (B)	–	–	–	–		1	0.53
H. cuvieri (C)	–	–	–	–		1	0.74
H. cuvieri (E)	18	3.85–6.75 (5.1 ± 0.23)	8.87–13.1 (10.785 ± 0.3)	3.1–3.9 (3.278 ± 0.05)		11	0.18–0.98 (0.51 ± 0.08)
H. cuvieri (F)	5	4.91–6.32 (5.586 ± 0.24)	11–12.63 (11.824 ± 0.3)	3.2–3.4 (3.3 ± 0.03)		5	0.29–0.87 (0.6 ± 0.101)
H. cuvieri (G)	5	3.39–5.05 (4.154 ± 0.31)	8.19–9.78 (8.986 ± 0.32)	3–3.3 (3.2 ± 0.05)		3	1.08–1.31 (1.23 ± 0.075)
H. cuvieri (H)	6	3.34–4.43 (4.088 ± 0.17)	8.96–11.39 (10.145 ± 0.36)	3.2–3.4 (3.3 ± 0.04)		2	0.79–0.79 (0.79 ± 0.003)
H. mastersi (I)	10	3.76–5.58 (4.555 ± 0.18)	9.45–12.22 (10.506 ± 0.29)	3.4–3.5 (3.445 ± 0.02)		5	0.52–0.89 (0.66 ± 0.07)
H. mastersi (J)	10	4.06–5.73 (4.663 ± 0.2)	9.62–13.6 (11.336 ± 0.47)	3.2–3.7 (3.405 ± 0.05)		5	0.4–0.52 (0.44 ± 0.03)
H. mastersi (K)	10	4.27–5.92 (5.102 ± 0.18)	10.68–14.07 (12.205 ± 0.34)	3.2–3.7 (3.46 ± 0.04)		4	0.5–0.81 (0.62 ± 0.07)

Abbreviations: *N*, number of specimens; NW, number of whorls; P/E, penis-to-epiphallus ratio; SH, shell height; SW, shell width. Subclade letters are given in parentheses.

long penis (penis/epiphallis length of 0.53), with very few penial lamellae and a spermatophore with 15 robust spines.

Subclade C from Mitchell River NP consisted of small semislugs of a uniform speckled brown, with narrow shell lappets lacking pigmented warts. The penial complex was long, with a penis-to-epiphallus length of 0.74, and both penis and epiphallus were folded. It was not possible to measure shells owing to insufficient material; however, specimens appeared to be smaller than those belonging to subclades A, E and F.

Subclade G contained specimens from the northern Blue Mountains (Katoomba, Mt Wilson and Mt Tomah). These semislugs were significantly smaller

Figure 5. Maximum likelihood tree showing an ancestral character estimation of the proportion of penis length to epiphallus length. Species identifications are according to previous taxonomy.

than subclades A, E and F (SH, SD: $P < 0.05$) and ranged in colour from yellowish brown with dark markings to black. These populations had a very long penial complex, with a long penis relative to the epiphallus (penis-to-epiphallus length of 1.08–1.31) and very closely spaced penial lamellae. The bursa copulatrix duct was long and narrow, and the spermatophore had 14 spines.

A population from the Brindabella Ranges (subclade H) in western NSW consisted of grey speckled semislugs lacking pigmented warts on the shell lappets, significantly smaller than subclades A, E and F (SH, SD: $P < 0.05$). Members of this subclade had a distinct genital morphology consisting of a penis with two chambers separated by a slight constriction. The penis-to-epiphallus length was 0.79, and the epiphallus entered the penis laterally. The proximal chamber contained deeply V-shaped (close to longitudinal), less folded lamellae; the distal chamber had shallower V-shaped rows of heavily folded lamellae. The spermatophore had 19 spines, starting with two robust, complex spines with multiple branches and rapidly decreasing in size.

An additional lot from Wee Jasper (subclade D), at the northern extreme of the Brindabella Range, did not

group with subclade H but instead formed the sister group to sublade C. Morphologically, this population was very similar to the specimens of subclade H and shared the distinctive two-chambered penis. However, they differed in having fewer spermatophore spines, a shorter, straighter epiphallus and shorter penis, and less deep and distinct grooves in the distal portion of the penial interior.

Helicarion mastersi *complex*
Within this principal clade, three subclades could be defined morphologically: one from Macquarie Pass to southern Sydney (I), the second from Kangaroo Valley (J), and the third from around Kiama (K). The differences between the subclades included size, body coloration, penis-to-epiphallus length, penis shape and spermatophore morphology.

Populations from Macquarie Pass to southern Sydney (subclade I) were made up of small, greyish to orange-brown semislugs, with a contrasting cream sole and dark shell lappets. Members of this group were significantly smaller than the closely related subclade K from Kiama (SH, SD: $P < 0.05$). Members of subclade I had a penis-to-epiphallus ratio of 0.52–0.89, and the epiphallus entered the penis laterally, leaving a tiny

blind tip. The spermatophore had nine to 11 evenly spaced branching spines; the first three complex, and the remainder with simple bifurcations.

In contrast, specimens from Kangaroo Valley (subclade J) had a flatter shell (although no significant differences in shell shape or size were found), with very pale body coloration and a contrasting cream sole. The penis was narrower, with a small bulge at the proximal end, shorter relative to the epiphallus (penis-to-epiphallus ratio of 0.40–0.52), and entered the penis apically, leaving no blind tip. The spermatophore had more numerous and highly branched spines (12–14), and a small gap between the first and second spines.

Semislugs from around Kiama (subclade K), the type locality of *H. mastersi*, had deep pink coloration and a contrasting cream sole. The penis was broad and curving, with a more distinct blind tip than subclade I and a penis-to-epiphallus ratio of 0.50–0.81. The spermatophore of 11 spines was similar to that seen in subclade I.

TAXONOMIC PREAMBLE

GENERIC RELATIONSHIPS

Helicarion belongs to a monophyletic radiation of south-eastern Australian helicarionids, including genera *Mysticarion* Iredale, 1941, *Peloparion* Iredale, 1937, *Parmavitrina* Iredale, 1937, *Brevisentis* Hyman, 2007, *Ubiquitarion* Hyman, Lamborena & Köhler, 2017, *Cucullarion* Stanisic, 1998 and *Attenborougharion* Hyman & Köhler, 2017 (Hyman *et al.,* 2007, 2017; Hyman & Köhler, 2017). The key morphological characters defining this clade include the presence of at most a very short vagina, the absence of an epiphallic caecum and the presence of a flagellum with internal cryptae that produces a spinose spermatophore (Hyman *et al.,* 2017; Hyman & Ponder, 2010). *Attenborougharion*, which represents the most basal branch within this clade, is the only member of this radiation to have a small epiphallic caecum. Within this clade, the generic relationships remain largely ambiguous owing to low nodal support for principal splits in the mitochondrial phylogeny (Hyman *et al.,* 2017), making it impossible to ascertain the sister group of *Helicarion*.

Helicarion is distinguished from other Australian helicarionid semislugs by a unique genital anatomy, consisting of a reproductive system that is folded downwards into the foot, a penis without verge or internal pilasters and lined with diagonal rows of lamellae, and a flagellum that forms a spinose spermatophore with around nine to 20 branched spines that are arranged in a spiralling pattern. The manner in which the spermoviduct is folded into the foot cavity

is not seen in any other helicarionid genus, indicating that in this genus shell reduction has occurred independently from other helicarionid semislugs (Hyman & Köhler, 2017; Hyman *et al.,* 2017).

CRITERIA OF SPECIES DELINEATION

Species are the fundamental unit used in taxonomy and many other fields of biology, including biogeography, ecology and conservation biology. Yet there is still considerable controversy regarding the definition of what consitutes a species; therefore, many different species concepts exist (Mayden, 1997; Harrison, 1998; Sluys & Hazevoet, 1999; Hausdorf, 2011).

The application of different species concepts and the varying operational criteria used by these concepts to delimit species will, in many cases, result in a different outcome (Isaac, Mallet & Mace, 2004; Sites & Marshall, 2004). For example, the application of the phylogenetic species concept has resulted in higher species numbers than the use of the biological species concept (Agapow *et al.,* 2004; Isaac *et al.,* 2004; Hausdorf, 2011). This poses a real challenge for taxonomists working at the species level.

Given that in the present case we are dealing with sexually reproducing organisms, we consider the biological species concept of Mayr (1942) as an appropriate concept and therefore define species as groups of potentially inbreeding populations that are separated from each other through reproductive incompatibility. Although reproductive isolation in itself is difficult to confirm, we have applied operational criteria that relate to the patterns of phenotypic and genotypic distinctiveness (Sites & Marshall, 2004) that arise as the result of a lack of gene flow and ensure that reproductive barriers are maintained in the event of secondary contact between biological species. Accordingly, species are more than morphologically distinguishable entities, and we consider populations to represent distinct species only when differences in their morphology, anatomy and/or mitochondrial make-up are considered indicative of the absence of gene flow between them and/or that reproductive isolation is probable (i.e. when populations exhibit consistent differences in anatomical or morphological characters, particularly in traits relating to reproduction, whereas intermediates are absent and when they also form well-differentiated, monophyletic sequence clusters).

We are aware that our approach is more stringent than that of many contemporary taxonomists and concede that we may not recognize evolutionarily young and/or morphologically cryptic species that share ancestral polymorphisms in morphological or genetic characters. However, in such cases of poorly differentiated species with ambiguous or potentially

permeable species boundaries, reliable species delineation requires a denser geographical sampling, especially through potential contact zones, and rigorous population-level genetic analyses that are suited to estimate the amounts and direction of gene flow between populations. Such detailed investigations are beyond the scope of the present study.

As speciation is an evolutionary process, intermediate stages are predicted to exist where reproductive isolation may not have been achieved despite (putatively) on-going morphological or mitochondrial lineage diversification owing to lack or scarcity of genetic exchange. Therefore, we consider populations that have acquired a certain amount of consistent distinctiveness from other such lineages, but might have retained the potential to interbreed for the lack of marked differences in their genital anatomy, as cases of incipient speciation.

Given that *Helicarion* semislugs offer few external characters to distinguish species, we initially grouped specimens into candidate taxa with respect to the clustering of the mitochondrial trees (i.e. subclades A–K). The phenotypic distinctiveness of these groups was then assessed by the use of basic statistics of morphometric characters and comparative reproductive anatomy. Available taxon names were assigned to the recognized species based on morphological similarity with types and/or topotypes and with reference to the type localities.

SYSTEMATIC SIGNIFICANCE OF MITOCHONDRIAL CHARACTERS

It is well understood today that, for various reasons, the evolutionary history of any gene may differ from the evolutionary history of the studied organism (Moore, 1995). It has been shown, in particular for mitochondrial genes, that phenomena such as ancestral polymorphisms or introgression may cause non-monophyly of species in mitochnodrial DNA (mtDNA)-based phylogenies (e.g. Funk & Omland, 2003).

There are ample examples where mtDNA lineage differentiation and anatomical and morphological variation correspond well to each other; thus, indicating that mtDNA can be a reliable taxonomic indicator (e.g. Köhler & Johnson, 2012; Hyman *et al.*, 2017). However, there are also cases in which morphologically indistinguishable populations have accumulated deep mtDNA divergences that render mtDNA a poor indicator for the delineation of species (e.g. Pinceel, Jordaens & Backeljau, 2005). Such deep intraspecific mtDNA divergences have been attributed to the effects of considerable historical fluctuations in the ranges of species (Pinceel *et al.*, 2005). Sauer & Hausdorf (2012) demonstrated that attempts to delineate species based on distance thresholds for single markers are

inevitably prone to substantial error and suggested using multilocus data instead. Based on these considerations, the mitochondrial data analysed here can provide only a first pass for the identification of candidate species, whereas any formal species delimitation requires validation from comparative morphology.

In a previous study, we found that the ranges of intraspecific and interspecific p-distances among Australian helicarionids overlapped to some degree (Hyman *et al.*, 2017). However, the average p-distances between morphologically distinct species of > 3.6% in *COI* and > 2.9% in 16S were outside the zone of overlap. The observed mitochondrial differentiation between the two principal clades recovered herein (> 7% in *COI* and > 5% in 16S; Table 2) is therefore well within the range of interspecific variation as documented for several closely related helicarionid genera from southeastern Australia. Even the genetic differentiation of subclades in the *Helicarion* mtDNA tree (A–K) was largely within this range (mean group-wise distances from 3 to 8.0% in *COI* and from 0.5 to 5.4% in 16S; Table 2). That said, these distances were at the lower end of the rather wide spectrum of interspecific differences observed across a variety of stylommatophoran land snails (Davison, Blackie & Scothern, 2009).

SYSTEMATIC SIGNIFICANCE OF REPRODUCTIVE CHARACTERS

Penis and epiphallus
With the penis being the primary copulatory organ, significant differences in its anatomy are usually associated with the presence of reproductive isolation (Gómez, 2001). In *Helicarion*, the two major clades (*H. mastersi* and *H. cuvieri* complexes) exhibited coherent differences in the anatomy of the penial wall that were consistent with the presence of reproductive isolation. However, within each of these two groups the differences in the internal anatomy of the penis were subtle and difficult to quantify, lending no support to the delimitation of species beyond this bifurcation between the two main clades. The lack of other penial characters, such as a penial verge or pilasters, rendered the penial anatomy rather uninformative. In contrast, within each of the main clades we observed substantial variation in the lengths of the penis and epiphallus, both in absolute terms and relative to each other. For example, the ratio of penis to epiphallus length varied between 0.40 and 0.89 in the *H. mastersi* complex and between 0.18 and 1.31 in the *H. cuvieri* complex (Table 4). However, this variation is only partly consistent with the pattern of mitochondrial differentiation. An ancestral state reconstruction of the penis-to-epiphallus ratio suggested that several subclades have undergone significant changes in this

ratio; (relatively) shorter penises have evolved at least four times (all of subclade E excluding the most basal offshoot *H. leopardinus*, the Errinundra lineage of subclade F, the Brown Mountain lineage of subclade A, and the Kangaroo Valley lineage of clade J). A significantly longer penis has evolved once, in subclade G (Fig. 5).

We consider it possible that these changes in the relative (and absolute) lengths of the penis and epiphallus are correlated with the evolution of reproductive isolation. There are even indications that reinforcement, i.e. natural selection that increases reproductive isolation, may drive the differentiation of this morphometric ratio. When considering the occurrences of groups with particularly divergent ratios, it is apparent that all of them occur in regions of potential contact between members of at least two subclades. One of these areas of potential contact is the western part of the Sydney Basin (Blue Mountains, Royal NP) where the Kangaroo Valley lineage of subclade J, the Blue Mountains lineage of subclade E, and subclades G and I occur in proximity to each other. According to our ancestral state reconstruction (Fig. 5), subclade I has maintained the ancestral penis-to-epiphallus ratio. In contrast, the Kangaroo Valley lineage of subclade J and the Blue Mountains lineage of subclade E have evolved short and even shorter penises, respectively, whereas subclade G has evolved a very long penis. Moreover, members of subclade E consistently have a short penis even where they do not occur in proximity to members of another subclade (in Victoria), but when in proximity to other subclades, their penis becomes even shorter. Correspondingly, the short penis of the Brown Mountains lineage of subclade A belongs to an isolated, high-altitude outlier population of this clade surrounded by members of subclade F. The final short penis lineage (subclade F) belongs to a group found in Errinundra NP and Fairy Dell NR. The geographical range of this subclade is unclear, being based on only two rather distant samples, but this lineage with a very short penis is likely to be in close proximity to members of subclade E (short penis) and subclade C (moderately long penis).

If indeed the dramatic differences in penis length could be explained by reinforcement, this would provide convincing support to the existence of reproductively isolated species in the above-mentioned cases at least (i.e. each of the subclades A, E, G, I and J might represent distinct species). However, our data are not sufficiently detailed to demonstrate exactly that this is the case. Alternatively, the penis-to-epiphallus ratio might simply be more variable within species than currently thought, but poorly sampled, and was in this case potentially a poor indicator for reproductive isolation. Consequently, it remains to be scrutinized in more detail whether the penis-to-epiphallus ratio and the absolute penis length are indeed suitable criteria for the delimitation of species.

Spermatophore
Within the hermaphroditic Helicarionidae, reproduction takes place via the internal transfer of a sperm package (spermatophore). The spermatophore is formed inside the epiphallus and the accessory epiphallic flagellum immediately after the initiation of courtship behaviour (Baminger & Haase, 2001; Sionek & Kozlowski, 2001). The completed spermatophores of copulating snails are reciprocally transferred to their partners by eversion of the penis. Among pulmonates, the shape and sculpture of the spermatophore is thought to be species specific (Wiktor, 1987; Gómez, 2001). However, although species specificity is well documented for penial anatomy, for example in Australian camaenids (e.g. Solem, 1981), few empirical data are available for the spermatophore. In part, this is attributable to the absence of a hard spermatophore in many groups. Even in taxa where a hard spermatophore exists, intact examples are recovered in dissections only if the animal has mated recently. In addition, although the spermatophore may be species specific, it is probably not important in mate recognition, because spermatophore exchange takes place when mating is at a stage when a withdrawal of one partner would be likely to result in a loss in fitness.

The spermatophore shape can, in theory, be inferred by an examination of the lumen of the epiphallus and epiphallic flagellum, where it is formed; this can be sufficient to establish gross differences in shape but is often not accurate enough on a finer scale. Despite these difficulties in obtaining information, the spermatophore is thought to be an informative character in species discrimination in Helicarionoidea (Winter, 2008; Hyman *et al.*, 2017), Milacidae (Wiktor, 1987) and some Camaenidae (Sutcharit & Panha, 2006).

In *Helicarion*, the spermatophore varied in the number and arrangement of spines and in the branching pattern. However, much of the variation was observed at the population level and may therefore have little taxonomic implication. For example, within *H. cuvieri*, populations from the Otway Ranges, Brown Mountain, Mt Gulaga and Tallaganda NP could all be distinguished based primarily on the shape of their spermatophores. Each of these is an isolated, high-altitude area that formed a monophyletic group in the phylogenetic tree, but was associated with insufficient genetic and morphological divergence for these populations to be considered as separate taxa.

This is in contrast to statements about the species-specific morphology of the spermatophore in other helicarionds, where different spermatophores have been found in sister species (e.g. *Mysticarion hyalinus* and *Mysticarion insuetus*; *Parmavitrina disposita* and

Parmavitrina flavocarinata; Hyman *et al.,* 2017). In general, in these cases the differences were more comprehensive than in *Helicarion*. In *Parmavitrina*, the spermatophores differed in the length and sculpture of the tail-pipe and in the size, arrangement and branching pattern of the spines. In *Mysticarion*, the same pattern of a single spiral of branched spines was apparent in both species, but one species had significantly fewer, simpler spines. In both genera, the differences in the spermatophore were significant enough to be reflected in the shape of the flagellum.

COMPARISON WITH PREVIOUS STUDIES

The primary characters previously used to separate *H. niger* from Victoria and *H. cuvieri* from Tasmania included the presence of a penial papilla in *H. niger* (Kershaw, 1979, 1981). Such a structure has not been observed in any *Helicarion* specimen dissected for the present study. However, in the penial interior, the opening to the epiphallus is surrounded by a series of small, elongate papillae. During dissection, particularly if any damage was sustained to this area, one of these papillae could easily have been mistaken for a small penial verge or papilla. Hence, we consider this reported difference between *H. niger* and *H. cuvieri* to be erroneous.

Another distinguishing character referred to by Kershaw (1981) was the internal penial wall, sculptured with transverse folded lamellate pilasters in *H. cuvieri* and longitudinal lines of papillae in *H. niger*. Our investigations revealed a very similar penial interior in all *Helicarion* taxa, best described as V-shaped rows of folded lamellae. These lamellae were sometimes papillose, and at the proximal end of the penis were sometimes replaced by rows of papillae. There was no clear difference observed between the types of structures found in Victorian and Tasmanian populations, although the Victorian populations tended to have fewer penial lamellae overall.

The spermatophore was described by Kershaw (1981) as having 15 complex branching spines in *H. cuvieri* and eight simpler spines in *H. niger*. In the present study, this difference was not observed; Tasmanian populations exhibited spermatophores with 13–16 branching spines, similar to the 13–15 branching spines seen in most Victorian and NSW populations. Specimens from the Otway Ranges in Victoria had spermatophores of nine to ten spines with less complex branches, similar to those described by Kershaw (1981) for *H. niger*. It is possible that Kershaw based his description of the spermatophore of *H. niger* exclusively on specimens from the Otway Ranges, which were at that time treated as members of *H. niger*.

Kershaw (1981) also referred to the predominance of spiral sculpture on the protoconch of *H. niger* as a distinguishing feature. Such sculpture has been observed in all members of *Helicarion*. Characters relating to the radula were also used as discriminating features, but could not be tested here because the radula was not examined in the present study. Finally, Kershaw stated that the ureter and rectum were shorter relative to the kidney in *H. niger* than in *H. cuvieri*. This character is hard to illustrate and to quantify, because the pallial wall to which these organs are attached is curved and does not lie flat. For this reason, it was not extensively documented in all specimens examined. A direct comparison of specimens from around the type localities of *H. niger* and *H. cuvieri* did reveal a small difference.

In summary, our study failed to confirm any of the previously reported anatomical and morphological differences between *H. cuvieri* and *H. niger*.

THE REVISED CLASSIFICATION

Based on the evidence presented herein, the taxonomic arrangement of *Helicarion* species as previously suggested is completely redefined. Under the revised species delimitations, we recognize only two well-differentiated species, *H. cuvieri* and *H. mastersi*. The range of *H. cuvieri* is greatly expanded and now includes Tasmania, Victoria and most of southern NSW. Conversely, *H. mastersi* has a much more restricted range, from north of Nowra to southern Sydney in NSW.

Both species clearly differ from each other in terms of their comparative anatomy and mitochondrial phylogenetics, but also exhibit significant lineage differentiation in all examined characteristics. To capture this lineage differentiation taxonomically is challenging, however. Firstly, we considered that each of the subclades A–K represented a separate species. However, although the genetic differences between these subclades are similar to those seen in other helicarionid species (> 6.5% in *COI* and > 3.5% in 16S), there are only slight morphological differences. In the absence of any recorded sympatry and with no information regarding contact zones, we cannot be certain that the groups are reproductively isolated. We cannot demonstrate that the observed variation is significantly larger than variation that one can expect when comparing relatively small samples from randomly chosen sites across the range of a widely distributed species. This holds true particularly when considering that some variation might be correlated with locally different ecological variables (i.e. ecophenotypism). The strongest argument in favour of recognizing subclades as reproductively isolated species comes from

the divergent penis-to-epiphallus ratio in populations within potential contact zones between different subclades. However, our sampling through such contact zones is too sparse to produce the compelling evidence required to ascertain the presence of reproductive isolation.

Alternatively, subclades A–K might represent situations of incipient speciation, which are best recognized as subspecies, between which reproductive isolation might be incomplete. However, the observed disjunctions in the distributions of some widespread subclades (e.g. in A, E, F and I) is in conflict with the standard concept of subspecies as geographical races between which gene flow may still occur.

Consequently, we redefine *H. cuvieri* and *H. mastersi* in the taxonomic section below, but refrain from formally describing any further taxa. We believe that the variation observed is indicative of incipient speciation, but that our present data set is insufficient to determine how this should be translated into an objective taxonomy. We therefore provide detailed descriptions and illustrations of the morphology of subclades A–K. We recommend broader sampling to fill in gaps in distribution, with a focus on contact zones, and the analysis of multiple nuclear markers to estimate lineage differentiation and possible gene flow. In addition, a denser sampling throughout the ranges of subclades is necessary to investigate whether variation in morphospace is correlated with environmental gradients, thus lending support to the presence of ecophenotypism.

TAXONOMY

FAMILY HELICARIONIDAE BOURGUIGNAT, 1877

Diagnosis: Shell present, complete or reduced, 5–35 mm in diameter; usually thin walled, glossy; spiral grooves present on protoconch and teleoconch. Mantle with accessory lobes lying over body and shell lappets of variable size lying over shell. Sole of foot tripartite; caudal apparatus present, formed from upcurled sole. Kidney unilobed; minor venation on roof of mantle cavity absent or present; mantle gland absent. Genital system oviparous; oviduct glandular. Bursa copulatrix variable in length; inserted on vagina or, if vagina absent, at junction of free oviduct and penis. Stimulator absent. Epiphallus enters penis through a simple pore, fleshy lips or verge; interior of penis variable. Penial tunica present, open at proximal end, attached by muscle fibres to epiphallus. Epiphallic retractor caecum absent or present; epiphallic flagellum absent or present; where present, flagellum contains an axial filament. Spermatophore a soft capsule with hard-walled tail-pipe.

Remarks: The Helicarionidae is distributed in Australia, islands of the Pacific, Southeast Asia, Madagascar and the Mascarene Islands. It is grouped in superfamily Helicarionoidea, along with the Asian Ariophantidae and the African Urocyclidae. The three families are unified by the presence of a flagellum with an axial filament, an epiphallic caecum, and mantle lobes (Hausdorf, 1998; Hyman & Ponder, 2010); however, the flagellum and epiphallic caecum are absent in some members of all three groups. The only character that reliably distinguishes Helicarionidae from the other two families is the presence of a penial tunica that is open at the proximal end rather than being fused to the penis (Hausdorf, 1998; Hyman & Ponder, 2010).

GENUS *HELICARION* FÉRUSSAC, 1821

Helicarion Férussac, 1821: 19, 67 [quarto edition], 23, 71 [folio edition]. Type species *Helix cuvieri* Férussac, 1821 (by subsequent designation in Gray, 1847: 169); masculine.

Helicarium Agassiz, 1842–1847: 174 (unnecessary replacement for *Helicarion* Férussac, 1821).

Differential diagnosis

External morphology: Small to medium-sized shell, ear shaped, flattened, thin, golden, sometimes with a greenish tinge, glossy, 3.2–3.9 whorls, whorls rounded, base membraneous. Protoconch with fine spiral grooves, teleoconch with very fine spiral grooves. Body colour varying from black to pale grey, fawn or cream; sole often a contrasting colour to body. Mantle lobes and shell lappets of moderate size, none fused; mucous network prominent; caudal horn well developed.

Genital anatomy: Ovotestis of two to four lobes, embedded in digestive gland. Talon and carrefour embedded in albumen gland. Spermoviduct curves in a U shape towards tail then folds behind and descends towards head. Free oviduct with indistinct capsular gland in proximal portion; internal walls of capsular gland smooth; remainder of free oviduct with longitudinal pilasters. Bursa copulatrix moderately long, approximately half spermoviduct length; sac portion often tear shaped with elongated tip; inserted on free oviduct. Vagina short. Penis long, slender, with internal sculpture of V-shaped rows of papillose lamellae. Penis tunica attached by muscle fibres to middle of epiphallus; epiphallus enters penis through simple pore; epiphallic caecum absent; epiphallic flagellum with axial filament present, containing spiralling rows of internal cryptae. Spermatophore a soft-walled capsule with hard tail-pipe; branching spines present in spiralling pattern along tail-pipe.

Remarks

Helicarion (masculine) is the conserved spelling for this genus name (ICZN, 1992), and *Helix cuvieri* is its designated type species (Gray, 1847). The original spelling 'Helixarion' by Férussac (1821–1822, p. 19 [quarto edition], 23 [folio edition]) was an inadvertent error for the correct spelling *Helicarion* (1821–1822: p. 67 [quarto], 71 [folio]) and has been supressed subsequently (ICZN, 1992). We follow Hyman & Ponder (2010) in excluding *Desidarion* Iredale, 1941, *Luinarion* Iredale, 1933, *Platycloster* Hasselt, 1824 and *Laconia* Gray, 1855 from synonymy with *Helicarion*.

   Helicarion is found throughout Tasmania, in parts of Victoria and in southeastern NSW (as far north as the Central Coast) (Fig. 6). No other helicarionid semislugs are found in the southern part of its range (with the exception of *Attenborougharion* in southern Tasmania). From Narooma northwards, *Helicarion* shares its range with the semislug *Mysticarion porrectus*, which is very similar to pale specimens of *Helicarion*. However, *M. porrectus* is generally arboreal and has a more globose shell than *Helicarion*. Furthermore, members of *Helicarion* generally have darker coloration on their tail and a contrasting pale sole, whereas *M. porrectus* is uniformly pale. Further north in NSW, semislugs belonging to *Parmavitrina* can be distinguished by their larger size.

HELICARION CUVIERI FÉRUSSAC, 1821
(FIGS 6–18)

Helixarion cuvieri Férussac, 1821: pl. 9, fig. 8 (published in January).

   Helicarion cuvieri Férussac, 1821: 20 (published in April). Pfeiffer & Clessin, 1881: 32; Semper, 1870: 31, pl. 3, fig. 7, pl. 6, fig. 11; Tryon, 1885: 168, pl. 38, figs 36–38; Iredale, 1937: 7; Baker, 1941: 263; Kershaw, 1979: 146; Smith, 1992: 233; Hyman & Ponder, 2010: 24, figs 5A, 7A, B, 8A, B, 9A, B, 10A, 11A, 12A, 13A, 14A, B; Stanisic, Shea, Potter & Griffiths, 2010: 206–207, 209.

   Vitrina nigra Quoy & Gaimard, 1832: 135–136, pl. 11, figs 8–9; Cox, 1868: 84, no. 204; Petterd & Hedley, 1909: 310; Cox & Hedley, 1912: 14; Gabriel, 1930: 85.

   Vitrina cuvieri – Reeve, 1862: pl. 3, sp. 15; Pfeiffer, 1876: 24.
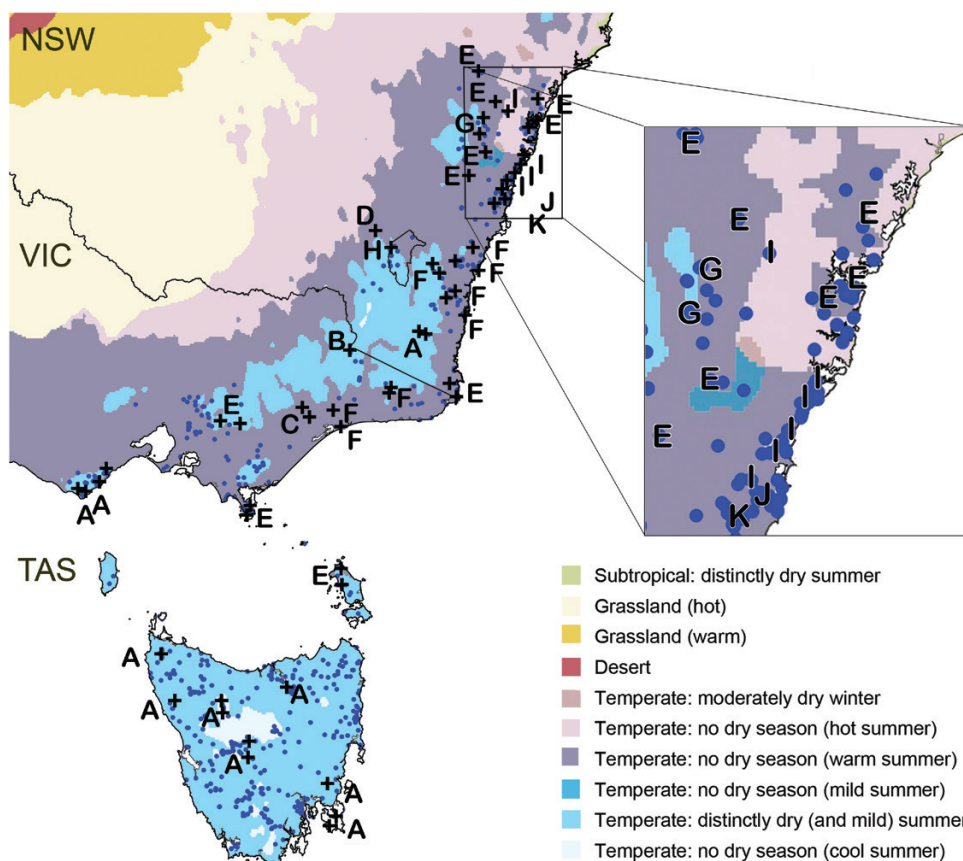
Figure 6. Occurrence records of *Helicarion* from the malacological collections of the AM, QM, NMV, TMAG and QVMAG. Symbols: +, specimens examined in the present study. Inset map shows details of specimens found in the Sydney region. Letters A–K refer to the subclades of the phylogenetic analysis. Subclades A–H, *Helicarion cuvieri*; subclades I–K, *Helicarion mastersi*.
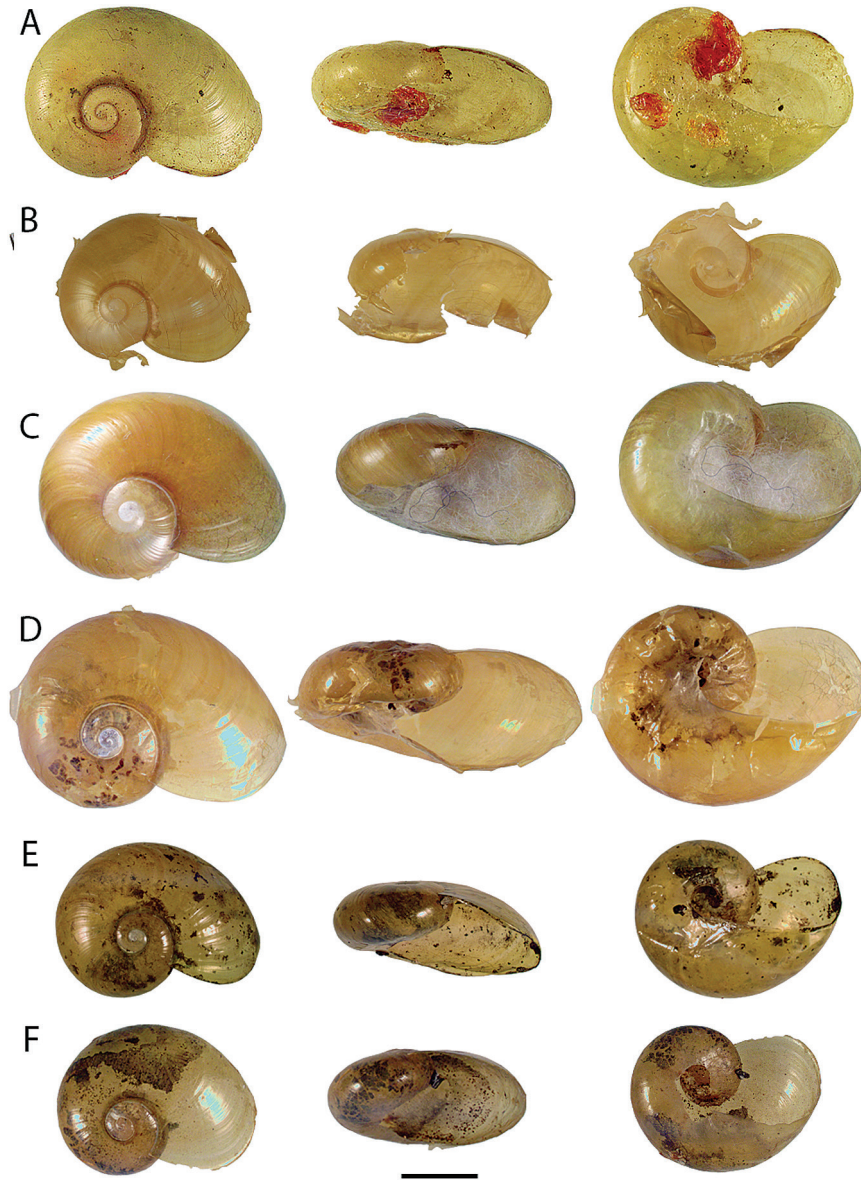
Figure 7. *Helicarion cuvieri* shells. A, clade A, AM C.443929, Mt Wellington, Tas (photograph: H. Barlow). B, clade C, MV590, Mitchell River NP, Vic. C, clade E (probable holotype of *Helicarion leopardinus*), AM C.101141, Ourimbah, NSW (photographer: H. Barlow). D, clade F, AM C.559126, Monga NP, NSW. E, clade G, AM C.500941, Blue Mountains NP, NSW. F, clade H, AM C.332848, Brindabella Range, ACT. Scale bar: 4 mm.

Nanina (*Helicarion*) *cuvieri* –Martens & Albers, 1860: 47.

Vitrina verrauxii Pfeiffer, 1850: 132; Martens & Albers, 1860: 44; Reeve, 1862: pl. 4, sp. 21; Cox, 1868: 83, pl. 14, figs 14, 14a; Pfeiffer, 1876: 24; Petterd, 1879: 49; Hedley, 1891: 24, pl. 2, fig. 10, 12, pl. 3, fig. 4.

Helicarion verrauxii – Pfeiffer & Clessin, 1881: 32; Tryon, 1885: 169, pl. 38, fig. 40.

Helix (*Paryphanta?*) *vitrinaformis* Legrand, 1871 [without page numbers].

Helix buttoni Petterd, 1879: 55 [unnecessary replacement name for *Helix* (*Paryphanta?*) *vitrinaformis* Legrand, 1871].

Helicarion niger – Iredale, 1937: 7; Kershaw, 1981: 19–24, figs 1–15; Stanisic, Shea, Potter & Griffiths, 2010: 206–207.

Helicarion mastersi callidus Iredale, 1941: 6; Smith, 1992: 234.

Helicarion leopardina Iredale, 1941: 6 [sic!]; Smith, 1992: 233–234; Stanisic *et al.,* 2010: 302–303.
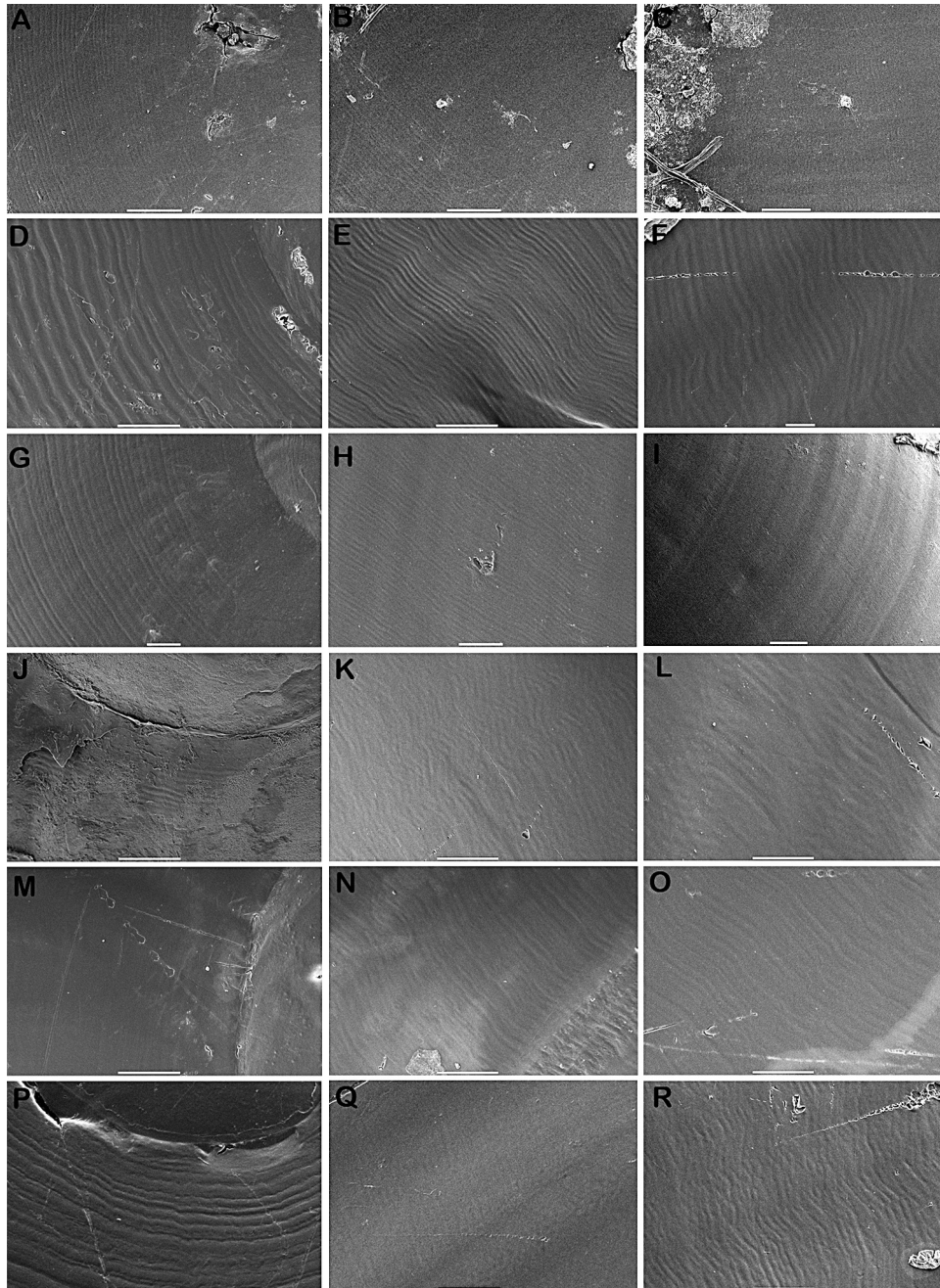
Figure 8. Microsculpture of *Helicarion cuvieri* shells: protoconch (first column), early teleoconch (second column) and late teleoconch (third column). A–C, clade A, AM C.443935. D–F, clade C, MV590. G–I, clade E, AM C.443902. J–L, clade F, AM C.559126. M–O, clade G, AM C.500941. P–R, clade H, AM C.332848. Scale bars: 100 µm (A–C, J), 50 µm (D, E, G, H, K–R), 20 µm (F), 200 µm (I).

Helicarion nigra – Smith, 1992: 234.

Material examined
Types: Neotype of *H. cuvieri*: MNHP no number (Needles picnic ground, S of Strathgordon Road, SW TAS) (Kershaw, 1979).

Lectotype of *Vitrina nigra*: MNHP no number (Western Port, Vic) (Kershaw, 1981).

Holotype of *H. leopardinus*: AM C.101141 (Ourimbah Scrubs, NSW).

Holotype of *H. mastersi callidus*: AM C101138 (Twofold Bay, NSW).
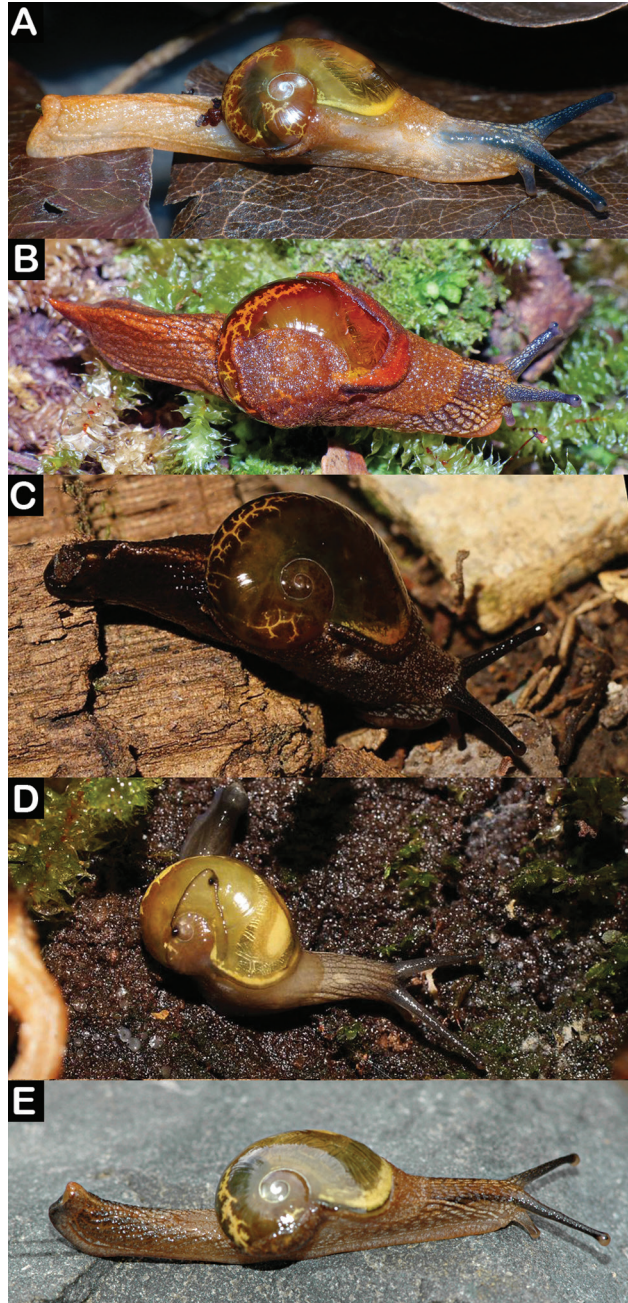
Figure 9. Live specimens of *Helicarion cuvieri*, clade A (not to scale). A, pale form, Central Plateau (Tas). B, orange form, Tarkine (Tas). C, dark form, Holwell (Tas). D, Otway Range NP, Victoria. E, Brown Mountain, NSW, AM C.483714. Photographs by Adnan Moussalli (A, B), Reiner Richter (C, D).

Non-type material: See Supporting Information (Table S1).

Description
External morphology: Shell greenish gold to orange-brown, 2.7–3.5 whorls. Body colour variable, ranging from cream to black, often speckled with orange, cream or brown. Shell lappets sometimes dark bordered, sometimes with two to three pigmented warts. Tail with a moderately strong keel, usually paler than body colour.

Genital anatomy: Penis generally slender, tubular, of variable length, occasionally slightly swollen
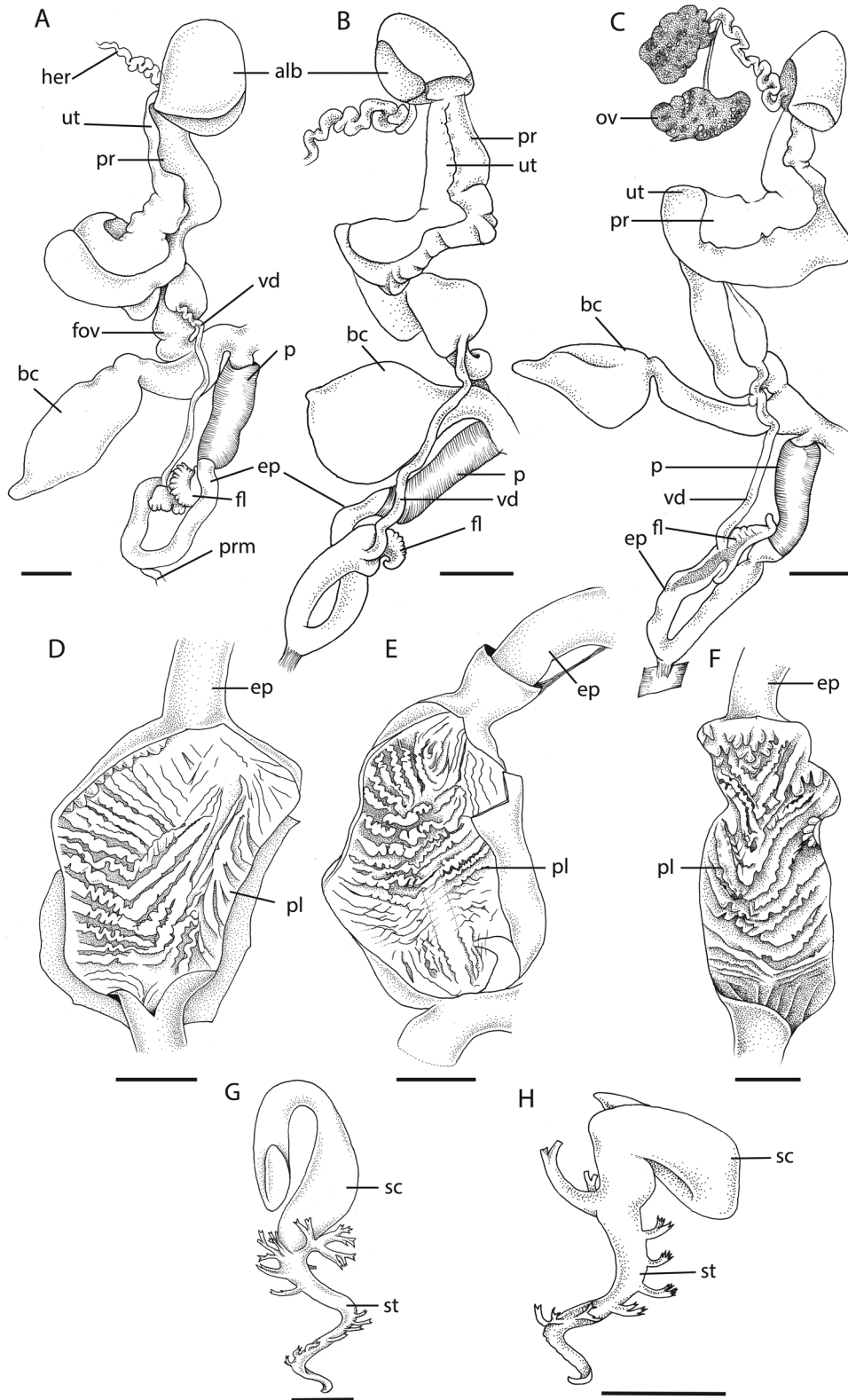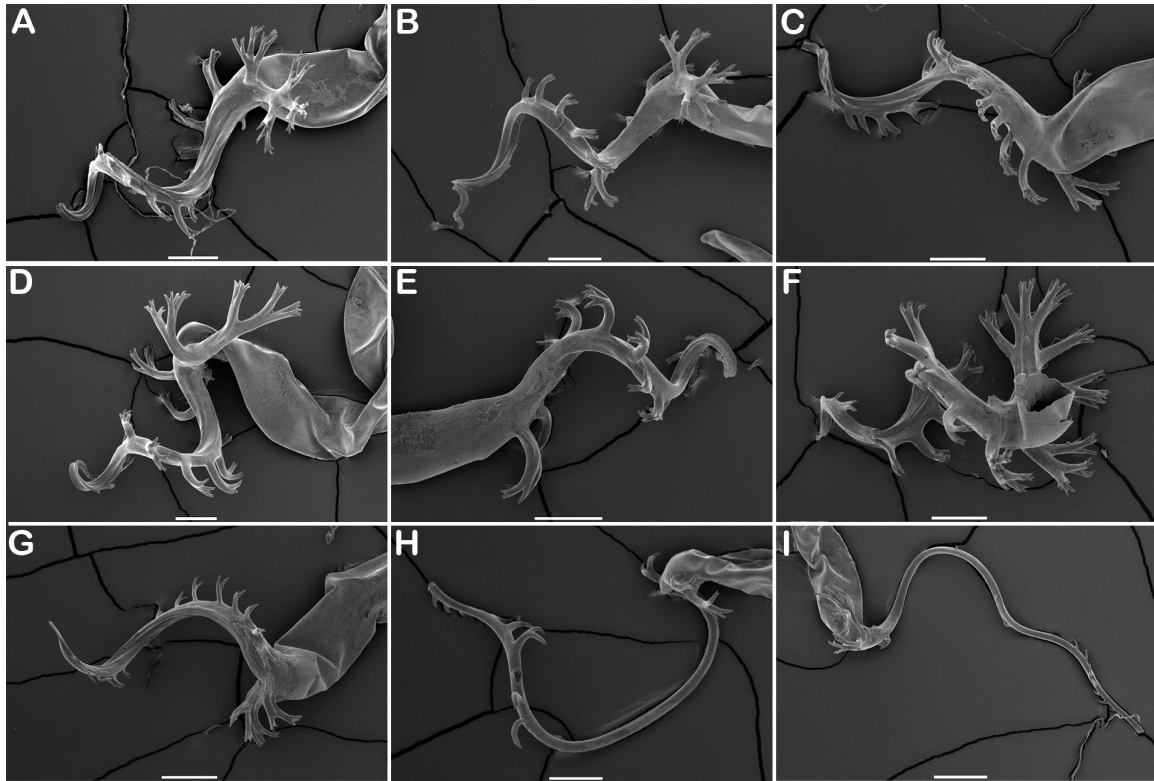
Figure 11. Spermatophores of *Helicarion cuvieri*. A–C, clade A. A, TMAG20673. B, TMAG30618. C, Brown Mountain, MO39992. D–F, clade E. D, Wilson's Promontory, AM C.166903. E, Blue Mountains, AM C.446482. F, Nadgee Nature Reserve, AM C.162824. G–I, clade F. G, Monga NP, AM C.559126. H, Mt Gulaga, AM C.358244. I, Tallaganda SF, AM C.358066. Scale bars: 500 µm.

proximally. Penial interior sculptured with nine to 50 rows of papillose ridges arranged in a deep V shape. Penis-to-epiphallus ratio variable, 0.18–1.31; epiphallus entering penis apically or laterally. Flagellum with internal cryptae. Spermatophore variable, with nine to 20 spines.

Remarks
Helicarion cuvieri has previously been recorded only from Tasmania, where it is widespread (Fig. 6). Here we expand the delineation of this species to include populations from eastern Victoria and southeastern NSW. This species and its congener, *H. mastersi*, are both highly variable in body size and colour, making it difficult to distinguish them reliably. *Helicarion cuvieri* generally has a uniform body and sole colour in darker shades ranging from grey to orange-brown and black, with dark eye tentacles. In specimens with a contrasting pale sole (clades E, F and G), the tail tip often has a dark vertical stripe. Three pigmented warts are often (but not always) present on the shell lappets, and the tail has a longer keel. *Helicarion mastersi* has no pigmented warts, generally paler coloration, with pale eye tentacles in shades of cream, pink, grey and brown, deepening on the tail, and has a pale sole contrasting with its body colour. The two species can be distinguished morphologically by the spacing of the internal penial lamellae; *H. cuvieri* has fewer, coarser lamellae arranged in a deep V shape, whereas *H. mastersi* has more numerous, finer lamellae arranged in a shallower V shape.

Within *H. cuvieri* there are eight morphologically distinct subclades (clades A–H), which are morphologically distinct; these are described below.

Figure 10. Reproductive anatomy of *Helicarion cuvieri* (clade A). A–C, genitalia. A, Buckland Area, TAS (TMAG E20673). B, Brown Mountain, NSW (AM C.483714). C, Otway Ranges, NMV F100667. D–F, penial interior. D, Buckland Area, TAS (TMAG E20673). E, Brown Mountain, NSW (AM C.483714). F, Otway Ranges, NMV F100667. G, H, spermatophore. G, Buckland Area, TAS (TMAG E20673). H, Otway Ranges, NMV F100667. Scale bars: 2 mm (A–C), 1 mm (D–H). In this and subsequent figures, for abbreviations see Material and Methods.
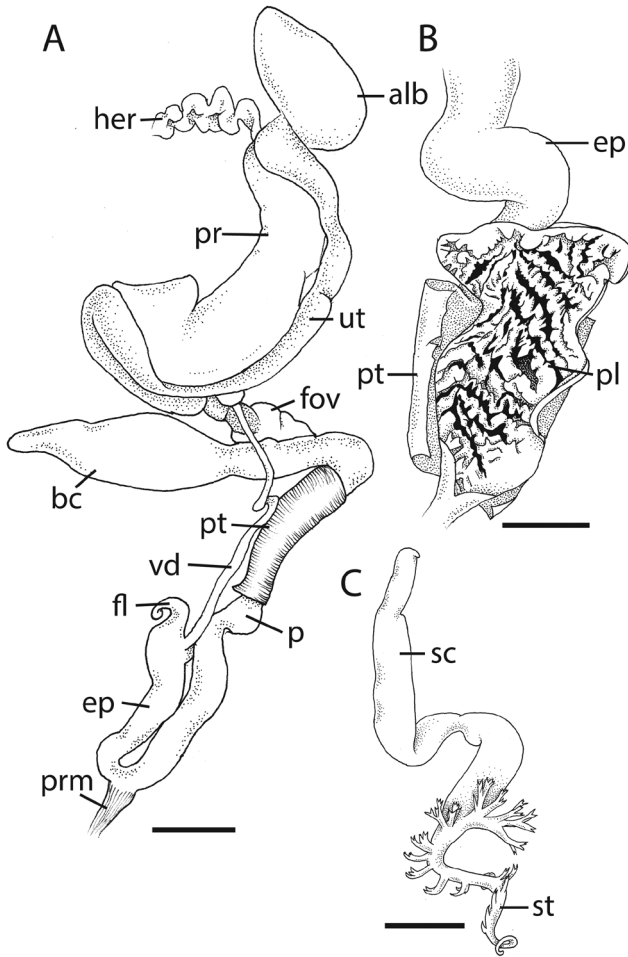
Figure 12. Reproductive anatomy of *Helicarion cuvieri*, clade B, F205184. A, genitalia. B, penial interior. C, spermatophore. Scale bars: 1 mm.

CLADE A
(FIGS 6, 7A, 8A–C, 9, 10, 11A–C)

Diagnosis
External morphology: Shell golden amber, 2.7–3.5 whorls (Figs 7A, 8A–C). Body cream, orange, grey or dark brown, often speckled with orange, cream or brown. Eyestalks blue-grey to black. Shell lappets dark bordered, with three pigmented warts, two on right lappet, one on left lappet, with black pigmentation in grey specimens and orange pigmentation in orange-brown specimens. Tail with a moderately strong keel, usually paler than body colour (Fig. 9).

Genital anatomy: Penis medium length, tubular, occasionally slightly swollen proximally. Penial interior with ~14–16 lamellae. Penis-to-epiphallus ratio of 0.41–0.84; epiphallus enters penis apically or slightly laterally. Spermatophore variable; Otway Ranges specimens with nine spines, formula (0, 7, 2, 0); Tasmanian

specimens with 13–16 spines, formula (2, 2, 9, 3); Brown Mountain specimens with 20 spines, formula (4, 11, 1, 4) (Figs 10, 11A–C).

Remarks
Clade A comprises all Tasmanian specimens along with one population from the Otway Ranges in Victoria and another population from Brown Mountain in NSW. There is a wide range of colour variation seen in clade A, including the only known orange forms of *Helicarion*. The two geographically isolated populations differ anatomically from the rest of the clade. Semislugs from the Otway Ranges have a much simpler spermatophore, with nine simple spines, in contrast to the ten to 16 more complex spines seen in Tasmanian specimens. Likewise, specimens from Brown Mountain in NSW have a much more complex spermatophore than Tasmanian specimens, with 20 spines in total, including a higher number of branching spines; this population also has a slightly longer penis. These differences probably reflect the isolation of these geographical areas. There may also be a connection with altitude, because both regions have a relatively high elevation.

Members of this clade are microsympatric with *Attenborougharion rubicundus* in southeastern Tasmania but can be distinguished by their smaller size and generally uniform cream to dark brown coloration, in contrast to the two-toned burgundy and green coloration of *A. rubicundus*.

CLADE B
(FIG. 12)

Diagnosis
External morphology: Shell golden amber, 3.1 whorls. Body and sole a uniform dark grey (in alcohol). Shell lappets darker, black bordered, with three pigmented warts, two on right lappet, one on left lappet. Tail keeled. Fig. 12

Genital anatomy: Penis moderately long, tubular, slightly swollen proximally. Penial tunica covering approximately four-fifths of penis. Penial interior with ~12 lamellae. Penis-to-epiphallus ratio of 0.53; epiphallus enters penis slightly laterally. Spermatophore with 15 spines, formula (3, 5, 5, 2).

Remarks
Clade B is represented by only a single specimen from Alpine NP in Victoria. This specimen is distinguished by its very dark body colour, the presence of pigmented warts on its shell lappets, and the moderately long penis with very few penial lamellae. Further collecting in the Alpine NP and adjacent high-altitude parts of Victoria and southern NSW is necessary to understand this clade better.

Figure 13. Live specimens of *Helicarion cuvieri*, clades C and E. A, clade C, Mitchell River NP (Vic), MV590 (photographer: Adnan Moussalli). B–D, clade E. B, Nadgee Nature Reserve (NSW), AM C.483711. C, Wollemi NP (NSW), AM C.572213.

CLADE C
(FIGS 6, 7B, 8D–F, 13A, 14)

Diagnosis
External morphology: Shell golden, 3.3 whorls (Figs 7B, 8D–F). Body and sole dark grey-brown (in alcohol); in life with a speckled appearance. Lappets narrow, lacking pigmented warts, dark bordered. Left lappet with dark streaks on inside. Mid field of sole slightly paler (Fig. 13A).

Genital anatomy: Penis long, slender, proximally swollen, swollen portion folded over. Penial tunica covering approximately three-quarters of penis. Penial interior with ~25 lamellae. Penis-to-epiphallus ratio of 0.74; epiphallus also folded. Spermatophore of 12 spines, formula (1, 4, 6, 1) (Fig. 14).

Remarks
Clade C is known only from Mitchell River NP (Fig. 6). No material was available to measure; however, based on the limited material available members of this clade appear to be relatively small, similar in size to members of clade G from the Blue Mountains. Clade C semislugs can be distinguished from other clades of *H. cuvieri* by their longer penis and epiphallus, both of which are folded.

CLADE D

See clade H below.

CLADE E
(FIGS 6, 7C, 8G–I, 11D–F, 13B, C, 15)

Diagnosis
External morphology: Shell amber to orange-brown, 2.8–3.4 whorls (Fig. 7C, 8G–I). Body grey or pale brown to black, colour deepening on tail and neck, with a vertical dark stripe at tail tip; sole generally paler than body. Shell lappets with three pigmented warts in Victorian populations (Fig. 13B, C).

Genital anatomy: Penial interior of few (nine to 13) lamellae arranged in deep V-shaped rows. Penis size and epiphallus entry variable: penis-to-epiphallus ratio of 0.44–0.50, epiphallus entering penis apically (Victorian populations and Flinders Island)' penis-to-epiphallus ratio of 0.18–0.36, epiphallus entering laterally (Nadgee NR, Burragorang, Mt Kelgoola and Mt Coricudgy); or penis-to-epiphallus ratio of 0.83, epiphallus entering laterally (Wyong, Wombeyan Caves). Flagellum with prominent internal cryptae, corresponding to ten to 13 robust branching spines on spermatophore; spermatophore formula (2–3, 4–10, 0–7, 0–2) (Figs 11D–F, 15).

Remarks
Clade E includes material from Victoria, including the type locality of *H. niger*. However, *H. niger* as previously understood contained all Victorian populations and Flinders Island (Tas). In contrast, clade E does not
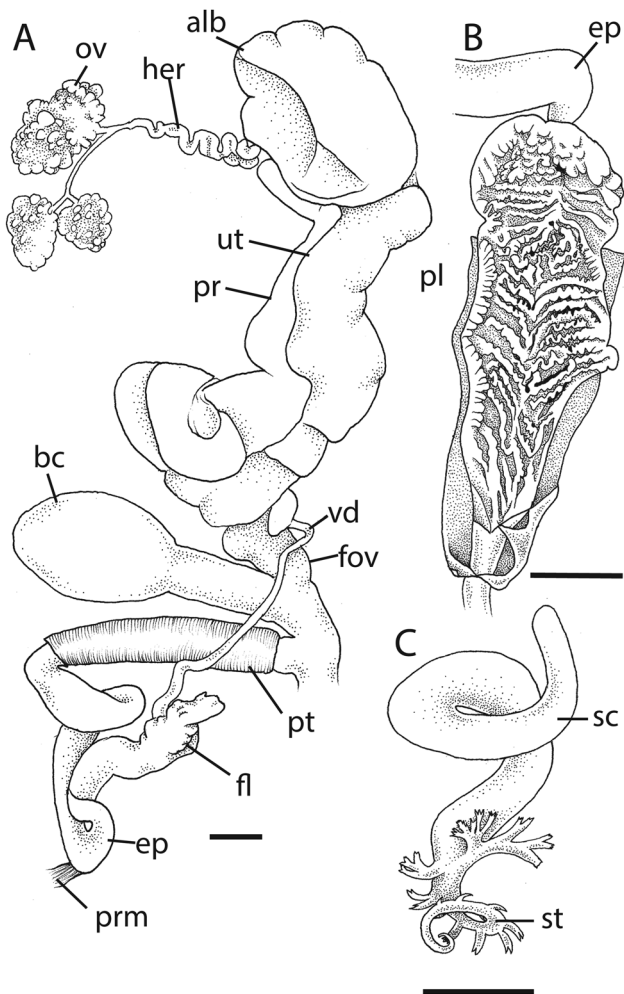
Figure 14. Reproductive anatomy of *Helicarion cuvieri*, clade C, MV590. A, genitalia. B, penial interior. C, spermatophore. Scale bars: 1 mm.

contain all Victorian populations; in fact, from Victoria, only populations from Wilsons Promontory (the type locality) and the Central Highlands are included. Several populations from south-eastern NSW are also included (Fig. 6). The resulting distribution, the largest of any *H. cuvieri* subclade, contains several large gaps, notably between Wilson's Promontory in Victoria and Nadgee Nature Reserve in NSW, and between Nadgee Nature Reserve and Wombeyan Caves. It is probable that further collecting will extend the known range of clade E still further and may somewhat reduce the size of the gaps in its distribution.

In addition to the largest distribution, this taxon also exhibits the most anatomical variation seen in any *Helicarion* clade. The Victorian populations (including Flinders Island; equivalent to *H. niger s.s.* and forming a monophyletic group) are significantly larger than the NSW specimens, and also differ in exhibiting

three pigmented warts on their shell lappets and in the presence of a medium-length penis with the epiphallus entering apically. Populations from Nadgee Nature Reserve (the type locality of *H. mastersi callidus*), Burragorang, Mt Kelgoola and Mt Coricudgy all have a very short penis, the shortest observed in *Helicarion* to date, with the epiphallus entering slightly laterally. Finally, specimens from Wyong and Gosford (representing *H. leopardinus*) and from Wombeyan Caves have a relatively long, broad penis with a slightly lateral epiphallus entry point. These differences are significant; however, there is only slight genetic differentiation, and one of the three groups thus delimited (the short penis group) is paraphyletic. Furthermore, there are some strong characters uniting the three groups, including a penial interior with a small number of relatively large internal lamellae, and the presence of a robust spermatophore with 13–16 spines, most highly branched. It is also possible that the penial differences are attributable to reinforcement, because the clades exhibiting a very short penis are all in close proximity to another clade (see Taxonomic Preamble).

Kershaw (1981) described a small penial papilla in *H. niger*; this has not been observed in any specimens in the present study.

CLADE F
(FIGS 6, 7D, 8J–L, 11G–I, 16A–C, 17)

Diagnosis
External morphology: Shell golden amber, 3.0–3.5 whorls (Figs 7D, 8J–L). Body cream to pale grey or brown, colour deepening on tail and neck, with a white to pale pink sole. Shell lappets with one to three pigmented warts (one or two on right lappet, one on left), less visible in pale specimens (Fig. 16A–C).

Genital anatomy: Penis long, slender, penis-to-epiphallus ratio of 0.29–0.87, epiphallus entering laterally or apically. Penial interior of 12–21 lamellae arranged in deep V-shaped rows. Spermatophore of 11–17 branches, with a gap after the second or third branch (Figs 11G–I, 17).

Remarks
Clade F is distributed in southeastern NSW and eastern Victoria. Members of this clade are very similar in external morphology to members of the neighbouring clade E and to pale specimens of clade A. All three taxa are large semislugs with two or three pigmented warts on their shell lappets. Anatomically, semislugs belonging to clade F can be distinguished from members of clade E by their longer penis relative to the epiphallus, with a larger number of penial lamellae.
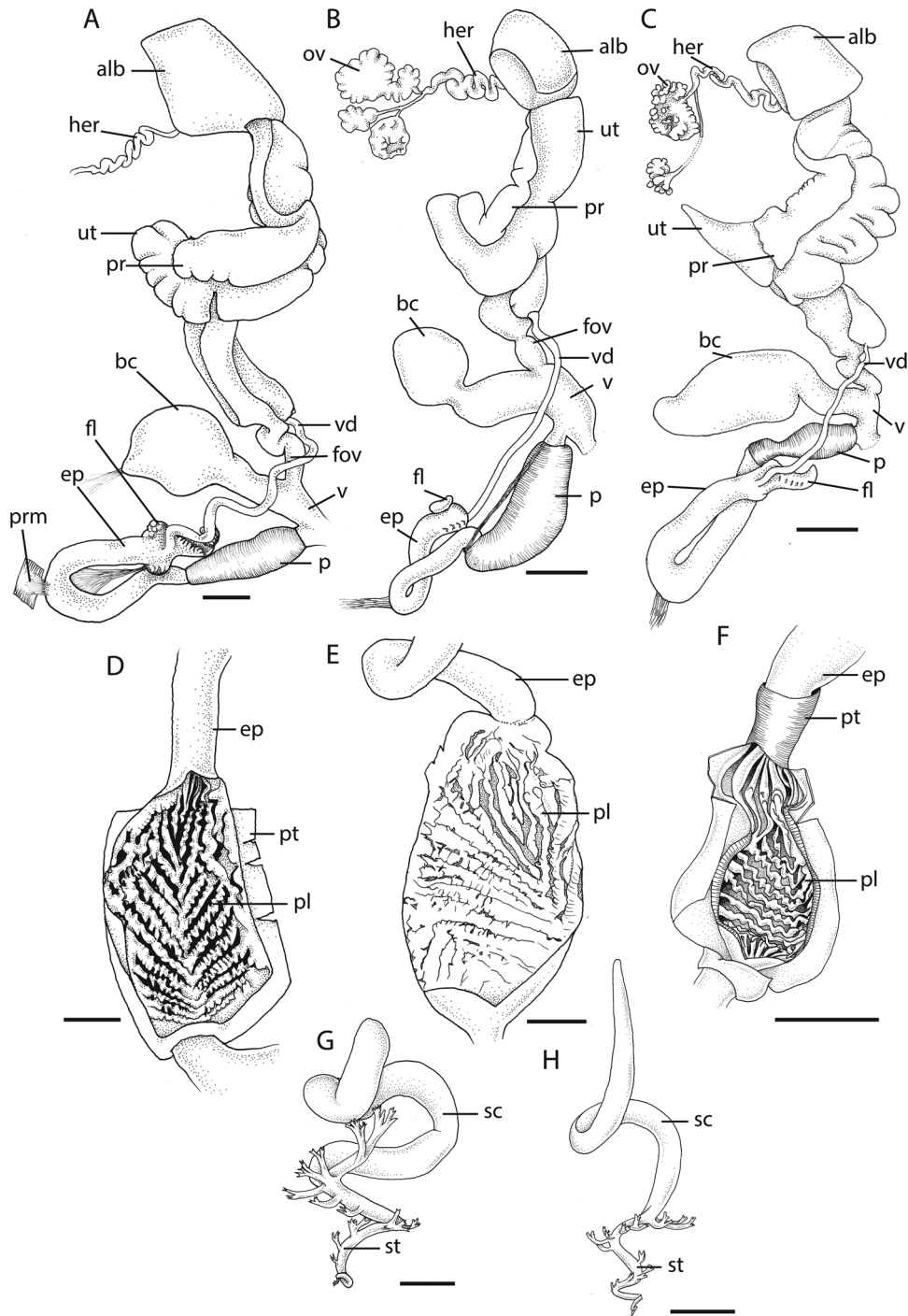
Figure 15. Reproductive anatomy of *Helicarion cuvieri* (clade E). A–C, genitalia. A, Wilson's Promontory, AM C.199603. B, Berowra Creek, AM C.446481. C, Lake Burragorang, AM C.446482. D–F, penis and penial interior. D, Wilson's Promontory, AM C.199603. E, Berowra Creek, AM C.446481. F, Lake Burragorang, AM C.446482. G, H, spermatophore. G, Wilson's Promontory, AM C.199603. H, Lake Burragorang, AM C.446482. Scale bars: 2 mm (A–C), 1 mm (D–H).

This clade contains four well-supported, morphologically distinct subclades. The major differences lie in the spermatophore and flagellum, particularly in the presence of a large gap in the pattern of spermatophore spines in populations from Mt Gulaga and Tallaganda NP, with some variation also present in
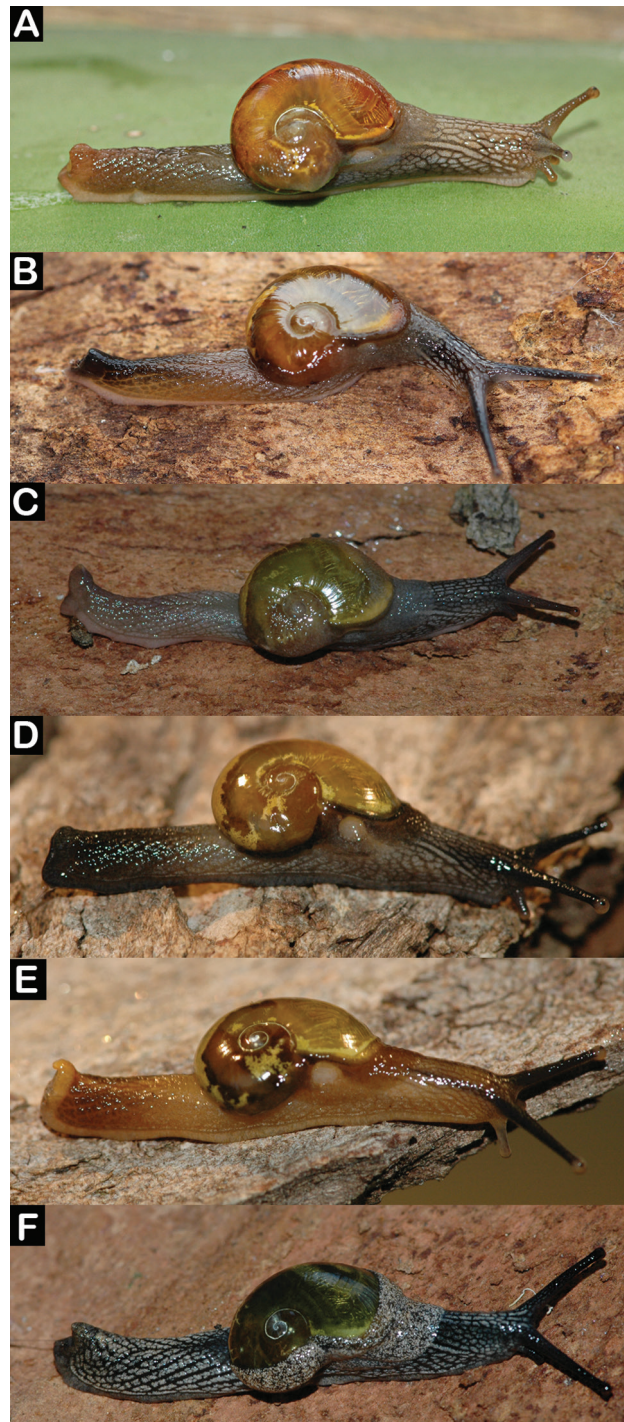
Figure 16. Live specimens of *Helicarion cuvieri*, clades F, G and H. A–C, clade F. A, Clyde Mountain, Monga NP, AM C.483704. B, Mt Gulaga NP, AM C.532843. C, Tallaganda NP, AM C.483744. D, E, clade G. D, Katoomba, AM C.500940. E, Mt Tomah, AM C.559128. F, clade H, Brindabella Ranges, AM C.483737.

the penis shape and length. However, the genetic distances between the subclades are low and indicative of their rather recent evolutionary origin and potentially incomplete lineage differentiation. We are also unsure whether the anatomical differences are truly indicative of reproductive incompatibility.
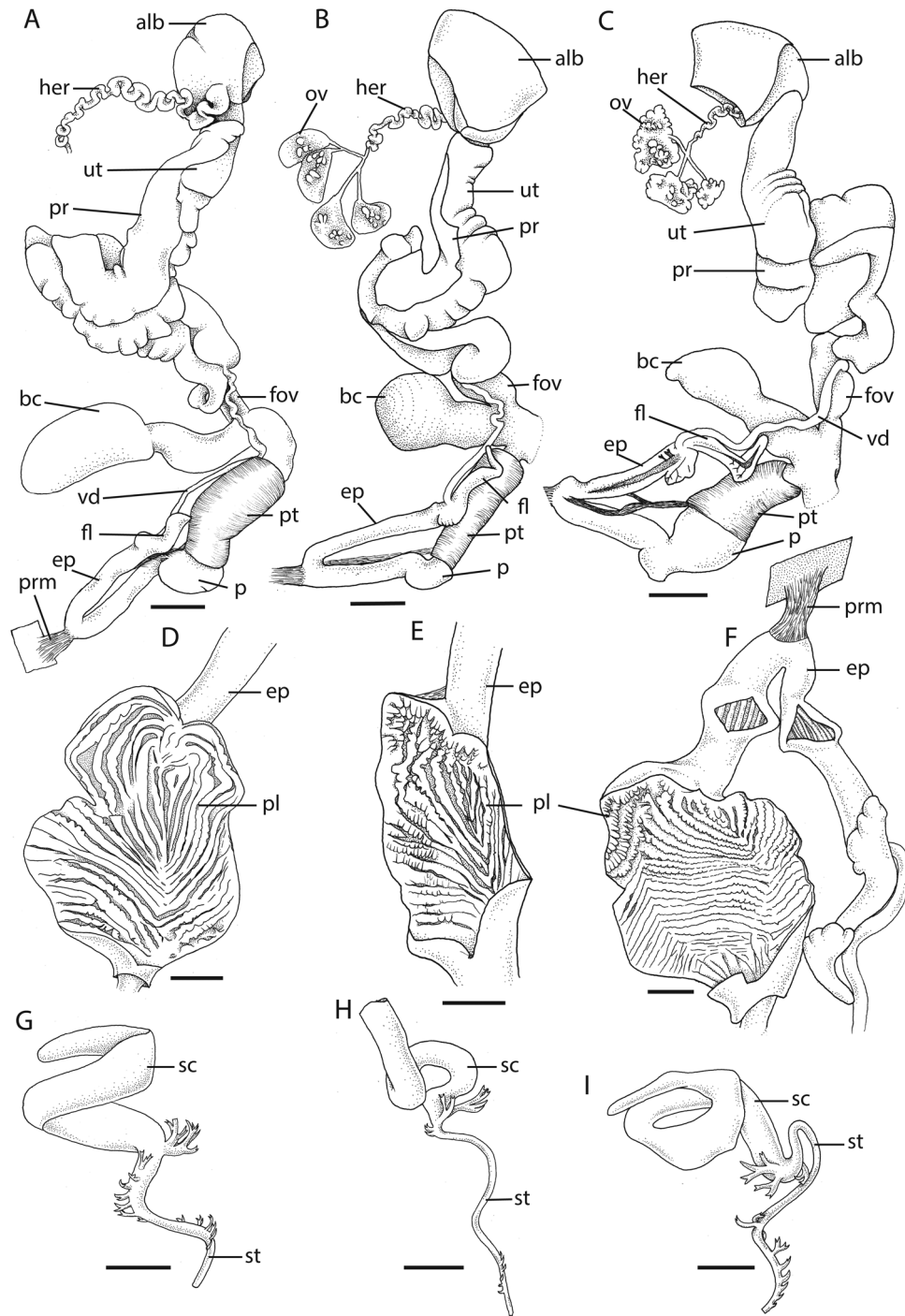
Figure 17. Reproductive anatomy of *Helicarion cuvieri*, clade F. A–C, genitalia. A, Monga NP, AM C.559126 (holotype). B, Tallaganda NP, AM C.358066. C, Mt Gulaga, MO39997. D–F, penis and penial interior. D, Monga NP, AM C.559126. E, Tallaganda NP, AM C.358066. F, Mt Gulaga, MO39997. G–I, spermatophore. G, Monga NP, AM C.559126. H, Tallaganda NP, AM C.358066. I, Mt Gulaga, AM C.358244. Scale bars: 2 mm (A–C), 1 mm (D–I).
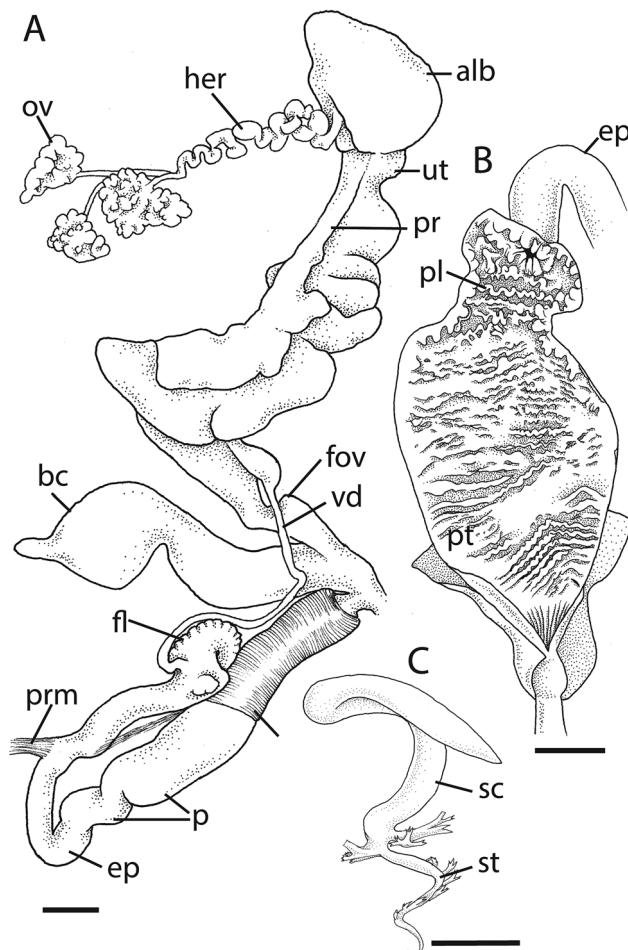
Figure 18. Reproductive anatomy of *Helicarion cuvieri*, clade G. A, genitalia (F219250-1). B, penial interior (F219250-1). C, spermatophore (AM C.559128). Scale bars: 1 mm.

CLADE G
(FIGS 6, 7E, 8M–O, 16D, E, 18)

Diagnosis
External morphology: Shell golden, 3.2–3.6 whorls (Figs 7E, 8M–O). Body cream to dark grey, darker on tail; lappets dark edged, lacking pigmented warts (Fig. 16D, E).

Genital anatomy: Penis very long, slender, swollen medially; proximal end similar in width to epiphallus; penis-to-epiphallus ratio of 1.08–1.31, epiphallus entering penis laterally through simple pore. Epiphallus with an extra fold. Interior of penis with numerous lamellae (~50), slightly deeper and more folded in narrow proximal portion. Bursa copulatrix with a very long duct, duct longer than bursa. Spermatophore with 14 evenly spaced branching spines of decreasing complexity; formula (1, 9, 2, 2) (Fig. 18).

Remarks
This clade is distributed in the Blue Mountains NP, including Mt Wilson, Mt Tomah Botanic Gardens and Katoomba (Fig. 6). Specimens from Jenolan Caves probably also belong to this clade. A separate taxon, clade E, is found further south in Burragorang.

Members of clade G are significantly smaller than members of clade A and *H. mastersi*. These semi-slugs can be distinguished from other subclades of *H. cuvieri* by their extremely long penis, with a distinctive shape and relatively numerous penial lamellae.

CLADE H
(FIGS 6, 7F, 8P–R, 16F, 19)

Diagnosis
External morphology: Shell greenish gold, 3.0–3.4 whorls (Figs 7F, 8P–R). Body grey, with deeper
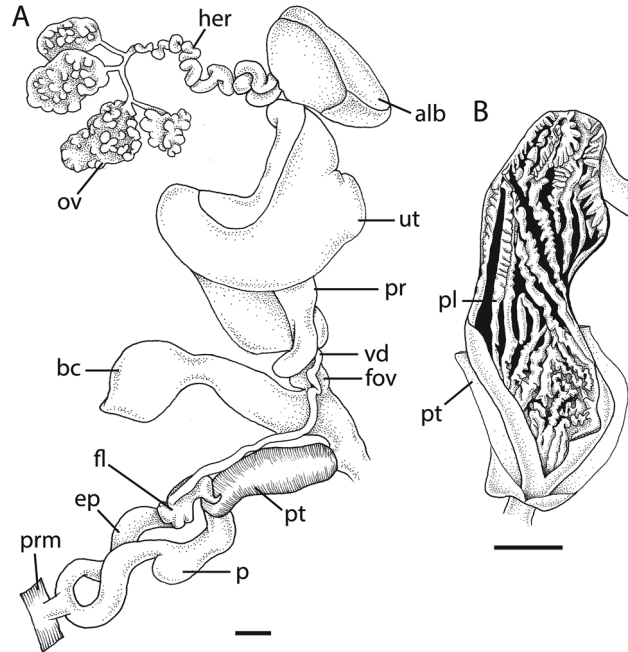
Figure 19. Reproductive anatomy of *Helicarion cuvieri*, clade H, AM C.559127. A, genitalia. B, penial interior. Scale bars: 1 mm.

Figure 20. Shells of *Helicarion mastersi*. A, holotype of *H. mastersi*, AM C.101139 (clade K). B, clade J, AM C.483702. C, clade I, AM C.374083. Scale bar: 4 mm.

grey-black wrinkles and black eyestalks. Shell lappets and mantle lobes speckled grey, dark bordered, without pigmented warts. Sole a uniform grey (Fig. 16F).

Genital anatomy: Penis long, slender, subdivided into two portions, with distal portion covered by penial tunica. Penial interior with wavy, pustulose lamellae
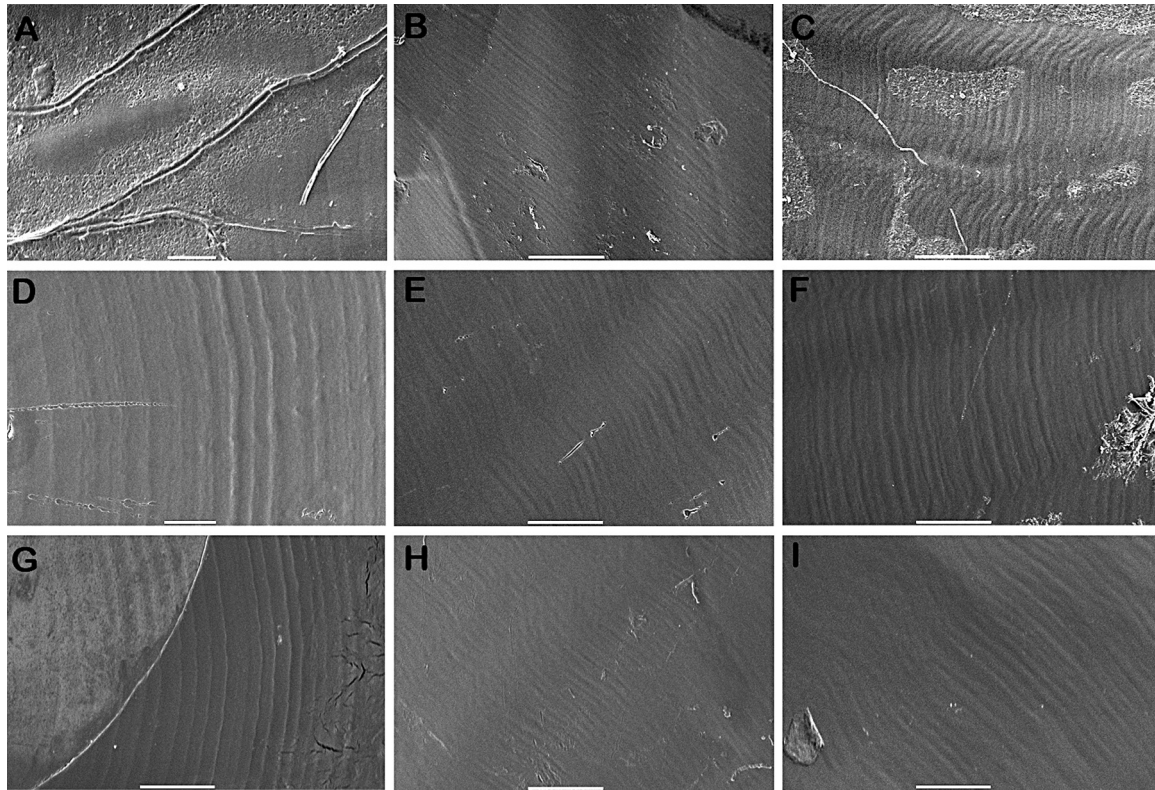
Figure 21. Microsculpture of *Helicarion mastersi* clades: protoconch (first column), early teleoconch (second column) and late teleoconch (third column). A–C, clade K, AM C.332815. D–F, clade J, AM C.483702. G–I, clade I, AM C.374083. Scale bars: 100 μm (A–C), 50 μm (D–I).

arranged in a V shape in distal portion; proximal portion with deep, distinct, less pustulose lamellae arranged in much deeper V shape (close to longitudinal). Total number of lamellae ~14–16. Penis-to-epiphallus ratio of 0.79; two arms of epiphallus twisted around one another. Flagellum with internal cryptae. Spermatophore of 19 spines, formula not recorded, most spines with multiple branches (Fig. 19).

Remarks

Clade H is known only from the Brindabella Range (Fig. 6). *Helicarion* specimens have also been collected at other sites in and around the ACT, including Tidbinbilla Nature Reserve, Namadgi National Park and Tantangara Mountain, but the identity of these specimens has not been confirmed. Specimens from Wee Jasper at the northern tip of the Brindabella Range are very similar in external morphology and in genital morphology, with the same overall pattern of a two-chambered penis but with a shorter penial complex and fewer spines on the spermatophore. This degree of variation is consistent with the population-level differences observed in other clades, and it is likely that the Wee Jasper population forms part of clade H despite not grouping together on the molecular tree.

Members of clade H are unique in their grey speckled appearance and greenish golden shell. They are significantly smaller than all other *Helicarion* semislugs. Their penial anatomy is highly distinct, particularly in the presence of very deep, nearly longitudinal lamellae in the proximal portion of the penial interior. The large number of spermatophore spines also helps to distinguish this clade.

HELICARION MASTERSI (COX, 1868)
(FIGS 6, 20–24)

Vitrina mastersi Cox, 1868: 86, pl. 14, figs 12, 12a; Pfeiffer, 1876: 24.

Helicarion mastersi – Cox, 1909: 6; Iredale, 1941: 6; Pfeiffer & Clessin, 1881: 32; Tryon, 1885: 170, pl. 38, figs 48–49; Hyman & Ponder, 2010: 24, figs 6A, B, 7C, D, 8C, D, 9C, D, 12B, 13B, 14C, 15A–C; Stanisic *et al.,* 2010: 302–303, 326.

Vercularion mastersi – Iredale, 1937: 9.

Helicarion mastersi mastersi – Smith, 1992: 234.

Material examined

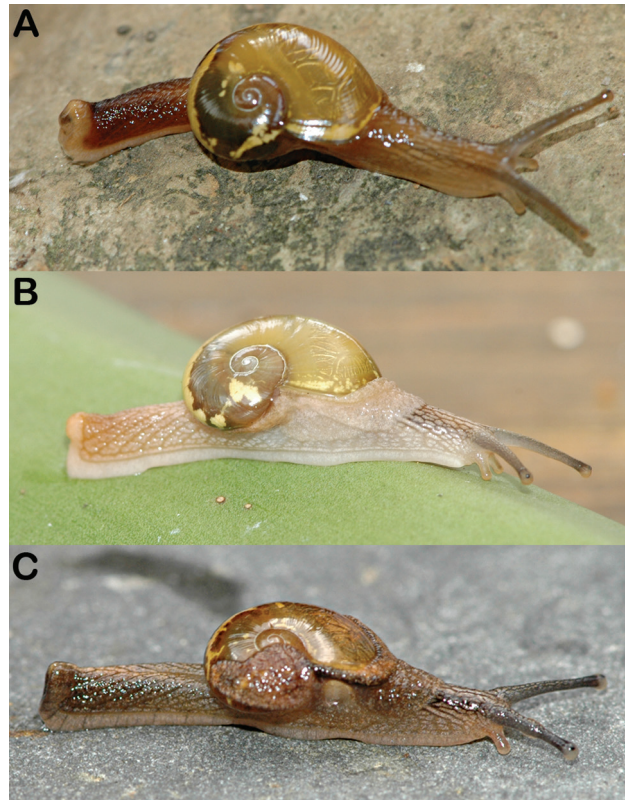*Holotype:* AM C.101139 (Kiama, 34°40′S, 150°51′E, leg. G. Masters, pre-1868).

Figure 22. Live specimens of *Helicarion mastersi* (not to scale). A, clade K, Minnamurra, AM C.483698. B, clade J, Kangaroo Valley, AM C.483702. C, clade I, Royal National Park, AM C.559129.

Paratypes: AM C.103648 (same data as holotype).

Non-type material. See Supporting Information (Table S1).

Description

External morphology: Shell golden brown to pale amber, 3.1–3.7 whorls. Body cream, greyish or fawn, with pink to orange-brown pink coloration, deepening on the tail; sole cream. Mantle lobes and shell lappets cream, sometimes lined with grey, with dark markings on underside.

Genital anatomy: Penis tubular, sometimes slender with a small bulge at proximal end, sometimes broad and slightly curving; epiphallus 1.5–3 times penis length, entering penis either apically or laterally (leaving a small blind tip) through a simple pore. Penial interior with > 20 closely spaced lamellae. Spermatophore with nine to 14 evenly spaced spines ($N = 5$) (in specimens from Kangaroo Valley, first branch is separated from remainder by a small gap).

Remarks

This species is delimited herein in a different manner from previous taxonomic studies, in that several populations from throughout NSW previously subsumed under the name *H. mastersi* are now recognized as members of *H. cuvieri*. This also applies to the two nominal species-taxa '*H. leopardinus*' and '*H. callidus*' introduced by Iredale (1941). See the description of *H. cuvieri* above for features that can be used to distinguish *H. cuvieri* and *H. mastersi*.

Helicarion mastersi is found from Kangarooo Valley in southern NSW to southern Sydney (Fig. 6). This species encompasses three well-differentiated subclades (I, J and K), described below. Differences between these three subclades include size and coloration, the presence or absence of a small blind penis tip and a varying number of spines on the spermatophore. To clarify the distinct status of these three forms further, denser sampling throughout the Upper Nepean dam catchment region and the Kangaroo Valley is necessary, particularly along potential zones of contact.

CLADE I
(FIGS 6, 20C, 21G–I, 22C, 23C, F, I, 24B)

Diagnosis
External morphology: Shell golden brown to pale amber, 3.1–3.5 whorls (Figs 20C, 21G–I). Body greyish
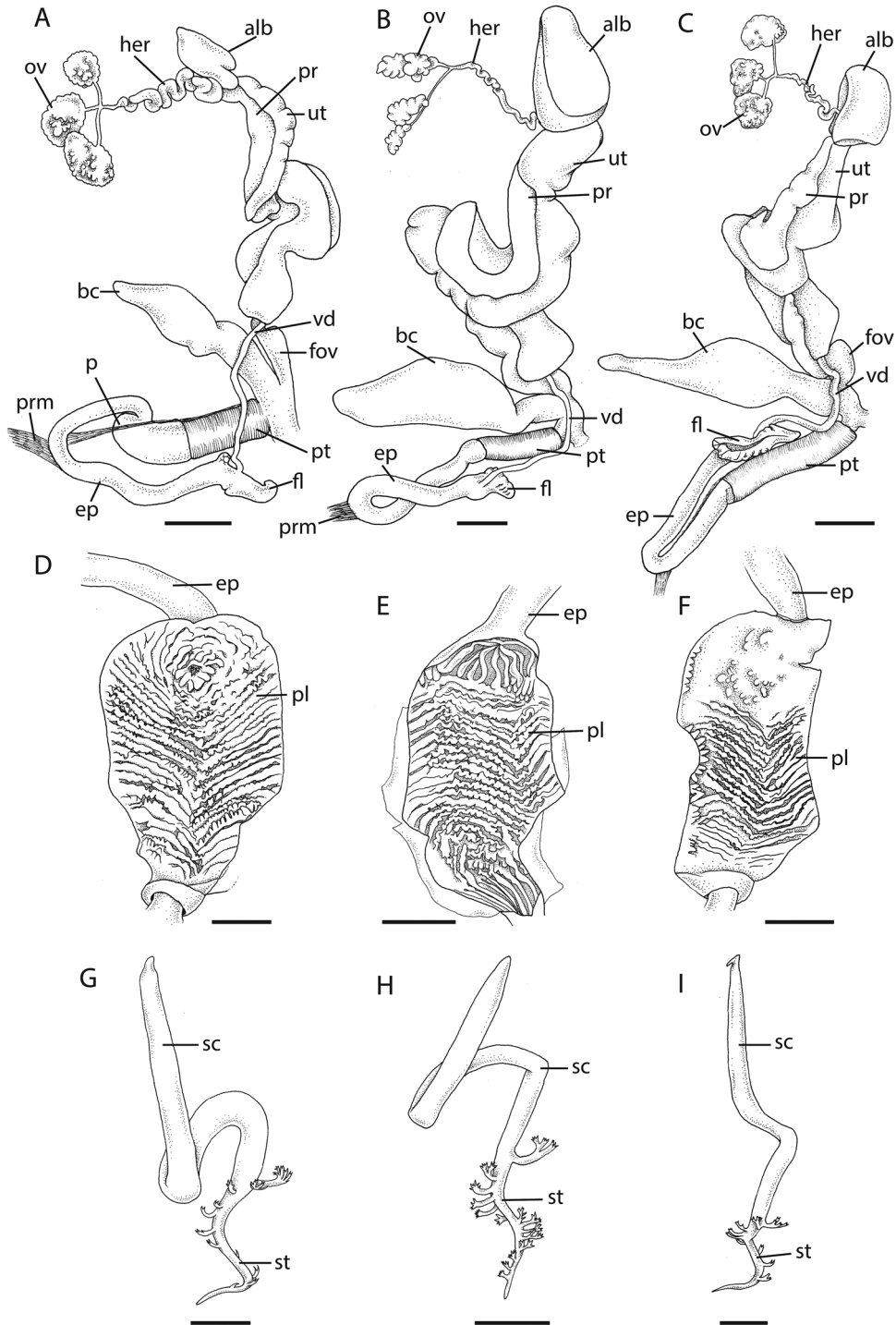
Figure 23. Reproductive anatomy of *Helicarion mastersi*. A–C, genitalia. A, clade K, Barren Grounds Nature Reserve, AM C.374836. B, clade J, Kangaroo Valley, AM C.472095. C, clade I, Royal National Park, AM C.385328. D–F, penis and penial interior. D, clade K, Minnamurra, AM C.483698. E, clade J, Kangaroo Valley, AM C.472095. F, clade I, Royal National Park, AM C.385328. G–I, spermatophore. G, clade K, Minnamurra, AM C.483698. H, clade J, Kangaroo Valley, AM C.559130 (holotype). I, clade I, Royal National Park, AM C.385328. Scale bars: 2 mm (A–C), 1 mm (D–I).
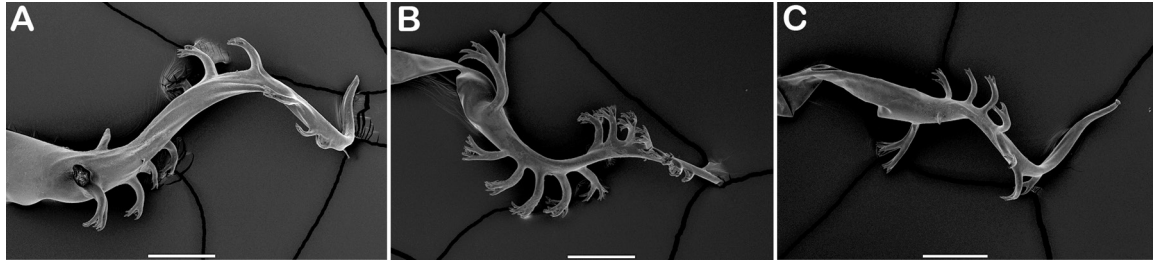
Figure 24. Spermatophores of *Helicarion mastersi*. A, clade K, AM C.365756. B, clade J, AM C.559130. C, clade I, AM C.385238. Scale bars: 500 μm.

or orange brown, darkening on the tail; sole cream. Shell lappets often lined with grey, with dark markings on underside (Fig. 22C).

Genital anatomy: Penis broad; penis-to-epiphallus ratio of 0.67–0.89, epiphallus entering penis laterally through simple pore. Spermatophore with nine to 11 evenly spaced branching spines of decreasing complexity; formula (1, 2, 6–8, 0–2) (Figs 23C, F, I, 24C).

Remarks

The range of this clade stretches from Macquarie Pass to southern Sydney (Fig. 6). The phylogenetic tree shows two clear groups: one from Razorback and Mt Kembla, and the other from Royal NP and Bulli Pass, but there are no significant anatomical differences.

Members of clade I can be distinguished from other *Helicarion* by their smaller size (they are similar in size to members of clades G and H but smaller than the other members of the genus) and from other NSW taxa by their grey-brown to orange-brown coloration. Like semislugs belonging to clades J and K, they have an interior penial sculpture of numerous rows of fine lamellae, but differ from these taxa in their broader penis, with a slightly lateral entry point, and spermatophore with fewer, less highly branched spines.

CLADE J

(FIGS 6, 20B, 21D–F, 22, 23B, E, H, 24B)

Diagnosis

External morphology: Shell golden, 3.2–3.7 whorls (Figs 20B, 21D–F). Body cream, with pale yellowish brown coloration on tail; sole cream. Mantle lobes and shell lappets pale (Fig. 22B).

Genital anatomy: Penis slender, tubular, with small bulge at proximal end; penis-to-epiphallus ratio of 0.40–0.52, epiphallus entering penis apically through a simple pore. Spermatophore with 13–14 branching spines; formula (1–2, 12, 0, 0); first branch separated from remainder by a small gap (Figs 23B, E, H, 24B).

Remarks

Clade J is known only from near Bellawongarah, north of Nowra (see Fig. 6). The majority of specimens have been collected along Kangaroo Valley Road as it crosses Berry Mountain. Very little material exists, reflecting the lack of collecting in this area; additional collecting might reveal a broader range.

Specimens belonging to clade J are similar in size to those of clade K but are slightly flatter in shape and can be distinguished by their paler body colouring. Individuals are similar in appearance to those belonging to clade F (part of *H. cuvieri*) but can be distinguished by the lack of pigmented warts on the shell lappets. Members of this taxon have a unique genital anatomy of a relatively short penis (penis-to-epiphallus ratio 0.4–0.52) with a small swelling at the proximal end and no blind tip, and a robust spermatophore with 13–14 heavily branched spines and a gap between the first and second spines.

CLADE K

(FIGS 6, 20A, 21A–C, 22A, 23A, D, G, 24A)

Diagnosis

External morphology: Shell golden, 3.2–3.7 whorls (Figs 20A, 21A–C). Body fawn, with pink coloration, deepening on the tail; sole cream (Fig. 22A).
Genital anatomy: Penis broad, curved, with small blind tip; penis-to-epiphallus ratio of 0.50–0.81, epiphallus entering penis laterally through a simple pore. Spermatophore with 11 evenly spaced spines ($N = 4$), formula (0, 3, 7, 1) (Figs 23A, D, G, 24A).

Remarks

Clade K is found in and around Kiama (the type locality of *H. mastersi*), at localities including Minnamurra NP and Barren Grounds Nature Reserve (Fig. 6). Its nearest relatives are distributed close by (clade I in Macquarie Pass to the north and clade J in Kangaroo Valley to the west), but the three taxa are allopatric. Members of clade K can be distinguished from other members of *H. mastersi*

by their pink body coloration and pale sole. These semislugs are similar in size to clade J but slightly larger than clade I. Anatomically, this taxon differs in the shape and size of the penis (particularly in the presence of a large blind penis tip) and the spermatophore type.

BIOGEOGRAPHY

The genus *Helicarion* is widespread and comparatively abundant in south-eastern Australia; a probable testimony to its fairly well-developed dispersal ability. It occurs throughout the zone of temperate forests and woodlands with mild to warm summers, but generally not in the zones of hot summers to the north and west of its current range and the zone of cold summers in the alpine areas of Tasmania (Fig. 6).

This entire region was exposed to more xeric conditions during the Plio-Pleistocene glacial cycles. A review of phylogeographical analyses has revealed that climatic fluctuations in this region have caused range contractions of mesic biota during the Quaternary, followed by expansions during wetter periods (Byrne *et al.,* 2011). Furthermore, it has been demonstrated that these contractions have produced high levels of intraspecific differentiation in a range of species that are restricted to wet forests and alpine areas, which has resulted from the long-term isolation of populations in refuges and from local extirpations of populations in areas with more xeric conditions (Byrne *et al.,* 2011).

A concordant history of historical range contraction into climatic refuges and subsequent range expansions is the probable cause of the observed genetic structuring in *Helicarion*. Such a scenario may also explain the existence of morphologically well-differentiated high-altitude groups in *Helicarion*, which may have undergone a long history of isolation, whereas lowland forms are deemed to have expanded their ranges relatively recently.

Based on the phylogeographical relationships documented here (e.g. Fig. 3), it is reasonable to postulate that *Helicarion* has originated on the Australian mainland and has colonized Tasmania subsequently. Quaternary climatic cycles have impacted Tasmania even more profoundly than the continental mainland as they caused repeated glaciations and created links to the continental Australia owing to sea level fluctuations. These processes have promoted both the isolation of populations in glacial refugia and an exchange between Tasmanian and mainland biota (e.g. McKinnon *et al.,* 2004). The signature of such fluctuations is seen in the phylogeny of *Helicarion* revealing significant lineage divergence among the Tasmanian populations and links with the continental fauna (sub-clade A).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Material examined. Abbreviations: AM, Australian Museum, Sydney; H, holotype; NMV, Museum Victoria, Melbourne; P, paratype; QM, Queensland Museum, Brisbane; TMAG, Tasmanian Museum and Art Gallery.