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Comparative diversity in glochidia of Australasian freshwater mussels

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Most freshwater mussels have larvae (glochidia in Unionidae, Margaritiferidae and Hyriidae) that are parasitic on fishes. This study describes and compares the diversity of glochidia among 17 species of Australasian Hyriidae. Here, scanning electron microscopy was used to illustrate shell morphology, while patterns of diversity in size, shape and morphological characteristics were analysed quantitatively and qualitatively with Principal Component, Linear Discriminant, and Multiple Correspondence Analyses to separate species with varying degrees of discrimination. Results showed shell lengths ranged from 50 to 390 μm. Shape varied, from sub-oval to sub-triangular, bilaterally symmetrical to scalene, and hook morphology varied from unicuspid, bicuspid, tricuspid or complex with varying length and structure. Unique observations of this study include the consistent variation in glochidial release mechanisms among Australian subfamilies and New Zealand genera. Hyridellini species and *Echyridella aucklandica* (Gray and Dieffenbach, 1843) are released either freely or as "mesoconglutinates" (presumed brood lures), whereas Velesunioninae and *Echyridella menziesii* (Gray and Dieffenbach, 1843) release glochidia in "amorphous mucous conglutinates". Hyridellini predominantly occur within perennially flowing rivers of coastal south-eastern Australia with generally low turbidity, whereas the Velesunioninae occur more typically in slower flowing, intermittent waters, many prone to extended periods of high turbidity. Thus, where Hyridellini occur, mesoconglutinates as visual brood lures may be a more efficient mechanism for infesting host fishes than the passive infestation strategy typical of velesunionine species frequenting more turbid waters. Finally, this study presents systematic descriptions of glochidia and a provisional key for identification of Australasian hyriid glochidia, making an important contribution to the current understanding of taxonomy and life history traits, both critical for hyriid conservation.

KEYWORDS

Unionoida, Hyriidae, conglutinate, glochidial release, reproduction, New Zealand, Australia, bivalve larvae

1 Introduction

Extant freshwater mussels (Bivalvia) of the order Unionoida occupy waterbodies on every continent except Antarctica, with a global diversity approaching 1,000 species (Bogan and Roe, 2008; Graf and Cummings, 2021). During reproduction, males release sperm into the water column that are then taken in through the inhalant siphons of females; eggs are fertilised internally and brooded in specialised chambers of the ctenidia called marsupia. Widely accepted as a dispersal strategy, the larvae of almost all freshwater mussel species are parasitic on fishes and rarely, amphibians (Howard, 1915; Kat, 1984). Larvae occur in two distinct forms: lasidia in the Etheriidae, Iridinidae and Mycetopodidae and glochidia in the Unionidae, Margaritiferidae and Hyriidae (Bauer and Wächtler, 2001). This study focuses on the glochidia of Australasian Hyriidae.

The mechanism of glochidial release and host infestation strategy varies widely. Some species broadcast individual glochidia directly to the water column to infest host fishes specifically during spawning migrations (Araujo et al., 2000; Hastie and Young, 2003; Soler et al., 2018). Other species have evolved modifications of the simple glochidia broadcast release strategy, such as *Unio crassus* Nilsson, 1822, that moves into shallow water to spurt water streams while releasing glochidia, presumably to attract potential hosts (Vicentini, 2005). In the Lampsilini (Unionidae), strategies for attracting hosts include mimicry via the production of various types of conglutinates, mantle lures and host-trapping (Barnhart et al., 2008). Functional conglutinates are structures that contain glochidia that often resemble food or prey items that infest hosts with glochidia when ingested; these vary in form and complexity (Barnhart et al., 2008; Watters, 2008). In amorphous, mucous conglutinates (or “mucous strands” or “mucous strings”), glochidia are released in a loose mucous matrix that dissociates in water (Watters, 2008). This type of glochidial release mechanism usually facilitates passive infestation where hosts contact glochidia as they swim through suspended mucus and glochidia (e.g., Matteson, 1948; Barnhart et al., 2008). The snuffbox mussel, *Epioblasma triquetra* (Rafinesque, 1820), has taken this further to actively trap the host between its modified shell valves whilst releasing glochidia for infestation (Barnhart and Roston, 2005; Barnhart et al., 2008).

Among species, glochidia vary widely in size, shape, and morphological features. Shell sizes range in length from 47.5 µm in *Margaritifera margaritifera* (Linnaeus, 1758) (Soler et al., 2018) to 500 µm in *Strophitus undulatus* (Say, 1817) (Hoggarth, 1999), while in shape outline they can vary from subtriangular (equilateral, isosceles, or scalene), suborbicular, hatchet-shaped (Ortmann, 1921; Kat, 1984; Jones et al., 1986; Hoggarth, 1999; Bauer and Wächtler, 2001; Sayenko, 2006, 2016; Pimpão et al., 2012) to an amorphous shape and bilateral asymmetry of opposing valves (Pfeiffer and Graf, 2015 and references therein). The glochidial stage of development in freshwater mussels is monomyarian, possessing a single adductor muscle that redevelops into a dimyarian state during metamorphosis to the juvenile stage (Bauer and Wächtler, 2001). Apart from Lillie (1895), there have been few studies of early development in freshwater mussels, but specific morphological features have been investigated, including sensory hairs, the larval mantle, ciliary fields and the larval filament, the latter of which is either present or absent, depending on species (Bauer and Wächtler, 2001; Nivischenko et al., 2022). The ventral apex of the glochidial valves in many species are equipped with hooks (as defined by Carter et al., 2012) of varying forms, also referred to as ‘larval teeth’, that assist in attachment to their host (Bauer and Wächtler, 2001). Morphological and morphometrical studies of glochidia and marsupia, as well as the mechanism and timing of glochidial release, have proven valuable in systematics and taxonomy (Ortmann, 1921; Parodiz and Bonetto, 1963; Heard and Guckert, 1970; Pimpão et al., 2012; O’Brien et al., 2019; Chernyshev et al., 2020), mode of parasitism (Barnhart et al., 2008; Huber and Geist, 2019), identification of host fishes (Shephard et al., 2021), conservation (Zając and Zając, 2021; Aldridge et al., 2022) and captive breeding programs (Benedict and Geist, 2021).

Among the glochidia-bearing families of freshwater mussels, extant Hyriidae are restricted to the Southern Hemisphere in Australasia (including Australia, New Zealand, Papua New Guinea, Solomon Islands, and Indonesian West Papua) and South America (Graf and Cummings, 2007; Bogan, 2008; Miyahira et al., 2017). The Australasian Hyriidae are split between two subfamilies: the Velesunioninae (genera: *Alathyria*, *Lortiella*, *Microdontia*, *Velesunio* and *Westralunio*), exclusive to Australia and New Guinea, and the Hyriinae (genera: *Cucumerunio*, *Hyridella* and *Virgus*), but the taxonomic position of the New Zealand genus, *Echyridella*, remains uncertain as representatives of this genus have not been included in recent molecular phylogenetic studies of the Hyriidae (Graf et al., 2015; Santos-Neto et al., 2016; Miyahira et al., 2017). Consequently, Graf and Cummings (2021) have assigned *Echyridella* as *incertae sedis* within the Hyriinae. The Hyriinae are partitioned into three tribes from South America, Hyriini, Castaliini and Rhipidodontini, and one tribe from eastern Australia and Papua New Guinea, Hyridellini (genera: *Cucumerunio*, *Hyridella* and *Virgus*) (Walker et al., 2014; Graf and Cummings, 2021). Glochidial morphology and morphometry, and in some cases, release mechanisms and host fishes, have been documented for several species of Hyriidae from Australia (McMichael and Hiscock, 1958; Parodiz and Bonetto, 1963; Atkins, 1979; Walker, 1981; Humphrey and Simpson, 1985; Jones et al., 1986; Widarto, 1993; Jupiter and Byrne, 1997; Klunzinger et al., 2012, Klunzinger et al., 2013; Klunzinger, 2020; Klunzinger, 2023a), South America (Bonetto, 1951; Bonetto, 1959; Bonetto, 1961; Bonetto and Ezcurra, 1963; Parodiz and Bonetto, 1963; Alvarenga and Ricci, 1977; Bonetto et al., 1986;Mansur, 1999; Mansur and Campos-Velho, 1990; Ricci et al., 1990; Semenas et al., 1994; Mansur and Silva, 1999; Viozzi and Brugni, 2001; Beasley et al., 2005; Pimpão et al., 2012) and New Zealand (Percival, 1931; Clearwater et al., 2014; Brown et al., 2017; Hanrahan, 2019; Melchior et al., 2021a; Melchior et al., 2021b). However, glochidial morphometry and morphology and their mechanism of release, remain unknown in many Hyriidae. This study incorporates published information, together with new observations from recent acquisitions of glochidia, to describe and illustrate behavioural, morphological and morphometrical data for a total of 17 species of Australasian Hyriidae from 24 localities of Australia, New Zealand, and Papua New Guinea, supplemented with a provisional dichotomous key for their identification.

TABLE 1 Provenance details of glochidia from Australasian Hyriidae included in this study. NSW—New South Wales , NT—Northern Territory, PNG—Papua New Guinea, Qld—Queensland, SA—South Australia, Vic—Victoria, WA—Western Australia. *Note: McMichael and Hiscock (1958) incorrectly identified *Hyridella drapeta* as *Hyridella australis* in their glochidia figure and Widarto (1993) reported data for *Velesunio ambiguus*, but the species does not occur in the Ross River, Queensland and is more likely *Velesunio angasi*. Coordinate system: WGS 1984 EPSG: 4326.

Taxon	Collection date	Collection locality	Latitude (° S)	Longitude (° E)	Source
Subfamily *incertae sedis*					
Echyridella aucklandica	January 2019	Ohautira Stream, New Zealand	37.7619	174.9802	Melchior et al., 2021a + this study
E. menziesii	pre-1932	Lake Sarah, Canterbury, New Zealand	-	-	Percival, 1931; McMichael and Hiscock, 1958
	December 2011	Waikato River, New Zealand	37.9466	175.5861	Jones unpublished, this study
	January 2019	Ohautira Stream, New Zealand	37.7619	174.9802	Melchior et al., 2021a + this study
Hyriinae: Hyridellini					
Cucumerunio novaehollandiae	May 1983	Macleay River, NSW, Australia	30.7548	152.1907	Jones et al., 1986 + this study
	June 2010	Gloucester River, NSW, Australia	31.8962	152.0974	this study (unpublished data from Jones, 2014)
	May 2021	Mary River, Qld, Australia	26.5475	152.7556	this study
Hyridella australis	April 1983	Macleay River, NSW, Australia	30.7548	152.1907	Jones et al., 1986 + this study
	2007	Williams River, NSW Australia	32.5587	151.7987	this study (unpublished data from Jones, 2014)
	April 2022	South Pine River, Qld, Australia	27.3500	152.9462	this study
*H. drapeta**	pre-1958	Williams River, NSW Australia	-	-	McMichael and Hiscock, 1958*
	2007	Williams River, NSW Australia	32.5587	151.7987	this study (unpublished data from Jones, 2014)
	March 2018	South Pine River, Qld, Australia	27.3500	152.9462	this study
H. sp. 'Diamond Creek'	December 2012	Diamond Creek, Vic, Australia	37.6762	145.1522	this study
H. depressa	1983	Macleay River, NSW, Australia	30.7647	152.3655	Jones et al., 1986 + this study
	1995	Lake Burragorang, NSW, Australia	33.9384	150.4291	Jupiter and Byrne (1997)
H. glenelgensis	December 2019	Crawford River, Vic, Australia	37.9308	141.3055	this study
H. narracanensis	December 2015	Ruby Creek, Vic, Australia	31.4319	145.9513	this study (unpublished data from Treby, 2016)
Velesunioninae					
Alathyria jacksoni	November 1977	River Murray, SA, Australia	34.1864	140.2920	Walker (1981)
A. pertexta pertexta	pre-1958	Larcom Creek, Qld, Australia	-	-	McMichael and Hiscock (1958)
	March 2018	Murray Lagoon, Qld, Australia	23.4022	150.4888	this study
	October 2018	Isaac River, Qld, Australia	22.6828	149.1800	Klunzinger, 2020 + this study
	August 2019	Mary River, Qld, Australia	26.5475	152.7556	
A. cf. *profuga*	pre-1986	Macleay River, NSW Australia	-	-	Jones et al., 1986 + this study
A. profuga	pre-1958	Williams River, NSW, Australia	-	-	McMichael and Hiscock (1958)
	October 2005	Mill Dam Falls (Williams River), NSW, Australia	32.5586	151.8043	this study
Lortiella froggatti	May 1988	Fitzroy River, WA, Australia	18.1116	125.7009	this study (AMS C.414981)
Velesunio ambiguus	pre-1958	River Murray, SA, Australia	-	-	McMichael and Hiscock (1958)
	November 1977	River Murray, SA, Australia	34.1864	140.2920	Walker (1981)

(Continued on following page)

TABLE 1 (*Continued*) Provenance details of glochidia from Australasian Hyriidae included in this study. NSW—New South Wales , NT—Northern Territory, PNG—Papua New Guinea, Qld—Queensland, SA—South Australia, Vic—Victoria, WA—Western Australia. *Note: McMichael and Hiscock (1958) incorrectly identified *Hyridella drapeta* as *Hyridella australis* in their glochidia figure and Widarto (1993) reported data for *Velesunio ambiguus*, but the species does not occur in the Ross River, Queensland and is more likely *Velesunio angasi*. Coordinate system: WGS 1984 EPSG: 4326.

Taxon	Collection date	Collection locality	Latitude (° S)	Longitude (° E)	Source
*V. angasi**	pre-1993	Ross River, Qld, Australia	-	-	Widarto (1993)
	October 2022	Mudginberri Billabong, NT, Australia	12.5926	132.8759	this study
Westralunio albertisi	June 2018	Levame Oxbow, Fly River, PNG	7.5484	141.4288	Klunzinger, 2023a + this study
W. carteri	December 2011	Canning River, Behind Fancote Park, Kelmscott, WA, Australia	32.0674	116.0102	Klunzinger et al., 2013 + this study
		Collie River, 100 m downstream from Southwest Highway, WA, Australia	32.3022	115.8175	

2 Materials and methods

2.1 Mussel collections and new observations

Details of collection localities and glochidial taxa included in this study are provided in Table 1. Distributions of focal taxa included in this study (based on Marshall et al., 2014; Walker et al., 2014; Klunzinger et al., 2022; Ponder et al., 2023) are provided in Supplementary Figure S1. Taxonomy follows Walker et al. (2014), Marshall et al. (2014), Klunzinger et al. (2022), Ponder et al. (2023) and Graf and Cummings (2023). Live samples of adult mussels of *Cucumerunio novaehollandiae* (Gray, 1843), *Hyridella australis* (Lamarck, 1819), *Hyridella drapeta* (Iredale, 1934), *Hyridella glenelgensis* (Dennant, 1898), *Hyridella narracanensis* (Cotton and Gabriel, 1932), and *Velesunio angasi* (Sowerby II, 1867) were hand-collected from multiple localities at varying times of the year; known times of brooding were gleaned from a combination of unpublished field observations and literature (Humphrey and Simpson, 1985; Jones et al., 1986; Playford and Walker, 2008; Jones, 2014; Treby, 2016). Atkins (1979) described the glochidia of *H. drapeta* from Diamond Creek, Victoria as having hooks, a larval filament and release mechanism resembling that of *Velesunio ambiguus* (Philippi, 1847) (as per Walker, 1981). Jones et al. (1986) found freshwater mussel specimens from the Macleay River, New South Wales, consistent with published and museum collections of *H. drapeta*, but the glochidia did not match the descriptions of Atkins (1979). Samples of glochidia from the species resembling *H. drapeta* were collected from adults in Diamond Creek (Table 1) to determine whether Atkins (1979) had potentially misidentified the species. *Velesunio ambiguus* was absent from the locality and subsequent investigations indicate that the species most recently collected has affinities with *H. drapeta*, but unpublished molecular sequence data indicate that it is an undescribed taxon, hereafter, *Hyridella* sp. 'Diamond Creek' (Klunzinger et al., unpublished data). Hand-collected mussels were retained in vessels containing water from each collection site, maintained at room temperature, and observed for glochidial release over several days, except for *H.* sp. 'Diamond Creek', where glochidia were collected directly from gravid females' ctenidia and observed for maturity under a compound microscope in the field. Glochidia are deemed mature when they expand their valves to rupture vitelline membranes and then begin "winking": rapidly opening and closing of the glochidial valves (cf. Jones et al., 1986; Kleinhenz et al., 2019). Glochidia of *E. aucklandica* and *E. menziesii* were collected and observed according to methods described by Melchior et al. (2021a). Glochidia of *Alathyria profuga* (Gould, 1851) were collected according to methods described by Jones et al. (1986). Samples of glochidia of *Lortiella froggatti* Iredale, 1934 were obtained, under license, from gravid females held in the malacology collection of the Australian Museum, Sydney (Museum Voucher No. C.414981).

Definitions of glochidial release mechanisms follow Watters (2008) and Haag (2012). Material released from exhalent siphons of live mussels was collected using a glass pipette, placed on a glass slide, and examined under light microscopy for the presence of glochidia with their behaviour observed. In *C. novaehollandiae*, *H. australis* and *H. drapeta*, brooding was confirmed by prying open the shell valves with reverse pliers or through dissection by inserting a scalpel between the two valves to cut adductor muscles to observe the inner pair of demibranchs for the presence of thickened, red-brown marsupia. Dissection was not performed in *H. glenelgensis* or *H. narracanensis* given these species have small sub-populations, restricted in geographic range, and listed as threatened (critically endangered or endangered) under the IUCN Red List, the Victorian *Flora and Fauna Guarantee Act 1988*, and the Commonwealth of Australia *Environment Protection and Biodiversity Conservation Act 1999* (Klunzinger, 2023b). Live individuals of each of these two species were returned to the wild after glochidia were collected.

2.2 Glochidial morphometry and morphology

Samples of live-released glochidia from *H.* sp. 'Diamond Creek' were collected by glass pipette, fixed in 2% glutaraldehyde following Jupiter and Byrne (1997) and rinsed and stored in Sorenson's sodium phosphate buffer for later study. Samples of live-released *V. angasi* (Mudginberri Billabong, Magela Creek, Northern Territory, Australia, Table 1) and *Westralunio carteri* Iredale, 1934 (from Canning River, Western Australia, Table 1) were separately collected by glass pipette, placed on a glass slide, and digitally photographed under compound light microscopy to capture details of soft tissues. Samples of glochidia from *L. froggatti* and *A. profuga* were flushed from the ctenidia of

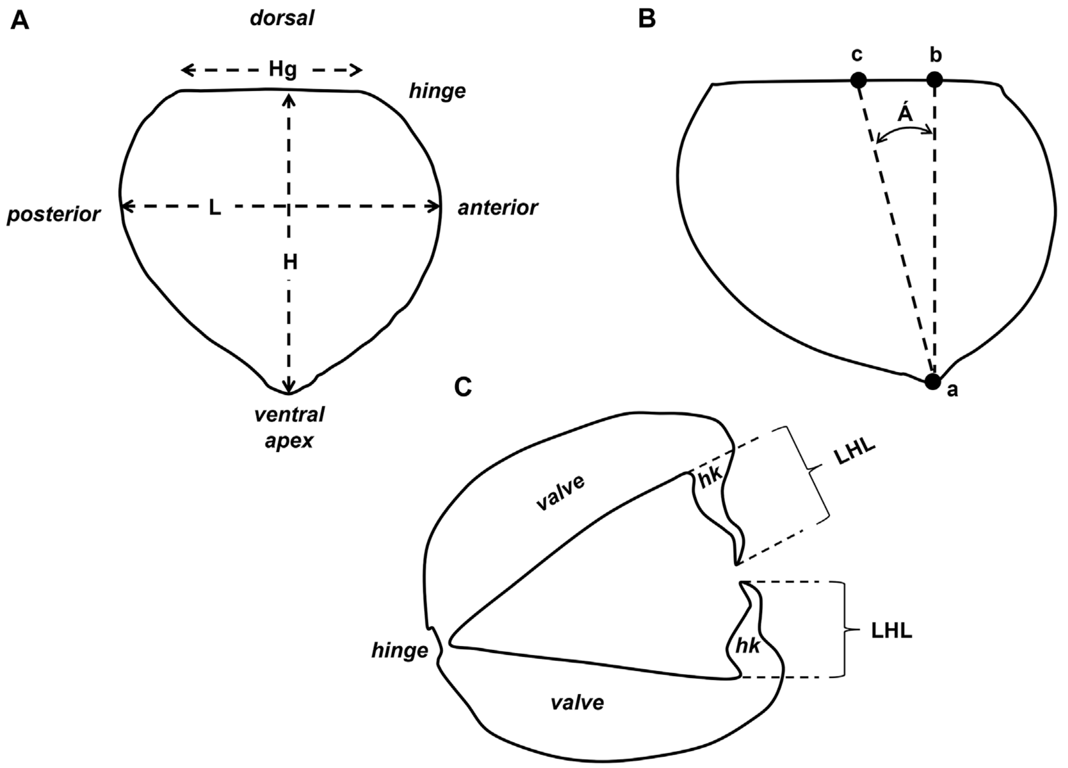

FIGURE 1
Morphometrical measurements of glochidial shells (right valve): **(A)** L, length, measured from anterior to posterior; Hg, hinge length; H, height, measured from dorsal hinge to ventral apex; **(B)** Á, angle of obliquity, measured in degrees (°), as the angle between the line that joins the ventral point (a) to the point perpendicular to the hinge (b) and the line that joins the ventral point (a) to the middle of the hinge line (c); **(C)** LHL, larval hook length, measured from the base of the junction with each valve to the terminal point of each larval hook (*hk*).

ethanol-preserved specimens of gravid females using a glass pipette. Samples of live-released glochidia for other species were collected by glass pipette and held in a glass jar with tap water for several days or up to a week, after which time glochidia had died and soft tissues had autolyzed, confirmed by examining a small sample of glochidia on a glass slide under light microscopy. Glochidial shells were then transferred by glass pipette into 5–mL microcentrifuge tubes containing 95% ethanol for preservation. Preserved glochidia were then deposited onto specimen stubs affixed with double-sided carbon tape using a glass pipette and allowed to air dry. Samples of *H. drapeta* and *H.* sp. 'Diamond Creek' were photographed on a Hitachi TM 3030 Plus Scanning Electron Microscope (SEM) and associated software at the Western Australian Museum in May 2019. Other samples were sputter-coated with platinum using a Safematic CCU-010 Compact Coating Unit and photographed using a Hitachi TM4000 Plus SEM and associated digital software at the University of Queensland Centre for Microscopy and Microanalysis between April 2022 and August 2023. Images or illustrations of glochidia from other studies (*Alathyria jacksoni* Iredale, 1934 and *V. ambiguus*, as per Walker, 1981; *Hyridella depressa* (Lamarck, 1819), as per Jones et al., 1986; *W. carteri*, as per Klunzinger et al., 2013; *H. narracanensis*, as per Treby, 2016; *Alathyria pertexta pertexta* Iredale, 1934, as per Klunzinger, 2020; *Westralunio albertisi* Clench, 1957, as per Klunzinger, 2023a; *H.* sp. 'Diamond Creek', taken at the Australian Centre for Microscopy and Microanalysis, University of Sydney, using a Zeiss EVO SEM (Jones, unpublished)) were also included for additional details of hook morphology.

Glochidia were measured to the nearest 1 µm from scale-imprinted SEM images for length (L), height (H), hinge length (Hg) and larval hook length (LHL) and to the nearest 1 degree (°) for angle of obliquity (Á) (see Figure 1). The Á was measured on just one individual valve that appeared to be resting flat on the specimen stub for each individual glochidium. The LHL values for each individual glochidium were measured either as the mean of two larval hooks (in the case where two opposing larval hooks could be accurately measured from an individual glochidium) or as an individual LHL (in the case where only one larval hook could be measured on an individual glochidium). Size was determined as the average of L and H. Additional data on shell size measurements of glochidia of various Australasian hyriid taxa were included from published literature and unpublished data of the authors.

2.3 Statistical analyses

All statistical analyses were carried out using the R statistical package (R Core Team, 2022). To understand the similarities of complex glochidial shapes across species, metrics of glochidial dimensions and shape (H, L, Hg, H/L, Hg/L, LHL and Á) were normalised and Principal Component Analysis (PCA) used to

TABLE 2 Definitions of categorical variables used in multiple correspondence analysis (MCA) of glochidia of Australasian freshwater mussels (Unionoida: Hyriidae). Each defined category for a particular variable is provided in italic font.

Variable name	Categorical definitions
glochidia size	*small*, glochidia <100 µm
	large, glochidia >100 µm
shape	*subtriangular*, ventral apex comes to a point and forms a triangular shape relative to the apices of the anterior and posterior ends of the hinge plane
	suborbicular, ventral apex rounded and overall shape oval to nearly circular
H/L ratio	*low*, <85%
	medium, 85%–110%
	high, >110%
valve symmetry	*bilateral*, ventral apex approximately mid-way between either end of the hinge line and mean Á <5°
	scalene, ventral apex off-centre relative to either end of the hinge line and mean Á >5°
cusp type	*unicuspid*, hooks come to a singular point (cusp) in either valve
	bicuspid, hooks with two cusps in each valve
	tricuspid, hooks with central well-formed tooth/blade bounded by two smaller cusps in at least one valve
	complex, hooks may have singular or multiple cusps and varying in form from blunt or rounded to pointed
hook form	*buttressed*, having distinctive supporting buttresses either side of the hook running from the ventral edge of the shell, gradually tapering off to below the cusp
	long, sigmoidal, LHL >20 µm and form an s-shape when viewed from the side
	reduced, hook reduced to a broad base from which a pair of hooks project, LHL <20 µm
larval filament	*present* or
	absent

reduce shape complexity into two or three explanatory dimensions. The closer two glochidial samples are in this reduced dimensional space, the more similar their overall shape. Samples within this space were then coded by their species affinity, highlighting species differentiation based on glochidial shape.

To further understand the specific morphometrical variables (i.e., discriminant functions) that best separated species, Linear Discriminant Analysis (LDA) was used (MASS package: Venables and Ripley, 2002); this analysis tests for group structure in multivariate data and identifies linear combinations of variables that best separate the input categories, in this case, the species.

Three measures of accuracy were then calculated to assess the classification of the glochidial samples into the species (as categories) based on morphological traits: 1) overall classification accuracy, 2) errors of omission (also known as producer's accuracy) and 3) errors of commission (also called user's accuracy) (Story and Congalton, 1986). An error of omission is the proportion of incorrectly classified glochidia for a known reference species, calculated as the number of correctly classified glochidia (on the major diagonal of the confusion matrix), divided by the number of samples of the reference species (i.e., the column total for the reference species). An error of commission is a measure of the reliability of the classification rule and addresses the question, "What percentage of glochidia classified as species *i* are actually species *i*?". This is calculated as number of correctly classified glochidia divided by the row total for species *i* (i.e., the number of glochidia classified as species *i*). Error matrices compare the allocation of glochidia among species using discriminant functions with their known species identities.

In addition to quantitative analysis of glochidial shell metrics, multiple correspondence analysis (MCA) was used to group species according to qualitative measures of glochidial morphology and to overlay species associations with morphological characters (Table 2) using the functions in the FactoMineR package (Lê et al., 2008). Multiple correspondence analysis is essentially PCA of qualitative (i.e., categorical) variables instead of quantitative measurements (Abdi and Williams, 2012). Qualitative glochidial characters were scored for the glochidia of all species of Australasian freshwater mussels for which there were data (Table 1), and MCA was used to ordinate species in two dimensions.

3 Results

3.1 Marsupial occupancy and glochidial release

Marsupia occupied the majority of the inner demibranchs of ctenidia in *C. novaehollandiae*, the inner four-fifths in *H. australis*, and the inner two-thirds in *H. drapeta* and *H.* sp. 'Diamond Creek'. Additionally, in *H. australis*, the dorsal one-third of the marsupia within the inner demibranchs were thickened and white in two dissected females. Marsupia occupied the upper four-fifths of the

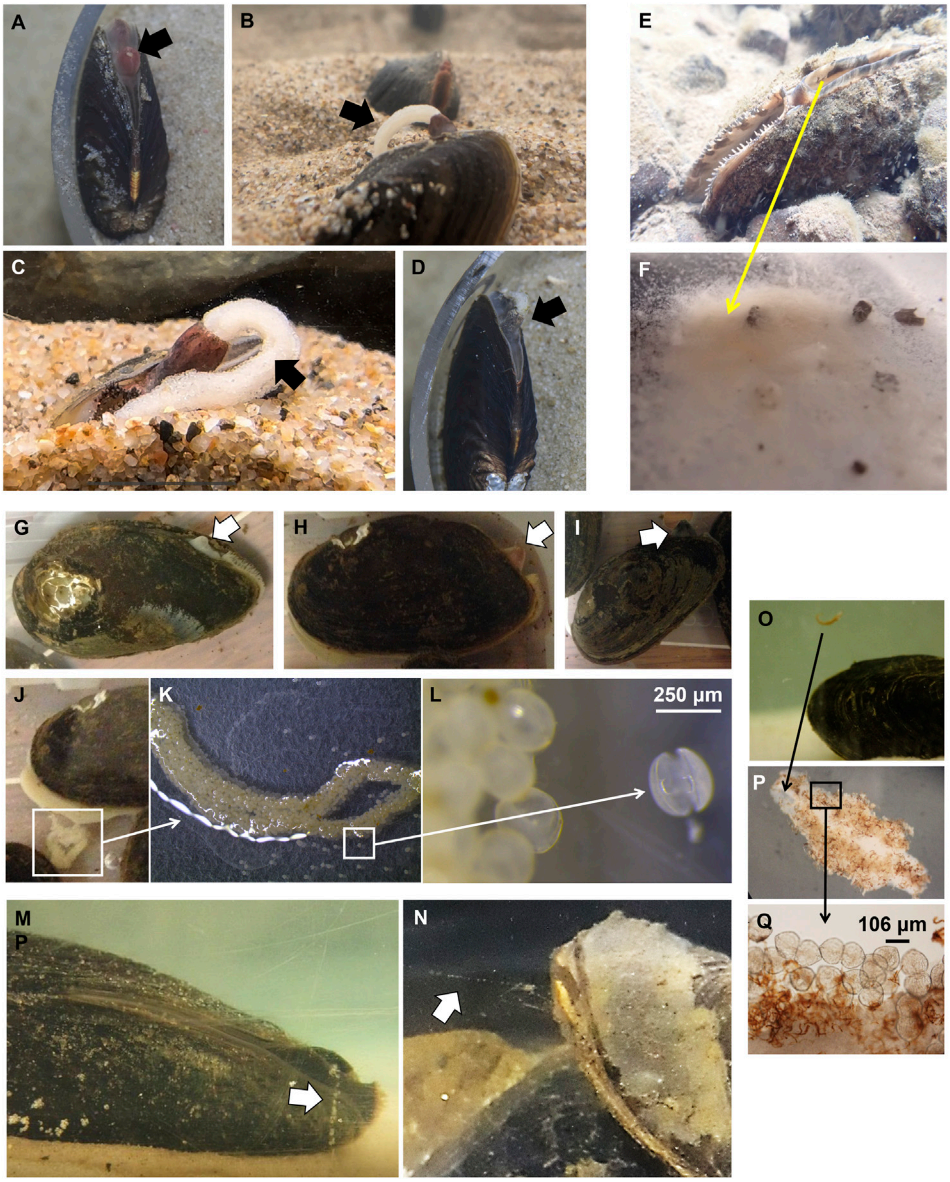

FIGURE 2
Glochidial release in ***Hyridella glenelgensis*** (Crawford River, Victoria, Australia): **(A)** exhalant siphon (block arrow) becomes enlarged and changes to bright pink in colour, followed by **(B, C)** release of thick white mesoconglutinates (block arrows), photos by Timothy Fernando, with permission, and **(D)** returning to normal size and mottled grey-brown (block arrow) post-release; ***Cucumerunio novaehollandiae*** (Mary River, Queensland, Australia): **(E)** Live gravid female in the wild in the burrowed position released, **(F)** individual mature, active glochidia as individuals, from the exhalant siphon (yellow arrow); ***Hyridella narracanensis*** (Ruby Creek, Victoria, Australia): siphon enlarges and changes colour from **(G)** white to **(H)** pink and **(I)** dark grey, followed by a waving motion and release of **(J, K)** conglutinates comprised of **(L)** individual glochidia held together by viscous adherent gel-like material; **(M)** ***Echyridella menziesii*** (Ohautira Stream, New Zealand; Melchior, 2021) and **(N)** ***Alathyria pertexta pertexta*** (Mary River, Queensland, Australia; Klunzinger, 2020), block arrows indicating amorphous mucous conglutinates; ***Echyridella aucklandica*** (Ohautira Stream, New Zealand; Melchior, 2021): **(O, P)** mesoconglutinates comprised of **(Q)** individual glochidia attached to conglutinate.

inner demibranchs of ctenidia in *E. aucklandica* (Melchior et al., 2021a) and the inner one third in *E. menziesii* (McMichael and Hiscock, 1958; Melchior et al., 2021a). The marsupia of members of the Velesunioninae generally occupy the inner two-thirds of the inner demibranchs (Humphrey and Simpson, 1985; Walker, 1981; Klunzinger et al., 2013; Klunzinger, 2020; 2023a; Jones, *pers. obs.*). Reproductive output for *H. depressa* is positively correlated with nutrient status of ambient waters (Byrne, 1998), while under eutrophic conditions, the marsupia may occupy the entire inner demibranchs in *V. angasi* (Humphrey and Simpson, 1985). As gravid adult females of *H. glenelgensis* and *H. narracanensis* were not dissected, the proportion of ctenidia occupied by marsupia was not determined.

Undissected *H. australis* released several white botuliform conglutinates, approximately 20–30 mm in length within 4 days post-collection. Glochidia released from *H. glenelgensis* and *H. narracanensis* released glochidia contained within sticky white to light yellow conglutinates, preceded by an enlargement of the

TABLE 3 Qualitative traits of Australasian glochidia used in multiple correspondence analysis (MCA). Definitions of each trait are provided in Table 2.

Taxon code	Taxon	Size	Shape	H/L ratio	Valve symmetry	Cusp type	Hook form	Larval filament
	Subfamily *incertae sedis*							
Eauc	*Echyridella aucklandica*	small	subtriangular	medium	bilateral	unicuspid	buttressed	absent
Emnz	*E. menziesii*	large	subtriangular	low	scalene	unicuspid	buttressed	present
	Hyriinae: Hyridellini							
Cnov	*Cucumerunio novaehollandiae*	small	suborbicular	high	bilateral	bicuspid	reduced	absent
Haus	*Hyridella australis*	small	subtriangular	high	bilateral	bicuspid	reduced	absent
Hdrp	*H. drapeta*	large	subtriangular	medium	scalene	bicuspid	long, sigmoidal	absent
Hdia	*"H."* sp. 'Diamond Creek'	large	subtriangular	low	scalene	tricuspid	long, sigmoidal	absent
Hdep	*H. depressa*	large	subtriangular	medium	scalene	bicuspid	long, sigmoidal	absent
Hgle	*H. glenelgensis*	large	suborbicular	medium	bilateral	bicuspid	reduced	absent
Hnar	*H. narracanensis*	large	suborbicular	medium	bilateral	bicuspid	reduced	absent
	Velesunioninae							
Ajac	*Alathyria jacksoni*	large	subtriangular	medium	bilateral	unicuspid	long, sigmoidal	present
Aper	*A. pertexta pertexta*	large	subtriangular	medium	scalene	unicuspid	long, sigmoidal	present
Aprf	*A. profuga*	large	subtriangular	low	scalene	unicuspid	long, sigmoidal	absent
Lfrg	*Lortiella froggatti*	large	subtriangular	medium	scalene	unicuspid	long, sigmoidal	present
Vamb	*Velesunio ambiguus*	large	subtriangular	medium	scalene	unicuspid	long, sigmoidal	present
Vang	*V. angasi*	large	subtriangular	medium	scalene	unicuspid	long, sigmoidal	present
Walb	*Westralunio albertisi*	large	subtriangular	medium	scalene	unicuspid	long, sigmoidal	present
Wcar	*W. carteri*	large	subtriangular	low	scalene	complex	long, sigmoidal	present

exhalent siphon that turned from pale grey to dark grey or reddish brown and exhibited a "waving" motion prior to the release of conglutinates (Figure 2A–D, Figures 2G–L for *H. glenelgensis* and *H. narracanensis*, respectively); glochidia were only released after disturbance by squirting water in short bursts against the siphons of *H. glenelgensis* or tapping the shells of *H. narracanensis* with a blunt probe. Glochidia were released from *C. novaehollandiae* freely and were not contained in amorphous mucous conglutinates or any other form of conglutinate within 2 days post-capture (Figures 2E, F). Glochidia of *H. drapeta* were extruded from the exhalent siphons of brooding females as clear, amorphous, mucous conglutinates. *Hyridella* sp. 'Diamond Creek' are also thought to release glochidia as amorphous mucous conglutinates (Atkins, 1979, see discussion), although glochidial release of this taxon was not observed in the present study. Live samples of glochidia of *H.* sp. 'Diamond Creek' examined during the present study did not possess larval threads, unlike Atkins (1979) who described them as being present, presumably from the same taxon collected from the same stream. *Westralunio carteri*, *A. pertexta pertexta* and *V. angasi* release glochidia in amorphous mucous conglutinates (Humphrey and Simpson, 1985; Klunzinger et al., 2013; Klunzinger, 2020). *Echyridella menziesii* has been observed to release glochidia singularly and in amorphous mucous conglutinates (Melchior et al., 2021a). This form of glochidial release is illustrated in Figures 2M, N for *E. menziesii* and *A. pertexta pertexta*, respectively. Glochidia are released as functional conglutinates resembling vermiform macroinvertebrate prey items of fishes in *E. aucklandica* (Melchior et al., 2021a, Figures 2O–Q), similar in form to conglutinates released by those of *H. australis* (Jones et al., 1986; see also Section 4.1 below).

3.2 Glochidial morphology

Of the species studied, glochidia of *E. menziesii* and all velesunionine taxa except *A. profuga* possess larval filaments (Table 3). None of the glochidia in the Hyridellini in this study possessed larval filaments. Glochidia varied in size and shape among species and to a lesser extent within species. A summary of traditional morphometrical measurements obtained by this study, in comparison with other species and populations of Australasian glochidia for which published information is available, is provided in Table 4. The raw data from which this summary was tabulated are provided in Supplementary Table S1. To illustrate the variation in size and shape of glochidia, SEM images of shell valves are provided in Figure 3. Hook morphology is illustrated in Figure 4 with more detailed views and additional images of glochidia provided in the

TABLE 4 Glochidial dimensions in Australasian freshwater mussels (Bivalvia: Hyriidae) included in this study. Values presented are means ± standard errors. Taxonomy follows Walker et al. (2014) and Marshall et al. (2014); note, unpublished preliminary analysis of genetic sequences suggest *Hyridella* sp. 'Diamond Creek' is an undescribed species. Abbreviations: H, height; L, length; Hg, hinge length; LHL, larval hook length; Á, angle of obliquity; Australia: NSW—New South Wales, NT—Northern Territory, Qld—Queensland, SA—South Australia, Vic—Victoria, WA—Western Australia; NZ—New Zealand; PNG—Papua New Guinea. Data of individually measured glochidia are provided in Supplementary Material. *Note: data reported for *Hyridella drapeta* was originally misidentified by McMichael and Hiscock 1958 as *Hyridella australis* and data reported for *Velesunio angasi* was originally misidentified as *Velesunio ambiguus* by Widarto (1993).

Taxon	Larval thread	n	L (µm)	H (µm)	Size (µm)	Hg (µm)	H/ L (%)	Hg/ L (%)	LHL (µm)	Á (˚)	Locality	Source
Subfamily *incertae sedis*												
Echyridella aucklandica	absent	-	360	280	320	-	77.8	-	-	-	Lake Sarah, NZ	Percival (1931)
		-	323	277	300	-	85.8	-	-	-		McMichael and Hiscock (1958)
		28	101.9 ± 0.71	95.7 ± 0.74	98.8 ± 0.55	64.8 ± 0.66	94.1 ± 0.93	63.6 ± 0.59	13.8 ± 0.45 ($n = 4$)	2.1 ± 0.42 ($n = 20$)	Ohautira Stream, NZ	Melchior et al., 2021a; this study
E. menziesii	present	30	301.3±1.20	252.0 ± 0.90	276.0 ± 1.02	209.0 ± 1.60	83.6 ± 0.27	69.5 ± 0.60	81.3 ± 2.67 ($n = 15$)	15.2 ± 0.73 ($n = 5$)	Ohautira Stream, NZ	Melchior et al., 2021a; this study
Hyriinae: Hyridellini												
Cucumerunio novaehollandiae	absent	50	55.2 ± 0.08	64.1 ± 0.03	59.7	35.8 ± 0.75 ($n = 2$)	116.1	64.9	8.0 ($n = 1$)	-	Macleay River, NSW	Jones et al., 1986 + this study
		10	54.5 ± 0.76 ($n = 7$)	64.4 ± 0.50	59.0 ± 0.60 ($n = 7$)	-	116.7 ± 0.01 ($n = 7$)	-	-	-	Gloucester River, NSW	Jones, 2014 + this study
		20	51.1 ± 0.26	56.5 ± 0.31	53.8 ± 0.23	32.3 ± 0.41	110.7 ± 0.72	63.3 ± 0.80	5.0 ± 0.53 ($n = 10$)	0.9 ± 0.26 ($n = 20$)	Mary River, Qld	this study
Hyridella australis	absent	50	73.9 ± 0.07	94.7 ± 0.04	84.3	40	128	68	-	-	Macleay River, NSW	Jones et al., 1986 + this study
		20	75.6 ± 0.36	88.7 ± 0.45	82.1 ± 0.25	39.8 ± 0.27	117.3 ± 0.92	52.6 ± 0.38	17.1 ± 1.60 ($n = 7$)	0.9 ± 0.32 ($n = 20$)	South Pine River, Qld	this study
*H. drapeta**	absent	-	250	240	245	-	96.0	-	-	-	Williams River, NSW	McMichael and Hiscock (1958)
		1	250	233	241.5	155	93.2	62.0	-	-		Jones, 2014 + this study
		20	274.5 ± 1.70	250.0 ± 1.78	262.3 ± 1.43	192.0 ± 1.17	91.1 ± 0.70	70.0 ± 0.51	50.8 ± 1.49 ($n = 3$)	8.7 ± 0.63 ($n = 10$)	South Pine River, Qld	this study
H. sp. 'Diamond Creek'	present	30	330.0 ± 1.8	230.0 ± 1.8	280	248	70.6 ± 2.7	-	-	-	Diamond Creek, Vic	Atkins (1979)
	absent	20	331.0 ± 0.69	250.0 ± 0.00	290.5 ± 0.34	261.0 ± 0.78	75.5 ± 0.16	78.9 ± 0.15	71.5 ± 1.40 ($n = 11$)	-		this study
H. depressa	absent	50	253.0 ± 0.70	244.0 ± 0.70	248.5	152.0 ± 0.85	97.0	60.0	-	-	Macleay River, NSW	Jones et al., 1986 + this study
		5	243 ± 5.38	249 ± 1.79	246	-	102.5	-	44.4 ± 0.002 ($n = 2$)	-	Lake Burragorang, NSW	Jupiter and Byrne (1997)
H. glenelgensis	absent	20	249.3 ± 0.78	225.0 ± 1.20	236.9 ± 0.85	150.0 ± 0.25	90.1 ± 0.44	60.1 ± 0.19			Crawford River, Vic	this study

(Continued on following page)

TABLE 4 (*Continued*) Glochidial dimensions in Australasian freshwater mussels (Bivalvia: Hyriidae) included in this study. Values presented are means ± standard errors. Taxonomy follows Walker et al. (2014) and Marshall et al. (2014); note, unpublished preliminary analysis of genetic sequences suggest *Hyridella* sp. 'Diamond Creek' is an undescribed species. Abbreviations: H, height; L, length; Hg, hinge length; LHL, larval hook length; Á, angle of obliquity; Australia: NSW—New South Wales, NT—Northern Territory, Qld—Queensland, SA—South Australia, Vic—Victoria, WA—Western Australia; NZ—New Zealand; PNG—Papua New Guinea. Data of individually measured glochidia are provided in Supplementary Material. *Note: data reported for *Hyridella drapeta* was originally misidentified by McMichael and Hiscock 1958 as *Hyridella australis* and data reported for *Velesunio angasi* was originally misidentified as *Velesunio ambiguus* by Widarto (1993).

Taxon	Larval thread	*n*	L (µm)	H (µm)	Size (µm)	Hg (µm)	H/ L (%)	Hg/ L (%)	LHL (µm)	Á (°)	Locality	Source
									29.2 ± 0.52 (*n* = 20)	3.8 ± 0.31 (*n* = 20)		
H. narracanensis	absent	10	258.0 ± 2.49	236.0 ± 2.67	247.0 ± 2.00	150.0 ± 4.94	96.0 ± 1.18	58.3 ± 2.22	-	4.8 ± 0.76 (*n* = 10)	Ruby Creek, Vic	Treby, 2016 + this study
Velesunioninae												
Alathyria jacksoni	present	10	272.0 ± 2.97	210.0 ± 3.89	238.0	173.0 ± 2.94	93.0	71.0	50.0	5.0	River Murray, SA	Walker (1981)
A. pertexta pertexta	present	-	270	250	260	-	92.6	-	-	-	Larcom Creek, Qld	McMichael and Hiscock (1958)
		10	257.1 ± 2.47	242.5 ± 1.88	249.8 ± 1.95	175.8 ± 3.42 (*n* = 9)	94.4 ± 0.76	69.0 ± 0.96 (*n* = 9)	-	-	Murray Lagoon, Rockhampton, Qld	this study
		80	268.1 ± 1.21	243.0 ± 1.22	255.5 ± 0.96	191.0 ± 0.63	90.7 ± 0.52	71.3 ± 0.27	70.2 ± 1.04 (*n* = 29)	9.5 ± 0.78 (*n* = 20)	Mary and Isaac Rivers, Qld	Klunzinger, 2020 + this study
A. cf. *profuga*	absent	20	239.0 ± 0.89	204.0 ± 0.45	221.5	165.0 ± 0.89	85.0	69.0	-	-	Macleay River, NSW	Jones et al., 1986 + this study
A. profuga	absent	-	245	200	222.5	-	81.6	-	-	-	Williams River, NSW	McMichael and Hiscock (1958)
		17	238.8 ± 1.69	201.1 ± 1.21 (*n* = 16)	220.1 ± 1.24 (*n* = 16)	159.0 ± 1.61 (*n* = 15)	84.2 ± 0.01 (*n* = 16)	66.4 ± 0.06 (*n* = 15)	40 (*n* = 1)	8.5 (*n* = 2)	Mill Dam Falls (Williams River), NSW	Jones, 2014 + this study
Lortiella froggatti	present	46	260.6 ± 1.2	231.8 ± 0.7	246.2 ± 0.8	183.9 ± 0.8	89.0 ± 0.4	70.6 ± 0.4	73.0 (*n* = 1)	11.8 ± 0.35 (*n* = 20)	Fitzroy River, WA	this study
Velesunio ambiguus	present	-	250	220	235	-	88.0	-	-	-	River Murray, SA	McMichael and Hiscock (1958)
		10	247.0 ± 2.97	210.0 ± 3.89	228.5	173.0 ± 2.94	85.0	70.0	60.7	3.0		Walker (1981)
*V. angasi**	present	15	263	232	247.5	200	88.2	76.0	-	-	Ross River, Qld	Widarto (1993)
		60	254.0 ± 1.29	223.7 ± 1.10	238.9 ± 0.97	178.5 ± 1.16	89.3 ± 0.53	70.0 ± 0.41	50.8 ± 1.26 (*n* = 22)	9.6 ± 0.30 (*n* = 60)	Mudginberri Billabong, NT	this study
Westralunio albertisi	unknown	60	260.0 ± 1.11	233.1 ± 1.32	246.6 ± 0.99	183.6 ± 0.99	89.7 ± 0.53	70.6 ± 0.33	53.6 ± 0.84 (*n* = 3)	12.9 ± 0.28 (*n* = 60)	Levame Oxbow (Fly River), PNG	Klunzinger, 2023a + this study
W. carteri	present	120	307.8 ± 0.83	251.0 ± 0.73	279.4 ± 0.63	212.0 ± 0.78	81.6 ± 0.28	68.8 ± 0.27	53.9 ± 1.66 (*n* = 5)	19.8 ± 0.71 (*n* = 13)	Canning and Collie Rivers, WA	Klunzinger et al., 2013 + this study

Supplementary Material (Supplementary Figures S2-S18). Soft anatomy of glochidia was generally not available, owing to the methods of preservation in this study, but an example of soft anatomical features from a live *V. angasi* glochidium, including adductor musculature and sensory hairs, is provided in Figure 5A and an example of a larval filament from a live image of *W. carteri*

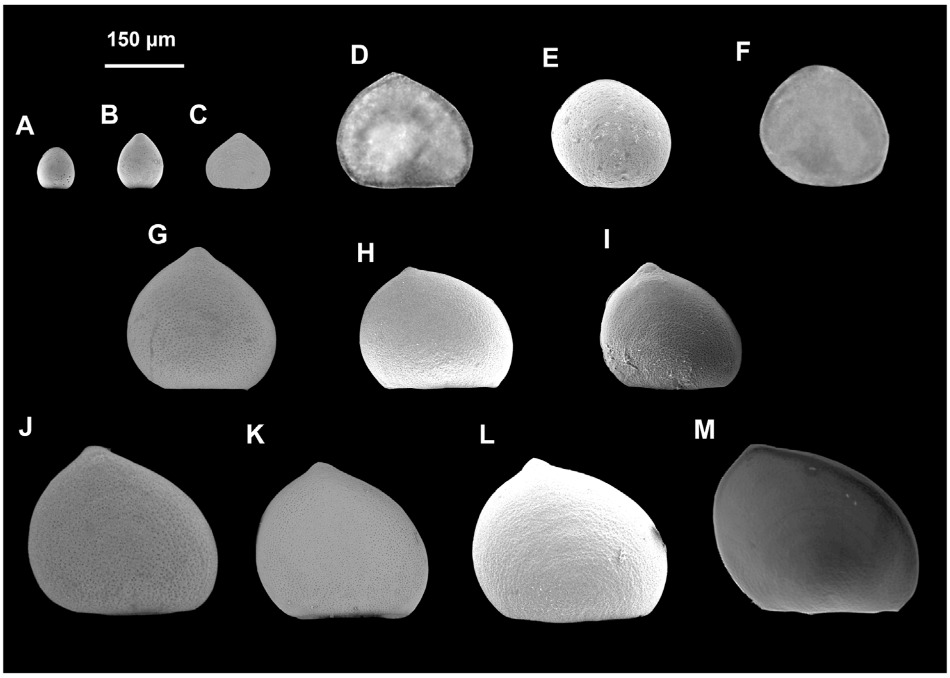

FIGURE 3
Size and shape variation in shell outlines of Australasian hyriid glochidia from this study: **(A)** *Cucumerunio novaehollandiae*, **(B)** *Hyridella australis*, **(C)** *Echyridella aucklandica*, **(D)** *Alathyria profuga*, **(E)** *Hyridella glenelgensis*, **(F)** *Hyridella narracanensis*, **(G)** *Hyridella drapeta*, **(H)** *Echyridella menziesii*, **(I)** *Lortiella froggatti*, **(J)** *Alathyria pertexta pertexta* (see also Klunzinger, 2020), **(K)** *Velesunio angasi*, **(L)** *Westralunio albertisi* (see also Klunzinger, 2023a), **(M)** *Westralunio carteri* (see also Klunzinger et al., 2013).

is shown in Figure 5B. Two valve shapes were apparent in the glochidia from this study (Table 3 and Figure 3): suborbicular in *C. novaehollandiae*, *H. glenelgensis* and *H. narracanensis* and subtriangular for all other species in this study. The H/L ratio, a measure of "squatness", varied from tall and narrow (H/L >100%) to short and broad (H/L <100%) with the two extremes in form represented by *C. novaehollandiae* and *H. australis* (H/L 110%–128%) compared with *E. aucklandica*, *A. profuga* and *W. carteri* (H/L 81%–85%). In terms of valve symmetry, most glochidia studied here are scalene, apart from *A. jacksoni*, *C. novaehollandiae*, *E. aucklandica*, *H. australis*, *H. glenelgensis*, *H. narracanensis* and one population of *V. ambiguus* that have ventral apices located approximately mid-way between either end of the hinge line (Á, 0–5°), imparting bilaterally symmetrical valves (Table 3). The most bilaterally asymmetrical species is *W. carteri* that has a much larger Á (19.8°) than other scalene species which ranged from about 8 to 10° in *A. profuga*, *H. drapeta*, *A. pertexta pertexta* and *V. angasi*, to about 12–13° in *L. froggatti*, *W. albertisi* and one population of *V. ambiguus*, with *E. menziesii* having the second largest Á at 15.2°.

The glochidia of Velesunioninae generally have unicuspid sigmoidal hooks, apart from *W. carteri* that has complex hooks which are variably uni-, bi-, or tricuspid (Table 3; Figures 4H–M). The hooks of *E. aucklandica* are distinctly unicuspid. In *E. menziesii* they are also unicuspid although the cusps of the opposing hooks in *E. menziesii* are not identical (Table 3; Figure 4C). In *E. menziesii* one hook terminates as a spatulate knob with a spinose tooth projecting from the end; the opposing hook is more claw-like but with two notches on each side of the hook (Figure 4G) that may allow the hook to snap off when the valves close.

Glochidial hook morphology in the Hyridellini is much more morphologically diverse than in the Velesunioninae (Table 3; Figures 4A, B, D, E, F). The Hyridellini generally have bicuspid hooks, apart from *H.* sp. 'Diamond Creek' that is tricuspid (Figure 4F). Hooks in *C. novaehollandiae*, *E. aucklandica*, *H. australis*, *H. glenelgensis*, and *H. narracanensis* are greatly reduced in length compared to other species studied (Tables 3, 4; Figures 4A, B, D, E; Supplementary Material), having a common base with two sharp cusps on either valve, but the length of teeth and degree of separation between cusps varies among taxa. In *C. novaehollandiae*, hooks are reduced to a pair of sharp spines just below the apex of each valve (Figure 4A) but in *H. glenelgensis* and *H. narracanensis*, the paired spines are longer, separated by a blade-like cutting edge (Figure 4D; Supplementary Material). The modified hooks of *H. australis* differ again and consist of a pair of curved, conjoined spines united on a common, slightly-raised base, that curve in the latero-dorsal plane (Figure 4B).

3.3 Comparative analyses

The five metrics of glochidial valve dimensions (L, H, Hg, H/L and Hg/L) were highly correlated, especially L, H, and Hg (Table 5). PCA separated the glochidial species into two distinct groups along the first principal component (Figures 6A, B), based mainly on size and H/L ratio along PC1, which explained 81% of the total variation (Table 6). Glochidia were further separated along the second and

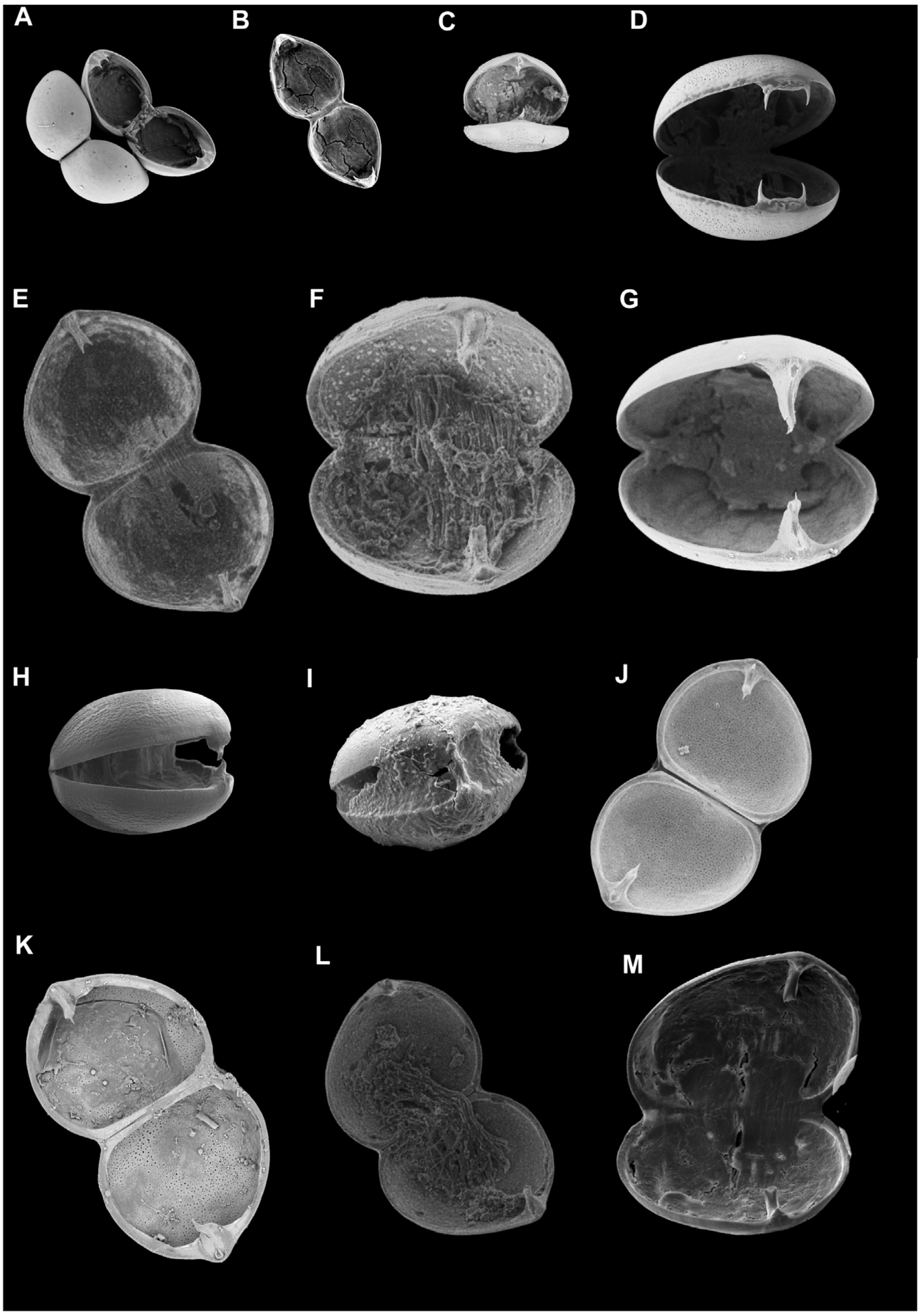

FIGURE 4
Open valves of glochidia showing hook morphology in species from this study. **Hyrrinae: Hyridellini and *Echyridella***: **(A)** *Cucumerunio novaehollandiae*, **(B)** *Hyridella australis*, **(C)** *Echyridella aucklandica*, **(D)** *Hyridella glenelgensis*, **(E)** *Hyridella drapeta*, **(F)** *Hyridella* sp. 'Diamond Creek', **(G)** *Echyridella menziesii*; **Velesunioninae**: **(H)** *Alathyria profuga*, **(I)** *Lortiella froggatti*, **(J)** *Alathyria pertexta pertexta*, **(K)** *Velesunio angasi*, **(L)** *Westralunio albertisi*, **(M)** *Westralunio carteri*. Images not to scale. Additional images and species are provided in the Supplementary Material.

third principal component axes (explaining an additional 15% of variation) according to shape (Á and H/L ratio) and LHL (Table 6). Principal Component 2 can be interpreted as contrasting scalene glochidia with those that have high H/L ratios and possess long sigmoidal hooks. Principal Component 3 contrasts glochidia that are tall (high H/L ratio) and bilaterally symmetrical with scalene glochidia that have short, wide valves. Many of the medium-sized and large glochidia could not be separated using these morphometrical criteria.

LDA showed that 97.5% of the between-group variance was accounted for by the first discriminant function (LD1), which reflected glochidial size and H/L ratio (Table 7). Only 1.8% of the between-group variance was accounted for by the second discriminant function (LD2), which further emphasized size but also separated

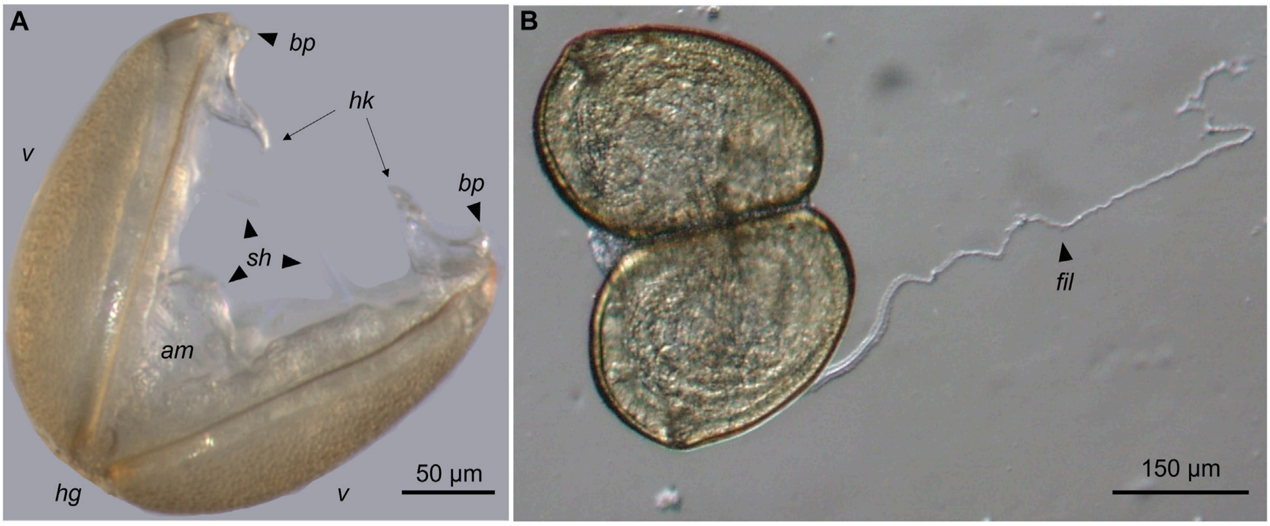

FIGURE 5
(A) Mature, live glochidium of *Velesunio angasi* (Mudginberri Billabong, Northern Territory, Australia) immediately post-release from a gravid female showing details of anatomical features: *am*–adductor muscle, *bp*–basal protuberance; *hg*–hinge; *hk*–hook, *sh*–sensory hairs, *v*–valves of shell. **(B)** Example of a larval filament (*fil*), this specimen of *Westralunio carteri* (Canning River, Western Australia).

TABLE 5 Pearson correlations between pairs of glochidial morphology variables.

	Length	Height	Hinge length	Angle of obliquity
Height	0.99			
Hinge length	0.99	0.97		
Angle of obliquity	0.75	0.70	0.80	
Height/Length ratio	−0.84	−0.78	−0.84	−0.67

species on hook length. The first two discriminant functions (LD1 and LD2) had an overall classification accuracy of 82.7%, a substantial improvement on the first discriminant function alone (57.5%). The three species with small glochidia, *C. novaehollandiae*, *H. australis* and *E. aucklandica*, were readily distinguished from all other species (Figure 6C) with no errors of omission or commission (Tables 8, 9). While there was considerable overlap in glochidial morphometrics of the remaining ten species, the first two discriminant functions classified most species with a high level of accuracy (Table 8). Linear Discriminant Analysis misclassified 27.5% of *A. pertexta pertexta* and 17% of *L. froggatti* glochidia. The largest errors of omission and commission were between *V. angasi* and *W. albertisi*, which show considerable overlap in size and shape metrics (Supplementary Table S2); 28% of *V. angasi* glochidia were misclassified as *W. albertisi* while 16.6% of *W. albertisi* were misclassified as *V. angasi* (Table 9).

Differences among species were also assessed using qualitative morphological traits by MCA (Figures 6D, E). Dimension 1 explained 40% of the variance in glochidial morphology; Dimension 2 explained 17%; and Dimension 3 explained 13% of the variance. Glochidia were partitioned into two broad morphological groups in the MCA biplots: i) large, subtriangular, scalene shells with a low to medium H/L ratio, and ii) small, suborbicular, bilaterally symmetrical shells with a high H/L ratio. The latter group also lacked a larval filament, and in the Australian Hyridellini, the hooks were bicuspid with a pair of short teeth projecting from a reduced base. Five of the velesunionine species shared the same morphological characters (Table 3) and were grouped together as "VELESUN" in the MCA biplots. The glochidia of Velesunioninae were typically large, subtriangular, and scalene with a sigmoidal hook terminating in a fluted, single pointed cusp (Figures 6D, E) except for *W. carteri* that had a complex cusp. The glochidia of all known members of the Velesunioninae possessed a larval filament except for *A. profuga*. The glochidia of several members of the Hyriinae, including *E. menziesii* from New Zealand, were similar in morphology to those of the Velesunioninae. Glochidia of the Hyridellini differed from those of the Velesunioninae in having bicuspid larval hooks or, in the case of *H.* sp. 'Diamond Creek', tricuspid. The hooks of *E. menziesii* were unicuspid, like those of the glochidia of the Velesunioninae but were also spinose and had more claw-like cusps, compared with the Velesunioninae that had hooks that were more fluted and had more blade-like cusps. In addition, the sigmoidal hooks of *Echyridella* spp. were buttressed and reinforced along their length.

4 Discussion

While adult freshwater mussels are moderately sedentary, the potential widespread dispersal is facilitated during the (usually)

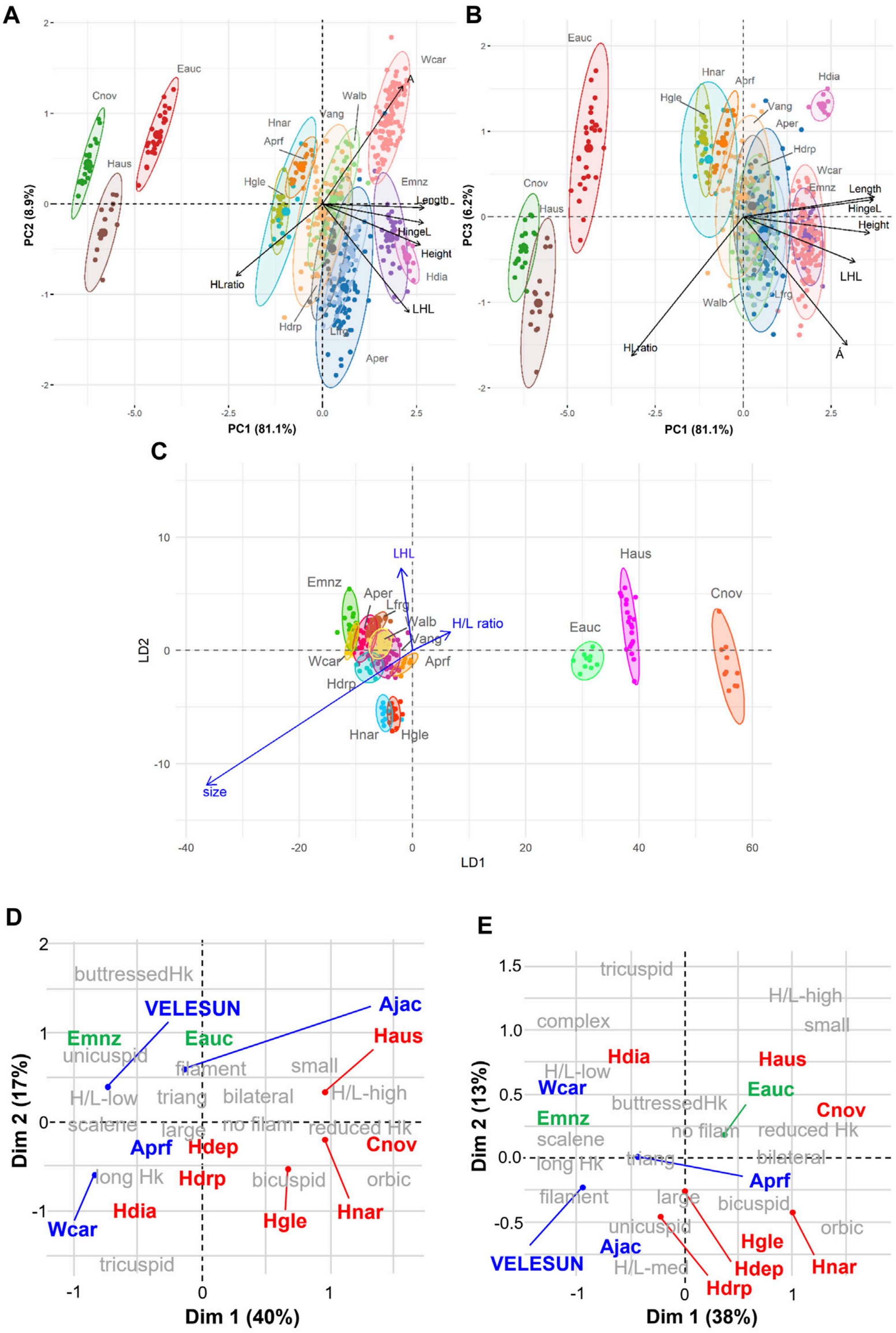

FIGURE 6
Principal component biplots for six morphometrical metrics of glochidial size and shape for **(A)** first and second principal components (PC1 and PC2, respectively), and **(B)** first and third principal components (PC1 and PC3, respectively). **(C)** Linear discriminant biplot for three morphometrics of glochidial size and shape for first and second linear discriminant functions (LD1 and LD2). Different colours in A-C represent different species. Multiple correspondence biplots for **(D)** Dimensions 1 and 2 (Dim 1 and Dim 2, respectively), and **(E)** Dimensions 1 and 3 (Dim 1 and Dim 3, respectively); subfamilies are colour-coded with Velesunioninae in blue text, Hyriinae in red text and the two *Echyridella* species in green; categorical data have grey text; 'VELESUN' includes five species of Velesunioninae (Vamb, Vang, Lfrg, Aper and Walb). See Table 3 for species abbreviations. Glochidial morphomertics: Á—angle of obliquity, H—height, L—length, HL ratio = H/L, LHL—larval hook length, Size = average of H and L. Abbreviations for categorical morphological traits (defined in Table 2): Hk—hook, med—medium, nofilam—larval filament absent, orbic—suborbicular, triang—subtriangular.

TABLE 6 Loadings on morphological variables for the first three principal components. The first three principal components accounted for 96% of the variation in glochidial morphometry (81.1%, 8.9% and 6.2% for PC1, PC2 and PC3, respectively).

Morphometric variable	PC1	PC2	PC3
Length	0.447	−0.021	0.102
Height	0.431	−0.226	−0.081
Hinge length	0.445	−0.105	0.087
Height/length ratio	−0.381	−0.396	−0.706
Larval tooth length	0.382	−0.598	−0.230
Angle of obliquity	0.354	0.651	−0.652

TABLE 7 Linear discriminant coefficients of Australasian glochidial morphometrics.

Variable	LD1	LD2	LD3	LD4
Variance explained	(97.5%)	(1.8%)	(0.5%)	(0.2%)
log(size)	−36.39	−11.91	−0.56	5.96
H/L ratio	6.69	1.64	5.93	24.65
log(hook length)	−2.03	7.25	3.54	−1.85
angle of obliquity	0.02	0.25	−0.38	0.16

parasitic larval phase, a comparatively rapid phase in their otherwise relatively long lifespan. The ecology of glochidial release from adult mussels and the morphology of the glochidia themselves are therefore integral components of freshwater mussel survival. Freshwater mussels globally are threatened and while the focus has often been on the adult life stage, an understanding of glochidial form, function and environmental requirements is also important for their conservation (Ferreira-Rodríguez et al., 2019; Aldridge et al., 2022; Geist et al., 2023). This study is the first to statistically compare the morphology and release mechanisms of glochidia from a large suite of species belonging to the Australasian Hyriidae. The addition of new data in the present study significantly expands the current knowledge of glochidial release mechanisms and glochidial morphological diversity among Australasian Hyriidae which are inherently important in taxonomy and conservation.

4.1 Glochidial release mechanism

The first description of glochidial release in functional conglutinates in Australasian Hyriidae (*H. australis*) was made by Jones et al. (1986); the present study now provides illustrations and descriptions of similar release mechanisms in a further two Australian hyriids (*H. glenelgensis* and *H. narracanensis*). Laboratory studies of the hyriid *E. aucklandica* provided the first photographic evidence of mesoconglutinate release (Melchior et al., 2021a) with further studies demonstrating that fishes are attracted to the mesoconglutinate as a food item that resembles prey (Melchior et al., 2021b); this supported the conjecture of Jones et al. (1986) that the conglutinate is a form of lure (i.e., functional conglutinate). Preliminary evidence indicates that glochidia of *H. glenelgensis* attach to the gills of *Galaxias maculatus* (Jenyns, 1842) (= *Mesites attenuatus* and *M. maculatus*) suggesting the mesoconglutinates may be ingested by host fish and thus, may indeed be a lure (Raadik et al., unpublished data). This phenomenon is not dissimilar to some species of North American Lampsilini (Unionidae) that release mesoconglutinates in a similar way (Barnhart et al., 2008). However, by comparison, the

TABLE 8 Confusion matrix summarising the classification accuracy of the first two linear discriminant functions in assigning glochidia to the correct species class. See Table 3 for species abbreviations.

	Observed species												
Predicted species	Aper	Aprf	Cnov	Eauc	Emnz	Haus	Hdrp	Hgle	Hnar	Lfrg	Vang	Walb	Wcar
A. pertexta	21	0	0	0	0	0	0	0	0	4	1	1	0
A. profuga	0	12	0	0	0	0	0	0	0	0	3	0	0
C. novaehollandiae	0	0	10	0	0	0	0	0	0	0	0	0	0
E. aucklandica	0	0	0	10	0	0	0	0	0	0	0	0	0
E. menziesii	0	0	0	0	14	0	0	0	0	0	0	0	1
H. australis	0	0	0	0	0	20	0	0	0	0	0	0	0
H. drapeta	1	0	0	0	0	0	8	0	0	0	0	0	0
H. glenelgensis	0	0	0	0	0	0	0	20	2	0	0	0	0
H. narracanensis	0	0	0	0	0	0	0	0	8	0	0	0	0
L. froggatti	5	0	0	0	0	0	0	0	0	29	0	0	0
V. angasi	0	0	0	0	0	0	0	0	0	0	39	10	0
W. albertisi	0	0	0	0	0	0	1	0	0	2	17	48	0
W. carteri	2	0	0	0	1	0	1	0	0	0	0	1	14

TABLE 9 Accuracy measures (%) for each species from an LDA classification based on two discriminant functions. See Table 3 for species abbreviations. Overall accuracy is 82.7%.

	Aper	Aprf	Cnov	Eauc	Emnz	Haus	Hdrp	Hgle	Hnar	Lfrg	Vang	Walb	Wcar
Producer's accuracy	72.4	100	100	100	93.3	100	80	100	80	82.9	65	80	63.3
User's accuracy	77.8	80	100	100	93.3	100	88.9	90.9	100	85.3	79.6	70.6	73.7

conglutinates of the hyriids are not as elaborate and sophisticated as seen in some Unionidae, such as the blackfly pupae mimics of *Ptychobranchus subtentum* (Say, 1829) (see Barnhart et al., 2008), complex baited brood lures of *Cyprogenia stegaria* (Rafinesque, 1820) or complex tethered pisciform brood lures of *Hamiota perovalis* (Conrad, 1834) (Hewitt et al., 2021).

The release of mesoconglutinates from *H. glenelgensis* and *H. narracanensis* was consistent with the observations of Jones et al. (1986) for *H. australis*, as well as *H. australis* from South Pine River, Queensland, observed in this study. Laboratory observations confirm that the glochidia of *H. glenelgensis* attach to the gills of fish but may not be retained on the fins (Fernando and Raadik, unpublished data). Although glochidial infestation on wild-caught fishes or from laboratory exposure studies have not been reported for these *Hyridella* species, the release of mesoconglutinates would suggest that glochidia of these species preferentially attach to gills in a manner like the glochidia of *E. aucklandica* (Melchior et al., 2021b). The colour change of the exhalant siphons in *H. glenelgensis* and *H. narracanensis* and pulsating or "waving" behaviour exhibited by *H. glenelgensis* and *H. narracanensis* (and by *H. australis* from Jones et al., 1986) may also provide additional stimulus to attract host fishes. Similarly, distinct morphological changes in siphon colour, size and shape prior to glochidial release have also been observed for the South American hyriid, *Castalia ambigua* Lamarck, 1819, that the authors suggested were host attraction mechanisms, although this species does not release conglutinates (Santos et al., 2021).

The present study also offers some contradictions on larval release to those reported in the literature; the glochidia from the Diamond Creek population of *H.* sp. 'Diamond Creek' did not possess a larval filament despite Atkins (1979) previously reporting its presence, and the glochidia of *E. menziesii* examined in this study does possess a larval filament (H.A. Jones, *pers. obs.*), although it was not reported by Melchior et al. (2021a). Larval filaments have also been reported in the velesunionine glochidia of *A. jacksoni* (Walker, 1981), *A. pertexta pertexta* (Klunzinger, 2020), *V. ambiguus* (Parodiz and Bonetto, 1963; Walker, 1981), *V. angasi* (Humphrey and Simpson, 1985), *W. carteri* (Klunzinger et al., 2013), and *L. froggatti* (this study). Amorphous mucous conglutinates are typically a passive entanglement mechanism and the larval filament may assist glochidia to remain in suspension over habitat substrates such as aquatic macrophytes, silty sediment, and woody debris, where potential host fishes are likely to frequent and subsequently contact glochidia from mucous webs (Matteson, 1948; Haag and Warren, 2003; Watters, 2008; Haag, 2012; Klunzinger et al., 2012; Klunzinger et al., 2013; Melchior et al., 2021a; Melchior et al., 2021b).

Glochidia arising from amorphous mucous conglutinates typically attach to the fins of host fishes, but occasionally also to gills and other body surfaces (Humphrey and Simpson, 1985; Barnhart et al., 2008; Klunzinger et al., 2012; Haag, 2012), whereas species that release glochidia via mantle lures, mesoconglutinates or freely into the water column, preferentially parasitise gills as a consequence of active host predation (Bauer and Wächtler, 2001; Strayer, 2008; Haag, 2012). While it was beyond the scope of this paper to explore host-fish relationships and deeper discussions about host fish use, the literature indicates that Australasian hyriid glochidia are host fish generalists, utilising a broad suite of both native and alien fishes that support metamorphosis to the juvenile stage, although not in equal proportions. The exception to this, however, is the glochidia of *E. aucklandica* that have only been found on the gills of New Zealand smelt, *Retropinna retropinna* (Richardson, 1848) and no other fishes, and indeed this has been the only host fish species to date shown capable of supporting metamorphosis of glochidia to the juvenile stage under laboratory conditions (Melchior et al., 2021b).

The glochidia of a larger group of hyriids, including *C. novaehollandiae*, *H. australis*, *H. depressa*, *H. drapeta*, *H. glenelgensis*, *H. narracanensis*, *A. profuga* and *E. aucklandica*, lack larval filaments. *Cucumerunio novaehollandiae* produces many more glochidia than other Australasian hyriids, owing to the small size of the glochidia and the large marsupia of adult female mussels (Jones et al., 1986; Jones, 2014). Jones (2014) found that some *C. novaehollandiae* produce broods of up to 4 million glochidia, whereas *H. depressa* has glochidial counts of less than 120,000 (Byrne, 1998). However, *C. novaehollandiae* reproduces once per year whereas other hyriids can produce multiple broods throughout the warmer months of the year (e.g., *H. depressa* from Jones et al., 1986; Byrne, 1998). *Cucumerunio novaehollandiae* is also a known gill parasite (Jones, 2014) and release of free glochidia into the water column and surrounding habitat (that tends to be free from fine sediments that could otherwise smother glochidia) as fish pass by would mean that their mode of release is passive. A strategy of release of mesoconglutinates for this species may be negated by scour and potential dislodgement from the stronger currents in which this species inhabits; thus, releases of free glochidia but in very large numbers may nullify losses in faster stream currents. On the other hand, *C. novaehollandiae* occupies microhabitats that are between or in the lee of large boulders, so the glochidia may accumulate within the protection of the separation zones and within the boundary layer of the stream where fishes may seek shelter from water currents, resulting in close contact with the large numbers of glochidia and infestation of the gills. At least some margaritiferids release their tiny glochidia in a similar way and occupy similar habitats elsewhere (Araujo et al., 2000; Hastie et al., 2000). Melchior et al. (2021a) reported a mean fecundity in *E. aucklandica* of 17,840 glochidia (range 1,737 to 34,570) compared to nearly double that exhibited by coexisting *E. menziesii* (mean 44,016, range 28,840 to 72,000). Here, through its specialised "brood luring" mesoconglutinates, *E. aucklandica* may face a trade-off in increased energy costs for reduced fecundity. Conversely, *E. menziesii*, as a host generalist, uses a more energy-efficient reproductive strategy, which may allow for greater reproductive output.

The Australian Hyriinae are almost exclusively associated with lowland sections of coastal rivers of Victoria, New South Wales, and southern Queensland (Walker et al., 2014) that arise steeply in the escarpment of the Great Dividing Range (Rustomji et al., 2009). By Australian standards, this region is relatively high in species diversity with as many as six species of freshwater mussel occurring within a river system. The rivers of southeastern, coastal Australia are perennial, with good water quality, relatively high in dissolved oxygen and water clarity (Jones and Byrne, 2014; Walker et al., 2014). *Echyridella aucklandica*, which produces mesoconglutinates, is almost exclusively a lotic habitat species, occurring primarily in relatively clear northern New Zealand coastal streams (Marshall et al., 2014; Melchior et al., 2021a). In contrast, velesunionine species of Australia and *E. menziesii* of New Zealand are widely distributed in lakes and rivers throughout much of each country and are typically resident in more languid and (periodically at least) turbid waters, many of which are characteristic of temporary and/or lentic waterbodies (Marshall et al., 2014; Walker et al., 2014). Studies on patterns of distribution and abundance of Australasian freshwater mussels are limited, but generally, predation pressures and food limitation are thought not to limit freshwater mussel distribution (Haag and Warren, 1998; Raikow and Hamilton, 2001; Hewitt et al., 2021). The available, albeit limited, data show Australian freshwater mussels to be host generalists, coinciding with studies pertaining to species that release glochidia freely or contained in amorphous mucous conglutinates; there is little evidence amongst these species that the distribution patterns of freshwater mussels, within drainages, are limited by host fish behaviour and movements (Walker, 1981; Humphrey and Simpson, 1985; Klunzinger et al., 2012; Jones, 2014). Rather, spatial patterns within rivers appear to be controlled by abiotic factors (Humphrey and Simpson, 1985; Brainwood et al., 2006, Brainwood et al., 2008a, Brainwood et al., 2008b; Jones, 2006, Jones, 2014; Jones and Byrne, 2010, Jones and Byrne, 2014; Klunzinger et al., 2015), but contemporary distributions have been fragmented by habitat loss (Jones and Byrne, 2010, Jones and Byrne, 2014; Klunzinger et al., 2015; Sheldon et al., 2020) and declines in fish populations in many waterbodies may be inhibiting recruitment of young freshwater mussels (Byrne, 1998; Walker, 2017). Haag and Warren (1998), however, found no evidence of partitioning amongst unionids in North American streams because of limited space, but highlighted the importance of the host-fish and glochidial release relationship, noting host-specificity for species that utilise lure mechanisms to attract host species. It is worth noting that the concept of host-specificity amongst unionid mussels using such glochidial prey-mimetic lures rarely applies to a single fish species, but rather to a very limited number of fish species (e.g., Hewitt et al., 2021). Hewitt et al. (2019) noted as well that many glochidial–host interactions still remain to be identified, making it difficult in some cases to assign particular freshwater mussel species as either host specialists or host generalists. In locations where Australian freshwater mussel diversity is relatively high, whether seasonality in glochidial release and host specificity may have evolved to partition host fish resources is debatable, particularly given the currently limited published data available to pursue this hypothesis. Nevertheless, in high clarity waters where most Australasian Hyriinae occur, mesoconglutinates may be a more efficient mechanism for infesting host fishes than the passive infestation strategy typical of velesunionine species that tend to occur in more turbid habitats (for which visual attractants would serve limited purpose). Only more complete data on field-infested fishes to identify hosts for species that release glochidia in mesoconglutinates may elucidate the underlying evolutionary advantage of this strategy amongst the Hyriinae.

4.2 Glochidial shell morphology

In agreement with Jones et al. (1986), glochidia from Queensland populations of *C. novaehollandiae*, *H. australis* and *H. drapeta* have bifurcated hooks, and similar shell size and shape. *Hyridella glenelgensis* and *H. narracanensis* also have hook bifurcation, although the separation between cusps is set wider apart than other species studied to date, apart from *H. australis*. Hooks are, however, much straighter, and perpendicular to opposing valves than *H. australis* that are more laterally splayed. Bifurcated hooks in glochidia of *H. depressa* were illustrated in each of Jones et al. (1986) and Jupiter and Byrne (1997) (see also Supplementary Figure S4).

Atkins (1979) illustrated *H.* sp. ‘Diamond Creek’ glochidia as having hooks similar in form to several members of the Velesunioninae, including *A. jacksoni* and *V. ambiguus* (Walker, 1981; Klunzinger, 2020), *L. froggatti* and *A. profuga* (this study), *V. angasi* (Humphrey and Simpson, 1985 and this study), and *A. pertexta pertexta* (Klunzinger, 2020), which typically have s-shaped hooks with singular pointed cusps on opposing valves. We questioned whether the species Atkins (1979) illustrated may have been *V. ambiguus* given this similarity and because research by Jones et al. (1986) revealed hooks that are bifurcated in *Hyridella* species, very different in form to velesunionine glochidia. Furthermore, the dimensions of this species’ glochidia are distinct from northern populations of *H. drapeta*. To investigate, specimens of gravid adults were collected from the same location as in Atkins (1979); however, whilst the adults were similar in form to other *H. drapeta*, unpublished genetic evidence has indicated that the taxon is not *H. drapeta* and is an undescribed species. The present study confirmed that this taxon does indeed have s-shaped hooks, in support of Atkins (1979); however, upon closer examination of hooks from SEM photography, the cusps were revealed to be tripartite rather than singular as illustrated by Atkins (1979), and thus different to the velesunionine glochidia, thereby supporting their position in the Hyridellini as opposed to Velesunioninae as originally implicated by the illustrations of Atkins (1979). Similarly, hooks of *E. menziesii* superficially resemble those of Velesunioninae, but the hooks are distinctively spinose with wide supporting buttresses and may also be mildly tripartite. *Westralunio carteri* has atypical and complex hooks in comparison to other Velesunioninae (Klunzinger et al., 2013 and this study). *Westralunio albertisi*, however, has glochidia that are morphologically similar to other Velesunioninae and although Klunzinger (2023a) described them as being divergent, there is the possibility that the specimens examined may have not been fully mature and hooks had not yet fully developed.

Glochidia from *C. novaehollandiae*, *H. australis* and *E. aucklandica* were exceptional in their smaller size compared to those of the other species included in this study. *Cucumerunio novaehollandae* has perhaps one of the smallest recorded glochidia globally, rivalled only by a few margaritiferid species, *Margaritifera dahurica* (Middendorff, 1850), *Margaritifera falcata* (Gould, 1851), *M. margaritifera* and *Cumberlandia monodonta* (Say, 1829) (see Harms, 1909; Howard, 1915; Murphy, 1942; Bauer, 1994; Nezlin et al., 1994; Pekkarinen and Valovirta, 1996; Baird, 2000; Ieshko et al., 2014; Vikhrev et al., 2019), all of which are similarly miniscule in shell length (<60 μm). It has been proposed that such small glochidia (<65 μm in size) may be an adaptation to enhance suspension in the water column (Barnhart et al., 2008). At the other extreme, glochidia of *E. menziesii*, *W. carteri* and *H.* sp. 'Diamond Creek' are the largest of the Australasian hyriids measured to date with mean shell lengths between 300 and 330 μm. While they are certainly not the largest in the world, they are comparable in size to several unionids including *Prolasmidonta heterodon* (Lea, 1859) (= *Alasmidonta heterdon*), *Pressodonta viridis* (Rafinesque, 1820) (= *Alasmidonta viridis*), *Utterbackiana suborbiculata* (Say, 1831) (= *Anodonta suborbiculata*), *Anodonta cygnea* (Linnaeus, 1758) and *Utterbackia imbecillis* (Say, 1829) (see Surber, 1912; Claes, 1987; Niemeyer, 1992 in Wächtler et al., 2001; Hoggarth, 1999; Kennedy and Haag, 2005). Among Hyriidae, few glochidia exceed shell lengths of more than 300 μm, although the South American hyriids *Diplodon charruanus* (d'Orbigny, 1835) (= *Diplodon trivialis*), *Diplodon martensi* (Ihering, 1893) (= *Diplodon decipiens* + *Diplodon suppositus*), *Diplodon delodontus* (Lamarck, 1819), *Diplodon multistriatus* (Lea, 1831) (= *Diplodon expansus*), *Diplodon garbei* (Ihering, 1910), *Diplodon uruguayensis* (Lea, 1859) (= *Diplodon hasemani*), *Diplodon iheringi* (Simpson, 1914), *Diplodon obsolescens* (Baker, 1913), *Diplodon paulistus* (Ihering, 1893) (= *D. paulista*), *Diplodon suavidicus* (Lea, 1859), *Diplodon rhuacoicus* (d'Orbigny, 1835) (= *Diplodon trivialis* + *Diplodon yaguaronis*) and *Triplodon corrugatus* (Lamarck, 1819) reach lengths of 300–320 μm (Bonetto, 1951; Bonetto, 1959; Bonetto, 1961; Bonetto and Ezcurra, 1963; Parodiz and Bonetto, 1963; Alvarenga and Ricci, 1977; Bonetto et al., 1986; Mansur, 1999; Mansur and Campos-Velho, 1990; Ricci et al., 1990; Semenas et al., 1994; Mansur and Silva, 1999; Viozzi and Brugni, 2001; Beasley et al., 2005; Pimpão et al., 2012), making the glochidia of *H.* sp. 'Diamond Creek' perhaps the largest among the Hyriidae.

Small glochidia that either lack hooks or have short hooks are typically gill parasites (Hoggarth and Gaunt, 1988; Araujo and Ramos, 1998; Bauer, 2001; Barnhart et al., 2008; Haag, 2012). The position of the adductor muscle in such glochidia results in long resistance arms, adapted for a large area of sweep that aids in attachment to host gill tissue (Hoggarth and Gaunt, 1988). In comparison, larger glochidia with longer hooks tend to parasitize the fins or epidermis of their hosts and are adapted for stronger gripping strength with a hook (or "larval tooth"), a large adductor muscle and depressed shell (Hoggarth and Gaunt, 1988; Bauer, 2001; Barnhart et al., 2008; Haag, 2012). Small glochidia that parasitise gills of their host fishes have also been shown to metamorphose to their juvenile form and grow considerably in size while still on the host (Araujo and Ramos, 1998; Bauer, 2001), whereas larger, hooked glochidia that typically parasitise fins and epithelia tend not to grow while on their host (Bauer, 2001). This is corroborated in Australasian studies of glochidial parasitism. The small, short-hooked glochidia of *C. novaehollandiae* and *E. aucklandica* parasitise gills of their hosts, grow while on the fish and are attached to their host for a considerable period (Jones, 2014; Melchior et al., 2021b). Indeed, Melchior et al. (2021b) showed that *E. aucklandica* larvae increased in size from 99.5 ± 4.7 μm SD to 449.2 ± 28.2 μm SD during metamorphosis while encysted on New Zealand Common Smelt *R. retropinna*, a quadrupling in size during the parasitic mussel stage. Similarly, *C. novaehollandiae* undergoes a considerable increase in size while attached to host fishes (Jones, 2014). In contrast, glochidia with larger shells that are equipped with larger hooks, e.g., *A. profuga*, *A. jacksoni*, *E. menziesii*, *H. drapeta*, *V. angasi*, *V. ambiguus* and *W. carteri*, do not appear to grow much on the host, if at all, and may be largely phoretic (Walker, 1981; Humphrey and Simpson, 1985; Widarto, 1993; Klunzinger et al., 2012; Jones, 2014; Melchior et al., 2021b; Melchior, 2021).

Most of the glochidia from species in this study are typical of those from other Hyriidae (i.e., South American Hyrrinae), in having shells with a subtriangular outline. *Hyridella glenelgensis*, *H. narracanensis* and to a lesser extent, *C. novaehollandiae*, are atypically round and more 'horseshoe' or nearly D-shaped. *Cucumerunio novaehollandiae* bears some resemblance in outline to South American glochidia of *C. ambigua* (Pimpão et al., 2012), albeit with much smaller glochidial shells. Although glochidia of *C. novaehollandiae* appear more rounded than most other hyriid glochidia, with a non-protruding ventral apex, their shells still come to a narrower point along the ventral edge than the wider hinge line, unlike D-shaped or horseshoe shaped glochidia such as North American *Lampsilis* or *Quadrula* species (Surber, 1912), for example, that have a much broader ventral edge, wider than their hinge line, giving a characteristic D-shape or horseshoe shape.

The soft anatomy of Australasian hyriid glochidia has not been examined in detail, but Jupiter and Byrne (1997), Percival (1931), and Parodiz and Bonetto (1963) have hinted at anatomical variation among species. The preservation techniques used in this study focused on glochidial shell examination for detailed SEM photography of shell structures. However, some preserved specimens show sensory hair arrangements and other soft anatomy in *H.* sp. 'Diamond Creek' and *H. australis* (see Supplementary Material), while a live image of *V. angasi* collected at the time of glochidial release shows sensory hair arrangements like those illustrated by Parodiz and Bonetto (1963). It would appear from the few images or illustrations available that *V. ambiguus* and *V. angasi* have sensory hairs located toward the dorsal portion of the shell, more basally to the adductor muscle, whereas *H. australis* and *H. depressa* have series of sensory hairs in several locations from just behind the larval hooks as well as medially and basally to the adductor muscle. The arrangement of sensory hairs may be a function of different attachment mechanism in gill versus fin parasitism (Hoggarth and Gaunt, 1988; Nivischenko et al., 2022).

4.3 Values and limitations of glochidial diversity in phylogenetic relationships

Although glochidial morphology and metrics of shell characters have some value as a tool to help identify some taxa (e.g., Kennedy and Haag, 2005; Pimpão et al., 2012), and indeed have been used for taxonomic re-arrangements in some instances (e.g., Pimpão et al., 2012; Pfeiffer and Graf, 2015; Cruz and Quesada, 2017; Lopes-Lima et al., 2018; Miyahira et al., 2019; Chernyshev et al., 2020), they are not always sufficiently unique within species or among taxonomic groups, as this study has demonstrated. Yet, this study also showed that for most taxa examined, certain characteristics were common at the subfamily and tribal level. For instance, larval hook bifurcation is a trait that appears to be restricted exclusively to the Hyridellini (*C. novaehollandiae*, *H. australis*, *H. depressa*, *H. drapeta*, *H. glenelgensis* and *H. narracanensis*). This is not yet fully resolved, however, given that glochidia of *Hyridella guppyi* (Smith, 1885), *Hyridella misoolensis* (Schepman, 1897) and *Virgus beccarianus* (Tapparone Canefri, 1883) have not yet been examined. Additionally, if *H.* sp. 'Diamond Creek' proves to be a member of the Hyridellini, it would be the only exception to this characteristic given its very different hook to other Hyridellini.

Glochidia of the vast majority Velesunioninae (*A. jacksoni*, *A. pertexta pertexta*, *L. froggatti*, *V. ambiguus*, *V. angasi*, *W. albertisi* and *W. carteri*) have a larval filament and subtriangular scalene shells of a relatively similar size, although *A. profuga* is the exception in being smaller and lacking a larval filament. In terms of hook morphology, most velesunionines have long, sigmoidal singularly pointed, fluted hooks whereas *W. carteri* has a complex hook structure. The glochidia of *W. carteri* are also much more scalene in shape and larger than the other glochidia within the subfamily. Again, however, glochidial characteristics are not yet fully resolved for the subfamily given that glochidia of additional taxa within the Velesunioninae remain to be examined (see note in provisional glochidia key below).

As for the New Zealand *Echyridella*, glochidia of the two species included in this study exhibit wide differences in several characters including size, shape, hook length, presence or absence of shell sculpturing, and the presence or absence of a larval filament. Both, however, have spinose hooks with wide buttressed supports. The glochidia of the third New Zealand hyriid, *Echyridella onekaka* Fenwick and Marshall, 2006, remains unknown. In a practical sense, identifying glochidia on host fishes could be problematic for *L. froggatti* and *Velesunio* species, *A. jacksoni* and *V. ambiguus*, and *A. pertexta* and *V. ambiguus* groupings, that may be sympatric in distribution and may produce glochidia around the same time of year (Walker, 1981; Humphrey and Simpson, 1985; Jones et al., 1986; Klunzinger, 2020). However, where LDA failed to accurately predict some species on glochidial shell metrics, in most cases this would be a moot point. For instance, where *W. carteri* was mistaken for *E. menziesii* or *L. froggatti* for *A. profuga* and *vice versa*, this would be irrelevant given these species are found in widely separate geographical ranges. Where uncertainty is an issue, genetic barcoding of glochidia on host fishes has proven useful for resolving such uncertainty (Kneeland and Rhymer, 2007; Boyer et al., 2011; Zieritz et al., 2012, 2018). Thus, using glochidial characteristics in species identification must be treated with caution.

5 Conclusion

(1) Of the 29 extant species of Australasian Hyriidae, glochidia are now described from 17 species (58.6%), five of which (17.2%) are described in this study for the first time.

(2) In combination with other published studies covered in the discussion, this study has added to the diversity of glochidia and mechanisms of release among Australasian freshwater mussels and this will be beneficial in helping to understand host fish use, dispersal mechanisms and designing captive breeding technologies in future work.

(3) Through statistical comparative analyses, this study highlights the utility of glochidial morphological diversity as an important aspect of systematics that, when coupled with genetic analyses, is likely to aid in modernizing the taxonomy of the Australasian Hyriidae. However, as statistical analyses showed in this study, reliance on glochidial characters alone for freshwater mussel systematics is not recommended, even though it revealed similarities and differences among and between taxonomic groups. Examining yet unknown Australasian hyriid glochidia and integrating glochidial characters with adult and juvenile morphology and molecular phylogenetic techniques will be a logical next step for modernizing the taxonomy of the Australasian Hyriidae and indeed for the Unionoida as a whole.

(4) Different glochidial release mechanisms were found between the Hyridellini and Velesunioninae. While this distinction appears to be related to different riverine typologies, the evolutionary significance remains to be fully explored among and between taxa.

Provisional key to the glochidia of Australasian Hyriidae

1. Glochidia small, length <100 μm; larval thread absent—2
- Glochidia large, length >100 μm, larval thread absent—4

2(1). Glochidial height/length ratio >105%; hooks unicuspid ……………………………………*Echyridella aucklandica*.
- Glochidial height/length ratio <105%; hooks bicuspid—3

3(2). Glochidial shape suborbicular; hooks reduced to a pair of spinose cusps set on a broad base (larval hook length <10 μm) ……………………………*Cucumerunio novaehollandiae*
- Glochidial shape subtriangular; hooks conjoined and arching (larval hook length >10 μm) ……………*Hyridella australis*

4(1). Glochidial shape suborbicular, larval hook reduced, length <20 μm; larval thread absent—5
- Glochidial shape subtriangular; larval hook length long, >20 μm; larval thread present or absent—6

5(4). Glochidial length <253 μm, height <230 μm and size <240 μm ……………………………………*Hyridella narracanensis*
- Glochidial length >253 μm, height >230 μm and size >240 μm ……………………………………*Hyridella glenelgensis*

6(4). Larval hook widely buttressed; larval thread present . . . .*Echyridella menziesii*
- Hook not widely buttressed; larval thread present or absent—7

7(6). Glochidial length <300 µm; larval thread present—8
- Glochidial length <300 µm; larval thread present or absent—9

8(7). Hinge length >220 µm; larval hook length >60 µm and cusps tripartite Hyridella sp. 'Diamond Creek'
- Hinge length <220 µm; larval hook length <60 µm . . . . *Westralunio carteri*

9(7). Larval hook bicuspid; larval thread absent—10
- Larval hook unicuspid; larval thread present or absent—11

10(9). Larval hook length >58 µm . . . .*Hyridella drapeta*
- Larval hook length <58 µm . . . .*Hyridella depressa*

11(9). Glochidial height/length ratio <86%; larval thread present or absent—12
- Glochidial height/length ratio >86%; larval thread present—13

12(11). Larval hook length >50 µm; larval thread present . . . .*Velesunio ambiguus*
- Larval hook length <50 µm; Larval thread absent . . . .*Alathyria profuga*

13(11). Larval hook length >60 µm—14
- Larval hook length <60 µm—16

14(13). Glochidial height <235 µm . . . . *Lortiella froggatti*
- Glochidial height >235 µm—15

15(14). Larval hook length <60 µm . . . .*Alathyria jacksoni*
- Larval hook length >60 µm . . . . *Alathyria pertexta pertexta*

16(13). Larval hook length <52 µm; mean Á <15° . . . .*Velesunio angasi*
- Larval hook length >52 µm; mean Á >15° . . . . *Westralunio albertisi*

Note: the following taxa of Australasian Hyriidae are excluded from this identification guide because no information on their glochidia is currently available:

Hyriinae: Hyridellini

Hyridella misoolensis (Schepman, 1897).
Hyridella guppyi guppyi (Smith, 1885).
Hyridella guppyi aipiana McMichael, 1956.
Hyridella sp. Jones et al., unpublished data (see Jones et al., 1986).
Hyridella sp. 'Southwest Victoria' Raadik et al., unpublished data

Velesunioninae

Velesunio wilsonii (Lea, 1859).
Velesunio sentaniensis (Haas, 1924).
Velesunio sp. B (see Baker et al., 2004).
Velesunio sp. D (see Baker et al., 2004).
Velesunio sp. 'Magela Creek' (see Kleinhenz et al., 2019).
Microdontia anodontaeformis Tapparone Canefri, 1883.
Alathyria pertexta wardi (Iredale, 1943a).
Alathyria pertexta magnifica McMichael and Hiscock, 1958.
Alathyria condola Iredale, 1943b.
Westralunio flyensis (Tapparone Canefri, 1883).
Westralunio inbisi inbisi Klunzinger, Whisson, Zieritz, Benson, Stewart & Kirkendale, 2022.
Westralunio inbisi meridiemus Klunzinger, Whisson, Zieritz, Benson, Stewart & Kirkendale, 2022.
Lortiella rugata (Sowerby, 1868).
Lortiella opertanea Ponder and Bayer, 2004.

Subfamily *incertae sedis*

Echyridella onekaka Fenwick and Marhsall, 2006.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

MK: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. HJ: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Visualization, Writing–original draft, Writing–review and editing, Validation. CH: Conceptualization, Data curation, Investigation, Methodology, Resources, Validation, Visualization, Writing–review and editing. MM: Conceptualization, Data curation, Investigation, Methodology, Resources, Validation, Visualization, Writing–review and editing. TR: Conceptualization, Data curation, Investigation, Methodology, Resources, Validation, Visualization, Writing–review and editing. ST: Investigation, Visualization, Writing–review and editing. LC: Investigation, Resources, Writing–review and editing, Visualization. FS: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing–review and editing.

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Conflict of interest

This study was conducted in a manner free from any activity that could be construed as a conflict of interest.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2023.1305077/full#supplementary-material

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