

# Description of a new genus and two new species of Darwinulidae (Crustacea, Ostracoda), from Christmas Island (Indian Ocean) with some considerations on the morphological evolution of ancient asexuals

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**ABSTRACT.** Darwinulidae is believed to be one of the few metazoan taxa in which fully asexual reproduction might have persisted for millions of years. Although rare males in a single darwinulid species have recently been found, they may be non-functional atavisms. The representatives of this family are characterized by a slow evolutionary rate, resulting in a conservative morphology in the different lineages over long time frames and across wide geographic ranges. Differences between species and genera, although often based on small details of valve morphology and chaetotaxy, are nevertheless well-recognizable. Five recent genera (*Darwinula*, *Alicenula*, *Vestalenula*, *Penthesilenula* and *Microdarwinula*) and about 35 living species, including also those left in open nomenclature, are included in this family. Previous phylogenetic analyses using both morphological characters and molecular data confirmed that the five genera are good phyletic units.

Here, we report on the results of a study on darwinulid ostracods from Christmas Island (Indian Ocean). The taxonomic investigation led to the description of a new genus and two new species, namely *Isabenula humphreysi* gen. nov. sp. nov. and *Vestalenula* sp. E, this latter being left in open nomenclature, because only one individual was available. A new phylogeny of the family, using 30 morphological characters of 23 described species, is here presented. A key to the genera of Recent Darwinulidae, based on the morphology of valves and soft parts in adult females, is also provided. The discovery of *Isabenula* gen. nov., which shares intermediate morphological features with the genera *Vestalenula* and *Penthesilenula*, opens new opportunities to critically reconsider previous hypotheses on tempo and mode of evolution in the family Darwinulidae and on the phylogenetic relationships between its representatives.

**KEY WORDS:** Ostracods, ancient asexuals, morphology, taxonomy, evolution, *Isabenula humphreysi* gen. nov. sp. nov., *Vestalenula* sp. E.

## INTRODUCTION

A few metazoan lineages, namely bdelloid rotifers, some groups in oribatid mites, some lineages in the stick insects, strains of the brine shrimp *Artemia salina* and darwinulid ostracods, are considered to be putative “ancient asexuals”, i.e. animal groups that have been reproducing without males (obligate parthenogenesis) over long geological time scales (see reviews in SCHÖN et al., 2009 and SCHWANDER et al., 2011). They represent evolutionary scandals (JUDSON &

NORMARK, 1996; SCHÖN et al., 2009), because they violate ruling evolutionary hypotheses which predict that fully asexual lineages are doomed to early extinction (MAYNARD SMITH, 1978). Nevertheless, in the Darwinulidae, the presence of long-lived asexual genera (e.g., >145 Myr for *Alicenula*) and species (20-25 Myr for the type species *Darwinula stevensoni*) is widely supported by fossil evidence (MARTENS et al., 2003; SCHÖN & MARTENS, 2003) although the asexual status of the family as a whole is still debated (MARTENS & SCHÖN, 2008)

Darwinulidae occur in freshwater (including lentic, lotic, and interstitial environments), but also in (semi-)terrestrial habitats and occasionally in brackish waters. Both ecological specialists and generalists, as well as intermediate forms, exist in this family, and taxa with the broadest ecological tolerance also have the widest geographical distribution (VAN DONINCK et al., 2002, 2003).

Darwinulidae are thought to reproduce apomictically (BUTLIN et al., 1998; SCHÖN et al., 1998, but see also GORELICK, 2003 for an alternative view). At least some species show extremely low rates of molecular and morphological evolution (SCHÖN et al., 2003) and a possible explanation of that could be the presence of highly efficient DNA repair mechanisms in this family (SCHÖN & MARTENS, 1998; 2003).

The existence of a continuous fossil record in the Darwinulidae is of the utmost importance for research on tempo and mode of biological evolution in organisms lacking sexual reproduction (SCHÖN et al., 2009). According to MARTENS et al. (2003), the presence of putative males in fossil Darwinulidae must be rejected for all post-Triassic (c. 208 Myr) records.

Rare males in a single darwinulid living species, *Vestalenula cornelia*, have recently been described by SMITH et al. (2006), but they may be non-functional, as they seem to have rudimentary reproductive organs, namely ovate structures that possibly represent vestigial or remnants of the Zenker organs, and hemipenes lacking a labyrinth and tubular structures differently from other Recent podocopid ostracods (Figs 3h and 3i in SMITH et al., 2006); in addition, no spermatozoa have been observed in either males or sympatric females (SMITH et al., *loc. cit.*).

The presence of rare males does not necessarily affect the status of “ancient asexuals” of the Darwinulidae. Long before the finding of those phenotypic males in living Darwinulidae, ROSSETTI & MARTENS (1996) stated that even if rare males would occur in this family, it is probable that they are atavistic and non-functional, as is

known in other asexual taxa such as *Artemia salina* (e.g., BROWNE, 1992; HALKETT et al., 2005). The occurrence of rare males has been reported in other parthenogenetic species of non-marine ostracods (GEIGER et al., 1998; YIN et al., 1999; NAMIOTKO et al., 2005), as well as in other groups of ancient asexuals, such as the oribatid mites (SCHÖN et al., 2008; HEETHOFF et al., 2009). These males may be dysfunctional, especially in older asexual lineages, since mutations are likely to accumulate in pathways necessary for exclusively male functions (BUTLIN et al., 1998). In some arthropod taxa, ancestral genes for male production can be maintained unexpressed over evolutionary time, and the loss of the complex process of sexuality may not be irreversible: for example, there is evidence that the Crotoniidae, a family of oribatid mites, have re-evolved sexuality from parthenogenetic ancestors (DOMES et al., 2007; but see GOLDBERG & ISIC, 2008). The discovery of males in *V. cornelia* does allow the decisive rejection of the presence of male specimens of *Darwinula stevensoni*, figured by BRADY & ROBERTSON (1870) and TURNER (1895), because the morphology of the copulatory appendages in *Vestalenula* clearly indicates that the presumed hemipenes of the *D. stevensoni* male constituted different parts of its anatomy (MARTENS & SCHÖN, 2008).

A worldwide taxonomic revision of the Recent Darwinulidae, based on both valve and soft part characters, has been performed by ROSSETTI & MARTENS (1998). Before that, all Recent darwinulid species were grouped in two genera, i.e. *Darwinula* and *Microdarwinula*. New synonymies were proposed and all the retained species were allocated to five genera, three of which were erected as new (*Alicenula*, *Penthesilenula* and *Vestalenula*, the latter two comprising two species-groups each).

In the subsequent years, new species of Recent Darwinulidae have been described, mainly from interstitial or semi-terrestrial habitats (ROSSETTI & MARTENS, 1999; MARTENS & ROSSETTI, 2002; PINTO et al., 2003, 2004, 2005; ARTHEAU, 2007; SMITH et al., 2006); these studies brought the total number of species of the family to

c. 35, representing roughly less than 2% of the global specific diversity of freshwater ostracods (MARTENS et al., 2008). Using the set of diagnostic morphological characters suggested by ROSSETTI & MARTENS (1998), all of these new species were easily accommodated into existing species-groups and genera, the latter being confirmed to be good phyletic units through cladistic analysis of both morphological characters and molecular data (MARTENS et al., 2005).

Here, we present new data on darwinulids collected in groundwater habitats of Christmas Island, a small, elevated coral-capped island of the Indian Ocean. The analysis of samples led to the description of a new genus and two new species; other material, consisting either of immature or incomplete specimens, was identified at the generic level. In particular, the new genus shows intermediate morphological features in the limb chaetotaxy between two extant darwinulid genera (*Vestalenula* and *Penthesilenula*), and its valve morphology is not sufficient to separate it from a species-group of the genus *Vestalenula*. This unexpected finding

opens intriguing questions on the patterns of morphological evolution and speciation in the Darwinulidae and on the origin and dispersal abilities of these ancient asexuals.

## MATERIALS AND METHODS

### *Study site and sampling locations*

Christmas Island (135 km<sup>2</sup>) is located in the Indian Ocean, about 350 km south-west of Java and 1200 km west of the Australian continent (Fig. 1), on an oceanic plate fixed to the northern flank of the Australian plate, with which it has drifted north at the same speed (in the Eocene, the northern edge of the Australian plate was about 2000 to 2800 km south of its current location) (LUNT, 2003). The island is the summit of a submarine mountain, formed by volcanic activity that began about 80 million years ago. It is an example of an isolated intra-plate island, resulting from a single volcano breaking off the ridge with part of the sub-ridge magma chamber beneath it (WHITTAKER, 1998). Its surface is mostly characterised by Tertiary carbonate



Fig. 1. – Map of Christmas Island. Its location in the Indian Ocean is shown by a star.

sediments deposited over the core of basaltic rocks. A detailed description of the island geology is provided by NAMIOTKO et al. (2004). Water drainage is mostly underground and karstic (GRIMES, 2001). The subterranean system of the islands includes a number of freshwater, marine, anchialine and terrestrial habitats. The island hosts a diversified fauna, with rare and endemic species (HUMPHREYS & EBERHARD, 2001; NAMIOTKO et al., 2004).

Ostracods used for this work were provided by W.F. Humphreys (Western Australian Museum) at Christmas Island in 1998 and preserved in alcohol. Four samples out of a total of ten were considered in the present study, i.e. only those containing darwinulids (as complete specimens, empty carapaces or isolated valves). The material was generally scarce and occasionally also damaged. In total, only three complete adult females were recovered. Sampling sites and collection methods are listed below.

- Grants Well, cave # CI-11, approx., cave # CI-64, approx. coordinates 10°28'08"S, 105°39'01"E. Sample BES 5740. Collected on March 28, 1998 by W.F. Humphreys, 24 hour filter on stream flow.
- Henderson's Spring, cave # CI-64, approx. coordinates 10°29'13"S, 105°40'40"E. Sample BES 5837. Collected on April 6, 1998 by W.F. Humphreys and R. Webb, net over outlet for 24 hours.
- Pump house at Ross Hill Gardens, cave # CI-64,, approx. coordinates 10°29'13"S, 105°40'40"E. Sample BES 5758. Collected on March 3, 1998 by W.F. Humphreys, outflow.
- Hugh's Dale (Dale No 2), Cave # CI-77, approx. coordinates 10°28'07"S, 105°33'40"E. Sample BES 5864. Collected on April 7, 1998 by S.M. Eberhard, hand net.

#### *Taxonomic analysis*

Dissections were done under a stereomicroscope. Valves were used for Scanning Electron Microscopy (SEM) and then stored dry in micropalaeontological slides; soft parts were dissected in glycerine and sealed in permanent glass slides. Descriptions of chaetotaxy used the

nomenclature proposed by DANIELOPOL (1968, 1970) and adapted by ROSSETTI & MARTENS (1998), PINTO et al. (2004) and SMITH & KAMYIA (2008). Only morphological features that allow discrimination between darwinulid genera and species were considered here. All the analysed material is lodged in the Ostracod Collection (OC) of the Royal Belgian Institute of Natural Sciences (RBINSc), Brussels.

#### *Phylogenetic analysis using morphological data*

Methods used for the construction of phylogenetic trees mostly conform to the criteria adopted by MARTENS et al. (2005); a new matrix was constructed from literature data to include additional species that have recently been described. Twenty-three species were retained, i.e. those with detailed description of both valve (characters 1-12) and soft part (characters 13-30) morphology (see Tables 1 and 2). Polarizations of character states are given in Table 1, but these are not necessarily the polarizations used by the programs during the tree-building. Phylogenetic trees were constructed for all morphological data combined. Input order of taxa was as in the matrix in Table 2. *Darwinula stevensoni* was used as outgroup, based on the results presented in MARTENS et al. (2005). A non-darwinulid would have been preferable as an outgroup, but the morphology of the Darwinulidae is too specialised to allow inclusion of non-darwinulid ostracod species and at the same time test for relationships within the Darwinulidae (HORNE et al., 2005). It must thus be understood that the topology of obtained trees is relative to the outgroup position of *D. stevensoni*.

Three different analyses were performed: (1) maximum parsimony analysis, without character weighting and without Dollo-up option; (2) neighbour joining analysis, without character weighting and without Dollo-up option; (3) neighbour joining analysis, with character weighing and with Dollo-up option. In the latter (3<sup>rd</sup>) analysis, all characters were designated 'Dollo-up', meaning that we assume that character states can change from plesiomorphic



TABLE 1

List of morphological characters and polarization of character states used for the phylogenetic analyses.

A. Valves	
1	Lateral view: sloping (0), sub-squarish (1) or rounded (2)
2	L/R (0) or R/L (1) overlap
3	Postero-ventral keel on RV absent (0), short (1) or long (2)
4	Ventro-frontal internal tooth (mostly in LV) long (2), short (1) or absent (1)
5	Posterior internal tooth (mostly in LV) in ventral position present (0) or absent (1)
6	Posterior internal tooth (mostly in LV) in caudal position present (0) or absent (1)
7	Hinge adont (0), with simple large cardinal teeth (1) or subdivided large cardinal teeth (2)
8	Size: >0.6 mm (0) or =<0.6 mm (1)
9	Le/H ratio >2.2 (0), between 1.8 and 2.2 (1) or <1.8 (2)
10	Brooding space externally visible (0) or not (1)
11	Position of Cms: towards the front (0) or central (1)
12	Muscle scars: average number of scars >8 (0) or =<8 (1)
B. Soft parts	
13	A1, first segment with 2 (0) or 1 (1) dorsal seta(e)
14	A1, "exopodite" with 3 (0) or 2 (1) setae
15	A1, second segment with (0) or without (1) dorso-apical seta
16	A1, third segment with (0) or without (1) ventro-apical seta
17	A1, fourth segment with (0) or without (1) ventro-apical seta
18	A1, fourth segment with 2 (0) or 1 (1) large dorsal seta(e)
19	A2, exopodite with 2 (0) or 1 (1) seta(e) (+spine)
20	A2, first segment of endopodite with 2 (0) or 1 (1) ventro-apical seta(e)
21	Md-palp, penultimate segment: seta <i>z</i> long (0) or short (1)
22	Md-palp, penultimate segment: seta <i>y</i> long (0), short (1) or absent (2)
23	Md-palp, last segment: «poil stevensoni» present (0) or absent (1)
24	Md-palp, last segment: number of apical claws: 5 (0), 4 (1) or 3 (2)
25	Md-palp, last segment: seta <i>a</i> claw-like (0), spine-like (1), absent (2)
26	Md-palp, last segment: seta <i>c</i> present (0) or absent (1)
27	T1, penultimate palp segment: number of seta(e): 2 (0) or 1 (1)
28	CR: number of seta(e): 2 (0), 1 (1) or absent (2)
29	P-abd: with projections (0), smooth (1) or absent (2)
30	Caudal seta present (0) or absent (1)

to apomorphic conditions, but that such changes are irreversible. Soft part characters were weighted five times more than valve characters, because the limb morphology appears to be more conservative and therefore more informative regarding deep-rooted relationships in Darwinulidae (MARTENS et al., 2005). The phylogenetic trees were validated using bootstrapping (10,000 replicates). Analyses 1 and 2 were done using PaupUp for Windows (CALENDI & MARTIN, 2005), the third analyses used PAUP\* 4.0b10 (SWOFFORD, 2002).

*Abbreviations used in the text and in the figures*

#### Valves

Cp	carapace
LV	left valve
RV	right valve
Cms	central muscle scar(s)
iv	internal view
lv	lateral view
H	height of valves
Le	length of valves

TABLE 2

Data matrix used in the phylogenetic analyses. Character numbers as in Table 1.

Taxa	Characters																																													
<i>D. stevensoni</i>	0	1	2	2	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3										
<i>A. serricaudata</i>	0	0	2	1	1	0	(01)	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	1	2	0	1	1	0	1	0	1	1	0	1	1	0	1									
<i>A. fuscabdominis</i>	0	0	2	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	2	1	1	2	1	1	1	0	1	1	0	1	0	1	1	0	1								
<i>A. inversa</i>	0	1	2	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	2	1	2	0	1	1	0	1	1	0	1	1	0	1	1	0	1	1								
<i>V. boteai</i>	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	0	2	0	1	1	1	1	1	1	1	1	1	1	1	1							
<i>V. botocuda</i>	1	0	1	1	1	1	0	1	1	0	0	1	1	0	0	1	1	0	0	1	0	0	1	0	2	0	1	0	2	0	1	0	0	1	0	0	0	0	0	0						
<i>V. irajai</i>	1	0	1	1	1	1	1	0	0	0	1	1	1	0	0	1	1	0	0	1	0	0	1	0	2	0	1	0	2	0	1	1	0	1	1	0	1	1	1	0	1					
<i>V. marmonieri</i>	0	0	1	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	0	1	0	1	0	2	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1					
<i>V. molopoensis</i>	0	0	1	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	0	1	0	1	0	2	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1					
<i>V. paglioli</i>	(01)	0	1	1	1	1	0	1	1	0	0	(01)	1	1	1	0	1	1	0	1	0	0	1	0	2	0	1	1	0	1	1	0	1	1	0	1	1	0	1	1	1	0	1			
<i>V. sp. D</i>	1	0	1	1	1	1	0	1	1	0	0	?	1	1	1	0	1	1	1	1	0	1	0	2	0	1	1	0	2	0	1	1	1	0	1	1	1	1	1	1	1	1	1			
<i>V. matildae</i>	1	0	0	0	1	1	1	0	1	0	0	(01)	1	1	1	1	1	1	1	1	0	1	0	2	0	1	1	0	2	0	1	1	0	1	1	0	1	1	1	1	1	1	0	1		
<i>V. sp. E</i>	1	0	0	0	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	0	1	0	2	0	1	1	0	2	0	1	1	0	1	1	1	1	1	1	1	2	1	1	1		
<i>V. cornelia</i>	1	0	0	0	1	1	0	1	1	0	0	(01)	1	1	1	1	1	1	1	1	0	1	0	2	0	1	1	0	2	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1		
<i>V. carveli</i>	1	0	0	0	1	1	0	1	0	0	0	1	1	1	1	1	1	1	1	1	0	1	0	2	0	1	1	0	2	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	
<i>I. humphreysi</i>	1	0	1	1	1	1	1	0	1	0	0	1	0	1	1	1	1	1	1	1	0	1	0	2	0	1	1	0	2	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	
<i>P. incae</i>	1	0	2	2	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	2	1	1	1	2	1	
<i>P. aotearoa</i>	1	0	2	1	1	0	0	(01)	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	2	1	1	1	1	
<i>P. kohanga</i>	(01)	0	2	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	1	0	1	1	1	1	1	1	1	2	1	1	1	1	1	1	
<i>P. brasiliensis</i>	1	0	2	(12)	0	1	0	1	(01)	0	0	(01)	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	(01)	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
<i>P. reidae</i>	(01)	0	2	1	0	1	0	1	(01)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1
<i>M. zimneri</i>	2	0	2	1	0	1	1	1	(12)	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	2	2	1	1	1	1	1	1	1	2	2	1	1	1	1
<i>M. inexpectata</i>	(12)	0	2	1	0	1	2	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	2	2	1	1	1	1	1	1	1	2	2	1	1	1	1	1

Limbs and soft parts	
A1	Antennula
A2	Antenna
Md	Mandibula
CR	caudal ramus
P-abd	post-abdomen
<i>b, c, w, x, y, z</i>	specific setae on Md-palp

## RESULTS

### *Taxonomic account*

Class Ostracoda LATREILLE, 1802  
 Subclass Podocopa G.W. MÜLLER, 1894  
 Order Podocopida SARS, 1866  
 Suborder Darwinulocopina SOHN, 1988

Superfamily Darwinuloidea  
 BRADY & NORMAN, 1889

Family Darwinulidae BRADY & NORMAN, 1889

Genus *Vestalenula* ROSSETTI & MARTENS, 1998

### *Diagnosis* (after ROSSETTI & MARTENS, 1998)

Small darwinulids, with valves elongate or short and subquadrate. RV with a posteroventral, external keel, the latter short (*boteai*-group) or elongate (*danielopoli*-group). LV with internal anteroventral tooth, no caudal or caudoventral internal teeth. Hinge adont. Mostly c. 0.6 mm or less in length. Valves with L/R overlap along anterior, ventral and posterior margins. Dorsal margin straight over part of length. Cms always situated towards the front in adult females. Large caudal brooding cavity externally visible. First segment of A1 with one dorsal seta, second segment with two ventral setae; fourth segment without ventro-apical seta. A2 with one long seta and a spine on exopodite. Seta *y* on penultimate Md-palp segment short; last segment with five apical claws. Adults mostly with CR, P-abd smooth or absent.

### *Vestalenula* sp. E

(Figs 2, 4F-H)

### *Material investigated*

One ovigerous female, with valves stored dry in

a micropalaeontological slide (used for SEM) and soft parts dissected in glycerine and kept in a sealed slide (OC3269), collected at Grants Well (see above for details).

### *Remark*

The genus *Vestalenula* includes four as-yet-unnamed species: sp. A and sp. B (DANIELOPOL, 1980) belonging to the *danielopoli*-group, and sp. C and sp. D (ROSSETTI & MARTENS, 1999) belonging to the *boteai*-group. Therefore, in the present paper we name our species in open nomenclature as *Vestalenula* sp. E. This species belongs to the *danielopoli*-group within this genus.

### *Diagnosis*

Small darwinulid (<0.4 mm). Valves subquadrate in lv, Le/H c 2.12. LV overlapping RV. Cp posteriorly enlarged to form a brooding pouch. Hinge adont. RV with an elongate ventral keel (*danielopoli*-group) relatively distant from the postero-ventral corner. LV with a long antero-ventral tooth. Cms with 6-7 spots arranged in a circular rosette. Third segment of A1 without ventro-apical seta. First endopodal segment of A2 with one ventro-apical seta. Penultimate segment of Md-palp with seta *y* short and seta *z* extending beyond last segment; this latter with five apical claws, setae *b* and *c* present. CR a hirsute base carrying a seta. P-abd absent. Males unknown.

### *Measurements*

Le(LV)=373 µm, H(LV)=176 µm, Le(RV)=358 µm, H(RV)=169 µm (n=1).

### *Differential diagnosis*

*Vestalenula* sp. E can be easily differentiated from most of its congeners by its tiny size. In fact, all the other species in this genus are 0.40-0.60 mm, apart from *V. matildae* (0.38 mm) (ROSSETTI & MARTENS, 1998, 1999; MARTENS & ROSSETTI, 2002; PINTO et al., 2003). This latter species is indeed the closest congener of *V.* sp. E, but it has a P-abd (not present in *V.* sp. E), slightly more elongated valves in lv (Le/H >2.2), and a higher number of spots (c. 9) in the Cms.

*Distribution*

The species is known from its type locality only.

Genus *Isabenula* gen. nov.

*Derivation of name*

We are pleased to name the genus after Dr Isabelle Schön (RBINSc, Brussels), the leading world authority in the application of DNA-based molecular techniques to non-marine ostracods,

for her outstanding contribution to the study of genetics and ecology of the Darwinulidae. The name is a combination of ‘Isabe’ (from Isabelle) and ‘nula’ for assonance with other darwinulid generic names. The etymology of the name Isabelle can be traced to Jezebel, which is transliterated from both the Hebrew Iyzebel and the Greek Iezabel, and means, among other things, “chaste”. This name seems most appropriate for a genus of supposedly ancient asexuals.

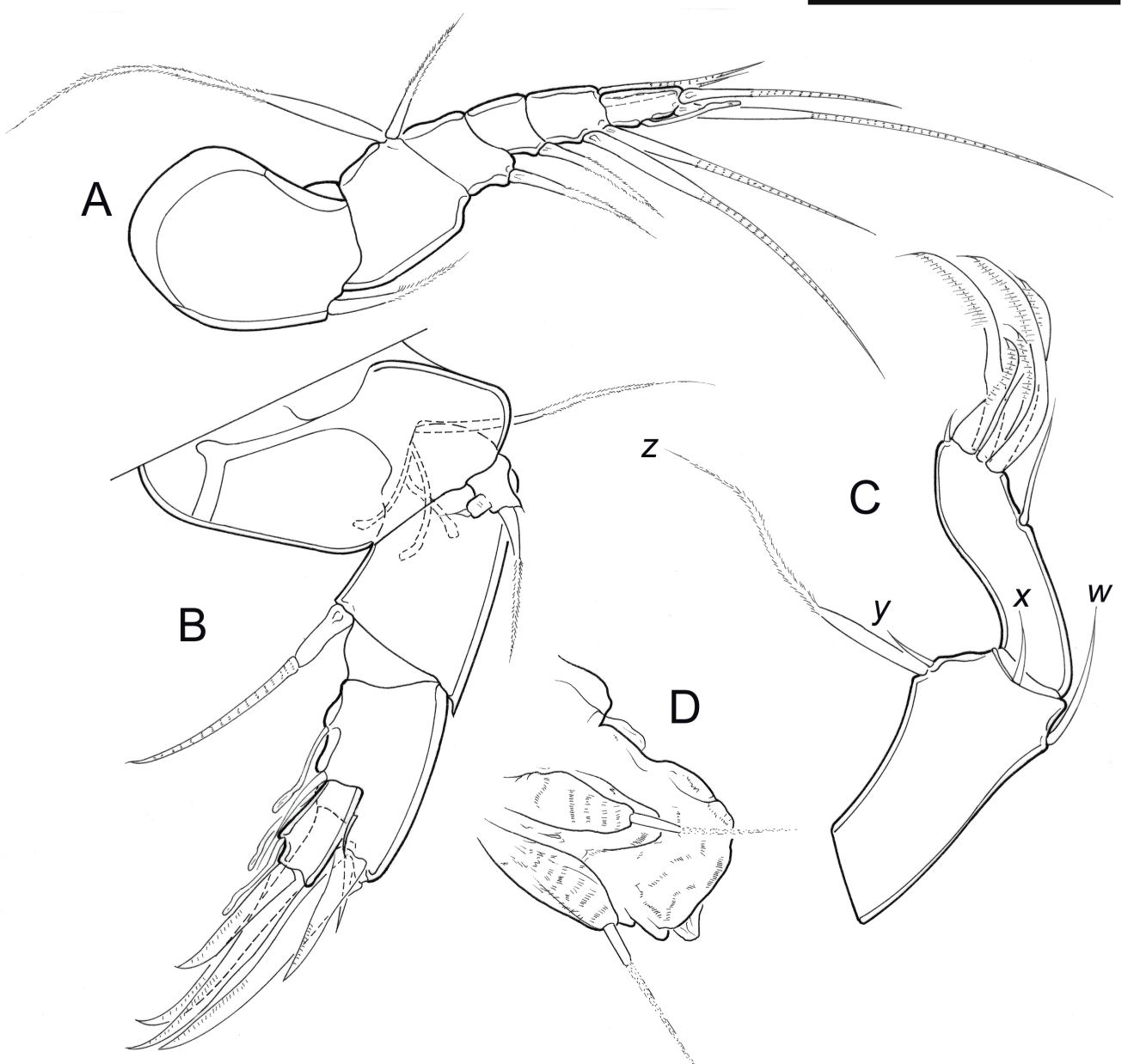


Fig. 2. – *Vestalenula* sp. E. A: A1 (OC3269). B: A2 (idem). C: Md-palp (idem). D: CR (idem). Scale bar: 50 µm.



TABLE 3

Differences in the soft part morphology of A1, A2 and Md-palp between the genera *Penthesilenula*, *Isabenula* n.gen., and *Vestalenula*. Shaded areas indicate more plesiomorphic states for the selected characters (see Table 1 and 2).

		<i>Penthesilenula</i>	<i>Isabenula</i> n.gen.	<i>Vestalenula</i>
A1, first segment	dorsal seta(e)	2	2	1
A1, second segment	ventral setae	3	2	2
	dorsal seta	present	present	absent
A1, third segment	ventral seta	present	absent	absent
A1, fourth segment	ventral seta	present	absent	absent
	dorsal seta(e)	2	1	1
A2, "exopodite"	long seta(e)	2	1	1
A2, third segment	ventral seta(e)	2	2	1 or 2
Md-palp, penultimate segment	setae <i>z</i> and <i>y</i> , relative length	$z \approx y$	$z \approx y$	$z \gg y$
Md-palp, last segment	seta <i>a</i>	present	absent	absent

#### Diagnosis

Medium-sized darwinulids, Cp sub-quadrate in lv, with externally visible brood pouch. RV with short postero-ventral keel, LV with antero-ventral internal tooth. A1 with two dorsal setae on first segment; second segment with dorso-apical seta. A2 exopodite with one seta and a lateral spine. Penultimate segment of Md-palp with setae *z* and *y* both long, last segment lacking seta *a*.

#### Type species (here designated):

*Isabenula humphreysi* Rossetti, Pinto & Martens sp. nov.

#### Differential diagnosis

*Isabenula* gen. nov. can be distinguished from the genera *Darwinula*, *Alicenula*, *Penthesilenula* and *Microdarwinula* by the presence of a short keel on RV. Since this feature is also found in the *boteai*-group of the genus *Vestalenula*, the valve morphology is not sufficient to separate *Isabenula* gen. nov. from *Vestalenula*. Nevertheless, given the apparent morphological stasis of the soft parts in the whole family Darwinulidae, *Isabenula* gen. nov. and *Vestalenula* can clearly be distinguished by the chaetotaxy of A1 (two dorsal setae on first segment and one dorsal seta on second segment in *Isabenula* gen. nov., one dorsal seta on first segment and dorsal seta absent on second segment in *Vestalenula*) and Md-palp

(penultimate segment with setae *z* and *y* both long in *Isabenula* gen. nov., seta *z* considerably longer than seta *y* in *Vestalenula*) (see Table 3).

#### *Isabenula humphreysi* sp. nov.

(Figs 3, 4A-E)

#### Type locality

Hugh's Dale (Dale No 2), Cave # CI-77 (see above for details).

#### Holotype

An ovigerous female, with valves stored dry in a micropaleontological slide (used for SEM) and soft parts dissected in glycerine and kept in a sealed slide. Material deposited in the Ostracod Collection of the Royal Belgian Institute of Natural Sciences, Brussels (OC3270).

#### Paratype

An adult female, with valves stored dry in a micropal slide (used for SEM) and soft parts dissected in glycerine and kept in a sealed slide (OC3271).

#### Derivation of name

This species is named after Dr William F. Humphreys (Western Australian Museum, Perth) for his important studies on the fauna of subterranean water systems and for providing us with the material described in the present paper.

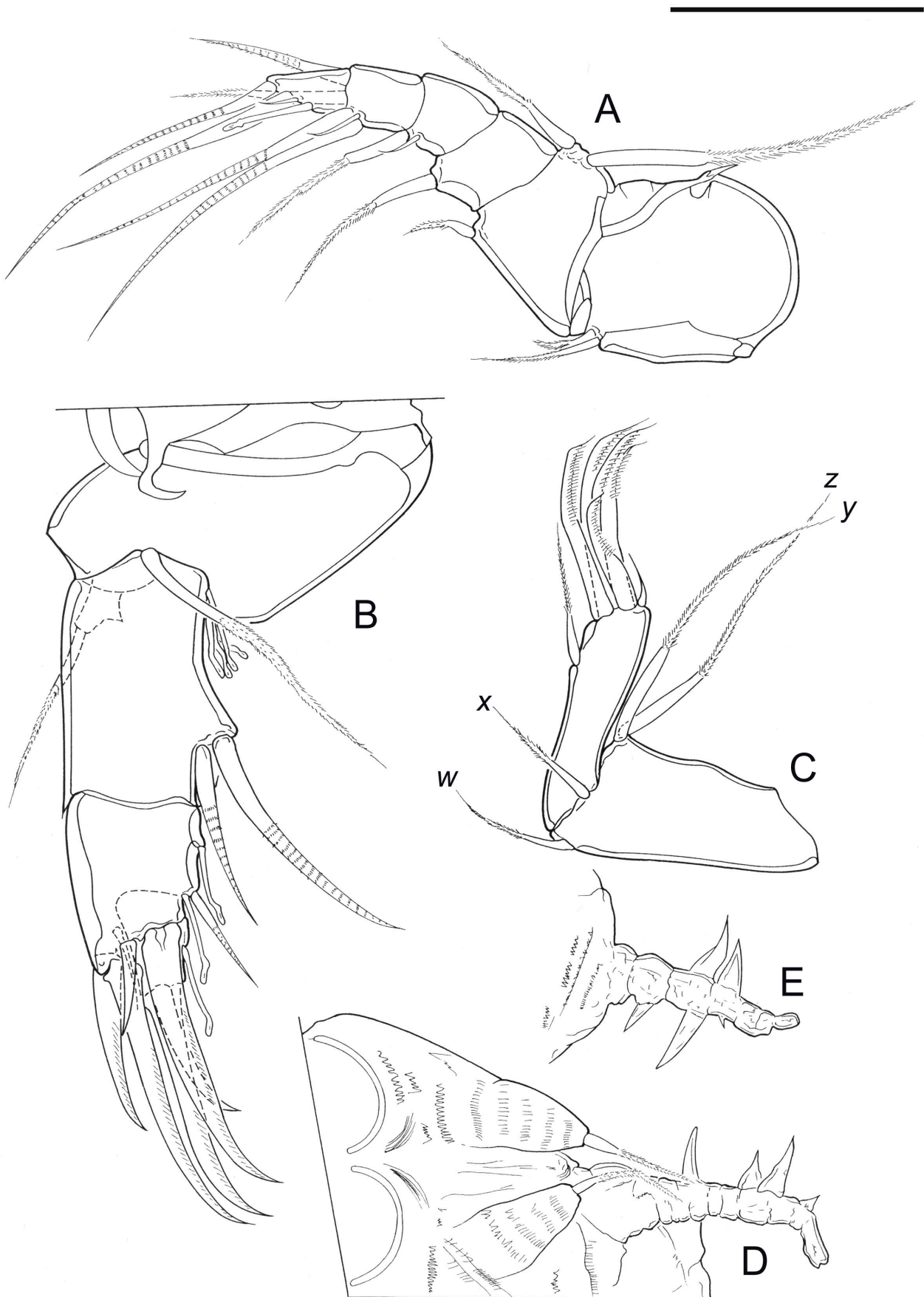


Fig. 3. – *Isabenula humphreysi* gen. nov.sp. nov. A: A1 (OC3270). B: A2 (idem). C: Md-palp (idem). D: CR and P-abd (idem). E: P-abd (OC3271). Scale bar: 50  $\mu$ m.

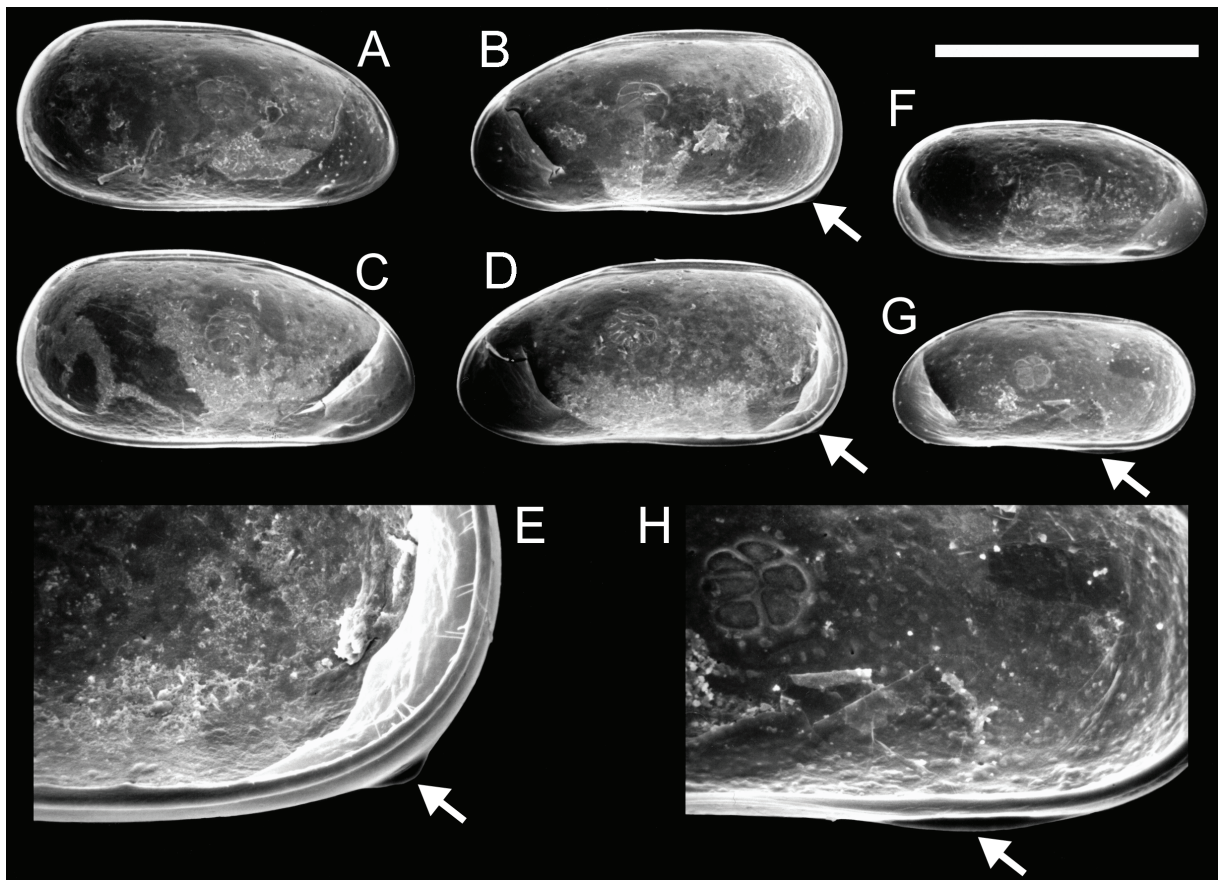


Fig. 4. – A-E: *Isabenua humphreysi* gen. nov. sp. nov. F-H: *Vestalenula* sp. E. A: LV, iv (OC3271). B: RV, iv (idem). C: LV, iv (OC3270). D: RV, iv (idem). E: RV, iv, detail postero-ventral corner (idem). F: LV, iv (OC3269). G: RV, iv (idem). H: RV, iv detail posterior part of ventral margin (idem). Arrows indicate the position of the keel on RV. Scale bar: 300  $\mu$ m for A-D, F,G; 100  $\mu$ m for E, H.

#### Diagnosis

Medium-sized species. Cp sub-quadrate in lv. LV overlapping RV. Hinge adont. Caudal brooding chamber externally visible. RV with short keel inserted at the postero-ventral corner, LV with antero-ventral internal tooth. Cms with c 7 spots arranged in a rosette. A1 with two dorsal setae on first segment, second segment with one dorso-apical seta and two ventral setae, one c half as long as the other; third and fourth segment with one long dorsal seta each. A2 exopodite with one seta and a lateral spine, first endopodal segment with ventral-apical setae. Penultimate segment of Md-palp with setae *z* and *y* both long, subequal; last segment with five terminal claws, setae *b* and *c* present. CR a broad conical base carrying a seta. P-abd long and cylindrical, with clear lateral projections. Males unknown.

#### Measurements

Le(LV)= 458-479  $\mu$ m, H(LV)=232-246  $\mu$ m.

Le(RV)=443-465  $\mu$ m, H(RV)= 224-229  $\mu$ m (n=2).

#### Distribution

The species is known from its type locality only.

#### Differential diagnosis

As for the genus.

#### Remarks

1. KLIE (1932, Figs 74 and 76) illustrated the Md-palp, CR and P-abd of a darwinulid species from Sunda Islands that he identified as *Darwinula malayica* MENZEL, 1923; this species was then transferred to the genus *Penthesilenula* by ROSSETTI & MARTENS (1998). In particular, KLIE (*loc. cit.*) figured the presence of two long *z* and *y* setae on the penultimate Md-palp segment, associated with the absence of seta *a*, i.e. a unique combination of characters now only known for *Isabenua humphreysi* sp. nov.



(Table 1). Actually, also the seta *c* on penultimate Md-palp segment appeared to be missing. Differences with *Isabenula humphreysi* sp. nov. can be found in the length of the CR setae and the shape of the P-Abd, which is respectively shorter and with lateral projections in this latter species. Due to the brief (and possibly erroneous) description by KLIE (1932) and the absence of reference material, the correct determination of this darwinulid species remains doubtful, but its synonymy with *Isabenula humphreysi* sp. nov. can almost certainly be excluded.

2. *Vestalenula flexuosa* was placed in the *boteai*-group by ROSSETTI & MARTENS (1999) because of the presence of a short keel on RV. However, after the description of *Isabenula* gen. nov., this species could belong either to *Vestalenula* or *Isabenula* gen. nov., as only the valves of this species are known. Only the discovery of animals with preserved soft parts will allow its precise allocation.

*Other material investigated (not illustrated)*

Two immature specimens (one with damaged LV) and an empty Cp of a juvenile from Henderson's Spring; one immature specimen from Pump house at Ross Hill Gardens; an isolated LV from Hugh's Dale (Dale No 2) (see above for details on sampling stations). All these specimens are provisionally assigned to the *africana*-group of the genus *Penthesilenula* ROSSETTI & MARTENS, 1998, due to the sub-quadrate shape of valves in lv and the presence of two rounded internal teeth (antero-ventral and postero-ventral) on LV. These teeth, however, can be also observed in larval specimens of the genus *Vestalenula*. Differences in valve morphology suggest the possible presence of two species. Due to the scarcity of the material, no further description is given here.

**Key to genera of Recent Darwinulidae based on the morphology of valves and soft parts in adult females**

1a. Cp rounded or slightly elongated in lv, no externally visible brood pouch, Cms centrally positioned ..... *Microdarwinula*

b. Cp elongate or sub-quadrate in lv, with externally visible brood pouch, Cms situated towards the front ..... 2

2a. RV without postero-ventral keel, LV with or without internal teeth. A1 with two dorsal setae on first segment; second segment with one dorso-apical seta and three ventral setae. A2 expodite with two setae and a spine ..... 3

b. RV with postero-ventral keel, LV with antero-ventral internal tooth. A2 expodite with one seta and a spine. Md-palp, seta *a* absent ..... 4

3a. Last segment of Md-palp with less than five claws (three or four), penultimate segment with seta *y* short or absent ..... 5

b. Last segment of Md-palp with five claws, penultimate segment with seta *y* long ..... *Penthesilenula*

4a. A1 with one dorsal seta on first segment; second segment with dorso-apical seta absent. Md-palp, penultimate segment with seta *z* long, seta *y* short ..... *Vestalenula*

b. A1 with two dorsal setae on first segment; second segment with dorso-apical seta. Md-palp, penultimate segment with setae *z* and *y* long ..... *Isabenula* gen. nov.

5a. RV overlapping LV,  $Le > 0.65$  mm. LV without internal teeth. Second segment of A1 endopodite with two large dorsal setae. Md-palp, penultimate segment with seta *z* long, seta *y* short; last segment with 'poil *stevensoni*'; setae *a* and *b* present ..... *Darwinula*

b. RV overlapping LV or LV overlapping RV,  $Le \leq 0.65$  mm. Md-palp, penultimate segment with seta *z* short, seta *y* absent; last segment without 'poil *stevensoni*', seta *a* absent ..... *Alicenula*

### Cladistic analysis of darwinulid species based on morphological features

Figure 5 shows the cladogram constructed with the parsimony criterion from all morphological data combined (valve and soft part characters), and using *Darwinula stevensoni* as outgroup. The

tree shows a separation into two main groups: *Alicenula* – *Vestalenula* – *Isabenula* gen. nov. on one hand, and *Penthesilenula* – *Microdarwinula* on the other, although bootstrap support for each of these two clusters is low. *Darwinula* remains outside of either group, even when it is not designated as outgroup (results not shown). In

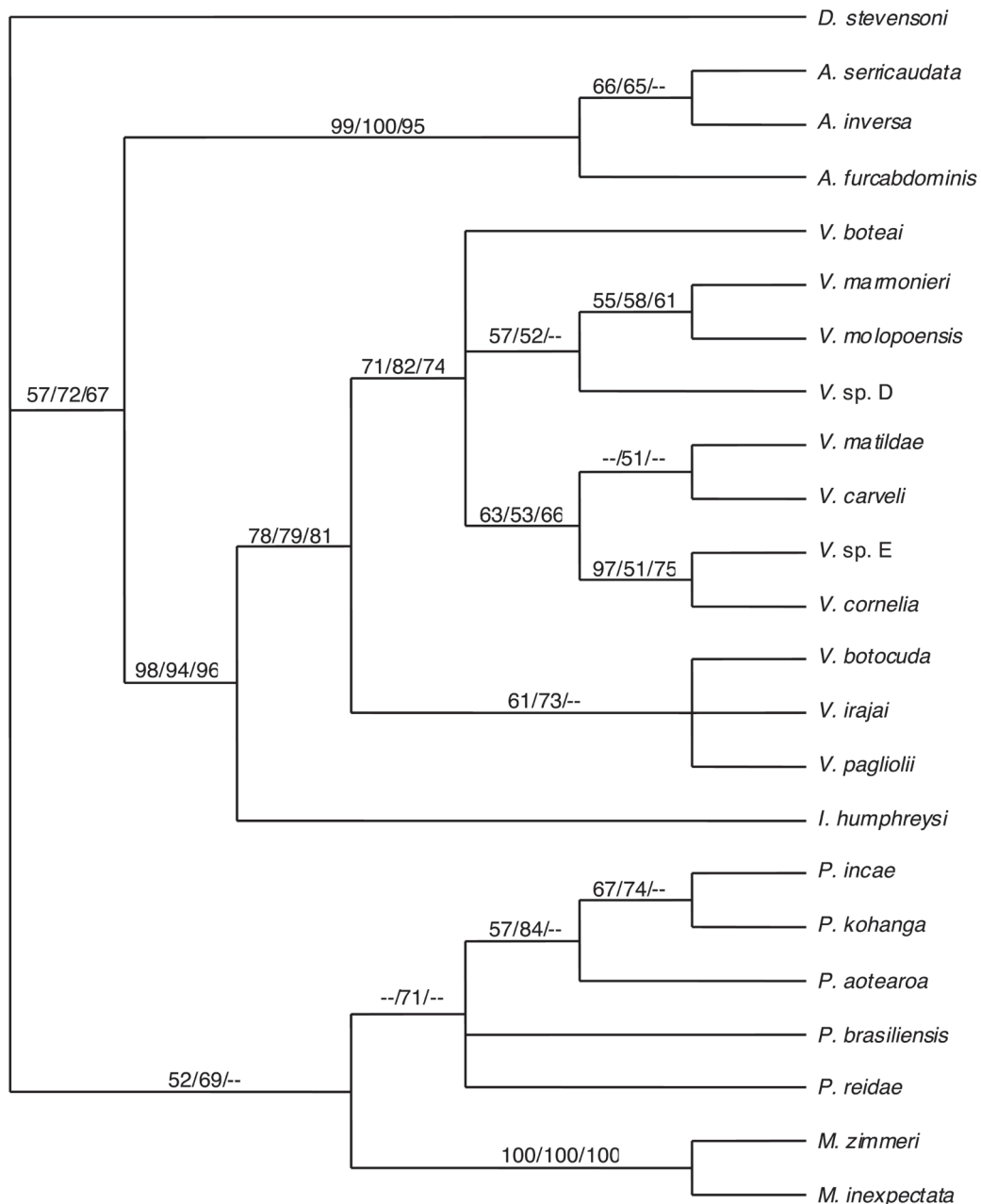


Fig. 5. – Cladogram constructed with parsimony criterion in PaupUp, using the morphological dataset in Table 3. *Darwinula stevensoni* is selected as outgroup. Numbers above branching events are % bootstrap values of 10,000 replicas as follows (1/2/3): (1) bootstrap (BS) values of neighbour joining (NJ) without special settings/ (2) BS values of NJ with character weighting and Dollo-up option/ (3) BS values of maximum parsimony analysis without special settings. ” --“ means the value was below 50%.



particular, *Isabenula* gen. nov. is a sister group to *Vestalenula*; the bootstrap values of the branch point for these two genera are 98/94/96 (see Figure 5 for explanation).

## DISCUSSION

### *Distribution and colonisation*

Samples from interstitial habitats of Christmas Island possibly yielded four different species of Darwinulidae. Such a high incidence of darwinulid species in a small area is quite uncommon, although coexistence of different species and genera has been previously reported, even from single locations (MARTENS et al., 1997; HIGUTI et al., 2007).

Darwinulids have a reduced mobility, and no dispersal mechanisms in the form of resting stages are known to date. Nevertheless, clonal reproduction theoretically allows the establishment of populations from a single individual; in addition, antiquity of the group and movement of land masses through geologic time could account for the intercontinental distribution of some species of darwinulids. Paleontological evidence confirms that the Darwinulidae, supposedly consisting entirely of non-marine genera (with the possible unique exception of the monospecific and highly enigmatic genus *Semidarwinula* (CHOE, 1988)), is the only surviving family of Darwinuloidea since the Permian-Triassic mass extinctions (MARTENS et al., 2003). On the other hand, in Christmas Island the main limestone deposits were laid down from the Late Oligocene onwards and a karst and underground drainage system developed only in the Miocene (GRIMES, 2001; NAMIOTKO et al., 2004). The origin of the island's darwinulid fauna is most likely a consequence of repeated events of colonisation, rather than of local speciation events, as we know that evolution in general in this group is slow (SCHÖN et al., 1998, 2003), while the availability of suitable habitats on this island is relatively recent in geological terms. Although accidental introduction through human

activity cannot in principle be discarded, long-distance transport by birds of living ostracod surviving the gut passage (e.g., PROCTOR et al., 1967; FRISCH et al., 2007) is the most probable way of dispersal. HORNE & SMITH (2004) documented that disjunctive distributions of freshwater ostracods may be associated with bird migration routes over broad geographic ranges. Distances of Christmas Island from both Indonesian islands and mainland Australia easily fall within the range of non-stop flights for many land- and seabirds (BERTHOLD, 2001). If we accept such a view, darwinulid ostracods found in subterranean habitats of Christmas Island could also inhabit (or have inhabited) epigeic waters of surrounding lands, where they can have been ingested by birds. Some darwinulid species are known to colonize both surface and subterranean aquatic habitats. For example, *Penthesilenula brasiliensis* was found in lakes, rivers and streamlets, interstitial and (semi-) terrestrial habitats (ROSSETTI & MARTENS, 1998; ROSSETTI et al., 1998; MARTENS & ROSSETTI, 2002; VAN DONINCK et al., 2003; PINTO et al., 2004).

Most of the known and described darwinulid species are assumed to be of Gondwanian origin and are more common in the Southern hemisphere. In particular, *Penthesilenula* and *Vestalenula* are by far the most speciose genera of the Darwinulidae (representing c. 80% of the known species of Recent darwinulids), and have numerous representatives in southeast Asia, Oceania and the West Pacific (ROSSETTI & MARTENS, 1998; MARTENS & ROSSETTI, 2002; MEISCH et al., 2007; REEVES et al., 2007). The discovery of *Isabenula* gen. nov., of which the phylogenetic relationships with *Penthesilenula* and *Vestalenula* are determined here, further reinforces the role of that geographic area as a biodiversity hotspot for the darwinulid fauna.

### *Characters used*

The revision of Recent Darwinulidae by ROSSETTI & MARTENS (1998) took into account both valve and soft characteristics, since it was conceived as a tool for neontological and

paleontological classifications of this family. However, it is clear that the identification at the genus/species level of fossils, exclusively based on valve morphology, may in some cases lead to incorrect conclusions. In fact, soft part morphology in the Darwinulidae is more conservative and thus more informative regarding phylogenetic and evolutionary relationships, while valve morphology is more plastic and more readily adaptable to changing environmental conditions. Therefore, chaetotaxy of limbs is generally used to characterise genera, while valve shapes are better suited as specific characteristics (MARTENS et al., 2005). For example, the presence of a keel on the RV, sub-quadrated valves and small Cp size are features found in *Isabenula* gen. nov. and in several species of *Vestalenula*. Extreme caution is therefore needed when synonymising fossil darwinulids with Recent taxa, as was for example done for *V. cylindrica* with *V. pagliolii* by GROSS (2008).

#### *Phylogenetic analysis*

MARTENS et al. (2005) performed a phylogenetic analysis of Recent Darwinulidae using both molecular and morphological data. The resulting trees showed a high degree of congruence, indicating that *Alicenula* and *Vestalenula* form one group, while *Penthesilenula* and *Microdarwinula* constitute another; the monospecific genus *Darwinula* is more closely related to the former cluster; exclusive morphological characters, mainly in the soft parts, seem to indicate that *Microdarwinula* has probably a monophyletic origin and is a relatively recent spin-off from a *Penthesilenula*-like ancestor, meaning that the latter genus has to be considered paraphyletic (MARTENS et al., 2005; PINTO et al., 2005).

Our phylogenetic analysis confirms the taxonomic separation of *Isabenula* gen. nov. from the other existing genera of Darwinulidae. Trees obtained combining valve and soft part characters indicate the presence of two main clusters (*Penthesilenula* + *Microdarwinula*, and *Alicenula* + *Vestalenula* + *Isabenula* gen.

nov.) and the isolated position of *Darwinula*, showing topologies that confirm those generated by MARTENS et al. (2005, Fig. 1) using morphological characters.

The diagnostic characters of the A1, A2 and Md-palp used to separate the genera *Vestalenula*, *Penthesilenula* and *Isabenula* gen. nov. (Table 3) show a clear pattern: they are plesiomorphic in *Penthesilenula* and apomorphic in *Vestalenula*, while in *Isabenula* gen. nov. there is a combination of ancestral and derived states (Table 1). Also the presence of an external keel on the RV in both *Vestalenula* and *Isabenula* gen. nov. (not present in *Penthesilenula*) as remnant of the outer list has to be considered a plesiomorphism (MARTENS et al. 2005). These morphological features and the results of the phylogenetic analysis thus indicate that *Vestalenula* could be a spin-off from an *Isabenula*-like ancestor, which in turn shares a common ancestor with *Penthesilenula*.

The new cladistic analysis does not allow us to identify species-groups (*boteai*- and *danielopolis*-groups in the genus *Vestalenula* and *incae*- and *africana*-group in the genus *Penthesilenula*), based on morphological features of the valves (relative length of the external keel in the RV and number and position of internal teeth in the LV) (ROSSETTI & MARTENS 1998). These species-groups have been created for practical convenience, and no taxonomic rank should be allocated to them.

#### *Phenotypic evolution in darwinulids*

Some changes in the phenotypic evolution of darwinulids can be seen as a result of natural selection. For example, miniaturization of the carapace may reflect an adaptation to reduce the risk of detection by visually-hunting predators and to permit the colonization of interstitial habitats. On the other hand, it is not immediately apparent how changes in chaetotaxy, often minimal (e.g., variation in the relative length or loss of setae), can be related to functional aspects, for example feeding and locomotion activities. In addition, in clonal organisms such morphological differences

cannot be the results of selective pressure on mate recognition systems or barriers to avoid cross breeding. Nevertheless, it is evident that the main causes of speciation invoked for sexual organisms, for example divergent selection due to niche-specific adaptations, also operate in ancient asexual lineages, although their rate and magnitude might differ (FONTANETO et al., 2007). As reported above, our present knowledge on darwinulids indicates that the soft part morphology is very conservative, also in long-lived taxa with intercontinental distribution. An alternative view states that natural selection may weed out variants that do not conform to a limited number of “models”, i.e. those arrangements of characters that are found and that can be used to unambiguously identify distinct taxonomic units. This is even more relevant in an animal group that reproduces fully asexually and for which any neutral or non-deleterious mutation might theoretically be clonally transmitted. Individuals with abnormalities in valve or limb morphology have been observed in some darwinulid populations. Such deviant morphologies are thought to be mostly epigenetic and due to developmental errors during the moulting process rather than be genetically transmitted (ROSSETTI & MARTENS, 1998, PINTO et al., 2007), but a genetic basis cannot be excluded *a priori* (see below).

A more detailed examination of data used for the present cladistic analysis (see Tables 2 and 3) reveals that in the Recent representatives of the Darwinulidae:

- 1) the body structure is very conservative, especially in the limb chaetotaxy;
- 2) few possible character states are known for each character;
- 3) morphological “gaps” are easily recognizable and allow the definition of genera, species-groups and species (see MARTENS et al., 1998; ROSSETTI & MARTENS, 1998 and FONTANETO et al., 2007 for a discussion on the applicability of the species concept and other taxonomic categories to clonal lineages);
- 4) in several clades, plesiomorphies and

apomorphies coexist (as do intermediate states), and characters seem to evolve independently and probably at different rates. In other terms, clades result from a mosaic of “ancestral” and more derived characters.

According to ROSSETTI & MARTENS (1998), two possible causes can be singled out to account for clear morphological gaps between species and genera in the Holocene and Recent Darwinulidae. Firstly, slower molecular evolution means that fewer intermediate forms originate. Secondly, it also means that natural selection has longer time spans to weed out the (potentially less fit) intermediate forms. The morphology of *Isabenula* gen. nov., although unexpected on the basis of existing knowledge, and intermediate between those of *Vestalenula* and *Penthesilenula*, can be simply viewed as a new combination of already described characters. It can be also hypothesized that the limited number of “attainable” morphological states reflects a condition of an evolutionary dead end, or at least of a low evolutionary potential, probably linked to obligate asexuality. An alternative view is that there are molecular mechanisms to slow down or even stop genetic (and probably also morphological) evolution in the Darwinulidae to preserve combinations of ecologically relevant genes which confer broad tolerance to diverse environmental conditions (VAN DONINCK et al., 2004). For example, highly efficient DNA repair has been invoked to explain the slow molecular evolution observed in some darwinulid species (SCHÖN & MARTENS, 1998, 2003). The wide geographical and ecological distribution of *Darwinula stevensoni*, the only representative of the genus that has persisted through geological time, seems to strengthen the hypothesis that natural selection actually acts to preserve the same successful, invariant model of organism (VAN DONINCK et al., 2002). The same argument can be extended at least to those darwinulid genera that are monospecific (*Isabenula* gen. nov.) or consist of few species (*Microdarwinula* and *Alicenula*).

It should also be evaluated, if for the

Darwinulidae, a pure phylogenetic approach is applicable to morphological data and under which conditions. Our cladistic analysis, which used the Dollo-up option, was based on the assumption that characters can change from plesiomorphic to apomorphic conditions, but are irreversible. DOMES et al. (2007) argue that Dollo's law is not applicable to putative ancient asexuals, because some oribatid mites might have regained the ability to reproduce sexually after an asexual period, but GOLDBERG & IGIC (2008) argued that both models, with and without reversal to sex, are equally likely.

There is some evidence that morphological evolution in Darwinulidae may not be completely unidirectional, or at least that some lineages could be an exception to general rules. Indeed, the absence of an externally-visible brooding pouch in the genus *Microdarwinula* can be seen as a pedomorphic feature (PINTO et al., 2005), and the occasional re-activation of genes regulating the expression of atavistic characters (e.g., the occurrence of rare males in *V. cornelia*) seems to be possible. If such silent genes are occasionally switched on, they can introduce strong bias into any phylogenetic scheme (ROSSETTI & MARTENS, 1998). However, the observed morphological abnormalities in the Darwinulidae seem to occur at the intraspecific level only, and are relatively easily recognisable amongst the various other features that do comply with the general diagnosis of species clusters (either genera or species-groups within genera), thus allowing the reconstruction of phylogenetically-valid taxonomies (ROSSETTI & MARTENS *loc. cit.*). The present phylogenetic reconstructions using two different criteria (maximum parsimony and neighbour joining) and with or without character weighting and Dollo-up options, all produce very similar topologies and bootstrap supports. Apart from demonstrating the robustness of the retained nodes, it may also be that the discussions on irreversibility of loss of character states (including the ability to reproduce sexually) are largely academic.

## CONCLUSIONS

In the last fifteen years, research on taxonomy, systematics, genetics and ecology of the Recent Darwinulidae has greatly expanded. Nevertheless, our knowledge is still far from sufficient in many fields. Further phylogenetic reconstructions using molecular data (nuclear and mitochondrial sequences) from a large number of darwinulid species are needed to give new insight into the still-puzzling evolutionary scenario of the Darwinulidae. We need to better calibrate the time of splitting of major lineages, and to assess the level of congruence between phylogenies constructed using morphological and molecular characters. A phylogeographic approach is severely hampered by the scarce information on the distribution of the Darwinulidae, most of them being known from their type locality only. A greater sampling effort in aquatic ecosystems that are generally scarcely studied, such as groundwater habitats, may lead to a more precise estimate of the taxonomic diversity of darwinulids. Finally, only a deeper integration of paleontological, morphological and molecular approaches will allow us to test hypotheses of the evolutionary processes in the Darwinulidae that remain partly speculative at this stage.

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