Living males of the ‘ancient asexual’ Darwinulidae (Ostracoda: Crustacea)

Robin J. Smith^{1,*}, Takahiro Kamiya² and David J. Horne^{3,4}

¹Lake Biwa Museum, 1091 Oroshimo, Kusatsu, Shiga 525-0001, Japan

²Department of Earth Sciences, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan

³Department of Geography, Queen Mary, University of London, Mile End Road, London E1 4NS, UK

⁴Zoology Department, Natural History Museum, Cromwell Road, London SW7 5BD, UK

Three living male darwinulid ostracods of a new species of the genus *Vestalenula* have been found in Yakushima, Japan. This is the first report of living darwinulid males for over 100 years and their morphology casts doubt on the two previous records from the late 1800s. The presence of male darwinulids also calls into question the hypothesis that the family Darwinulidae is an exclusively ancient asexual group, reproducing without sex for over 200 million years (Myr). Male carapaces are of similar size and shape to A-1 juvenile females of the same species, suggesting that males may have been dismissed as A-1 juveniles in other living and fossil species. The antennae and fifth limbs are sexually dimorphic: the male antennae have six segments compared with five in the female and a series of putative chemical receptors originating at the extra segment boundary, while the male fifth limbs have well-developed grasping hooks, as in males of many ostracod groups. The lack of Zenker's Organ and of complex internal structures within the hemipenis contradicts previous hypotheses of the phylogenetic position of darwinulids.

Keywords: Darwinulidae; males; sexual dimorphism; Ostracoda; *Vestalenula*

1. INTRODUCTION

Although established evolutionary theory holds that fully asexual species cannot avoid early extinction (Maynard Smith 1976), recent investigations have suggested that two metazoan lineages have been exclusively parthenogenetic for millions of years: the Class Bdelloidea of the phylum Rotifera (Mark Welch & Meselson 2000), and the family Darwinulidae of the crustacean class Ostracoda (Butlin *et al.* 1998). These so-called ‘ancient asexual scandals’ (Judson & Normark 1996) represent not only a challenge to theories about sex and parthenogenesis, but also model organisms for testing hypotheses that seek to explain why sexual reproduction is so prevalent in the animal kingdom.

Putative ancient asexuality in bdelloid rotifers (Mark Welch & Meselson 2000; Mark Welch *et al.* 2004) and darwinulid ostracods (Schön & Martens 2003), as well as (most recently) oribatid mites (Maraun *et al.* 2003, 2004) has been suggested by molecular studies of extant taxa. Darwinulids, comprising five living genera in which about 30 species have been described so far, additionally have an excellent fossil record of their carapaces (Rossetti & Martens 1998, 1999; Martens & Rossetti 2002; Pinto *et al.* 2003, 2004). They are the surviving family of the suborder Darwinulocopina, representatives of which were among the first ostracods to enter fresh waters, some 360 Myr ago in the Carboniferous period (Martens *et al.* 1998; Horne 2003). Analysis of sexual dimorphism of their shells (females have a posterior brood chamber that is speculated to be missing in males) has suggested that while more diverse Palaeozoic darwinulocopes were sexual,

darwinulids have been asexual since the Late Triassic, 208 Myr ago (Martens *et al.* 2003).

Much speculation has been generated by two previous reports of males (Brady & Norman 1889; Turner 1895) of *Darwinula stevensoni* (Brady & Robertson 1870), a species believed to have been fully asexual for the whole 20–25 Myr of its existence (Schön & Martens 2003). The two reports date from the late nineteenth century and were based on living material and led to Little & Hebert (1996) questioning the assertions about the ancient asexual status of darwinulids. However, as only one was illustrated (Brady & Norman 1889) and in neither case do any specimens remain in museum collections, it has been impossible to verify them and they have been generally regarded, at best, as functionless relicts (Rossetti & Martens 1996; Martens 1998). A recent molecular study demonstrated low allelic divergencies in DNA sequences for *D. stevensoni* (Schön & Martens 2003), contrary to high levels predicted for ancient asexuals (the ‘Meselson effect’; Mark Welch & Meselson 2000) and demonstrated for bdelloid rotifers, but ruled out rare syngamy or cryptic sex as explanations. Recently, another study has failed to find any evidence of the ‘Meselson effect’ in parthenogenetic oribatid mites (Schäfer *et al.* 2006) while emphasizing that its absence does not rule out long-term asexuality.

Sex is considered to be primarily genetically determined in non-marine ostracods, male heterogamy being the common pattern; as yet there is no evidence for reversals from asexual to sexual reproduction (i.e. the production of functional males from asexual females), although the rare appearance (e.g. 1 among 10 000 females) of probably non-functional males in at least one species *Limnocythere inopinata* (Baird 1843) is

* Author for correspondence (smith@lbm.go.jp).

acknowledged (Geiger *et al.* 1998; Schön & Martens 1998). Rare, non-functional males are also reported in parthenogenetic oribatids (Maraun *et al.* 2003).

Even rare, atavistic males can provide valuable morphological and phylogenetic information; the single illustration of male copulatory appendages of *D. stevensoni* (Brady & Norman 1889) has been tentatively and controversially interpreted (Maddocks 1973) as including Zenker's Organs (ZO; muscular sperm pumps), a synapomorphy that would link Darwinulocopina with the suborders Cypridocopina and Sigilliocopina. Horne *et al.* (2005) rejected Maddocks' view in their cladistic analysis of ostracod superfamilies and coded the ZO as absent in Darwinuloidea, but were unable to supply confirmatory evidence for their choice or utilize any of the new characters described herein.

Here, we report the occurrence of three males of a new species of *Vestalenula* from Japan. The morphology of the male provides significant information not only on the phylogeny of ostracods, but also on the status of Darwinuloidea as an ancient asexual lineage.

2. MATERIAL AND METHODS

Ostracods were picked from sediment collected from seven localities in Japan (see below) and stored in 70% ethanol. Specimens were dissected and soft parts mounted in 'Hydromatrix' mounting medium or glycerine on glass slides with a cover slip. Carapaces used for SEM investigation were coated with gold-palladium before being photographed with a Philips XL30 SEM. All latitudes and longitudes were recorded with a hand-held Garmin Etrex GPS unit using the Tokyo Datum. Water chemistry was measured at time of collecting using a portable Horita water chemistry unit. Units of measurements: conductivity (cond.), $S\ m^{-1}$; temperature (T), °C; total dissolved solids (TDS), $g\ l^{-1}$.

Terminology of the appendages follows that of Rossetti & Martens (1998) and Pinto *et al.* (2004). Abbreviations in text and figure captions: An1, antennule; An2, antenna; Md, mandible; MS, muscle scars; Mx, maxillula; L5, fifth limb; L6, sixth limb; L7, seventh limb; CR, caudal ramus; Hp, hemipenis; t, possible testes; P-abd, post abdomen; LV, left valve; RV, right valve.

(a) Localities, sampling dates and number of specimens collected

- (i) Yudomari, Yakushima Island, Kagoshima Prefecture, 30°13'48.7" N, 130°28'50.5" E. Gently flowing spring at foot of steep slope near coast. Sampled 28 March 2003, 98 females, 1 male, pH 7.5, T 19 °C, Cond. 33, TDS 0.21; 10 June 2003, 72 females, pH 6.67, T 24.5 °C, cond. 15, TDS 0.1; 18 September 2003, 24 females, pH 6.8, T 28.8 °C, cond. 45.1, TDS 0.29; 12 December 2003, 25 females, no water chemistry data; 8 March 2004, 64 females, pH 6.91, T 16.6 °C, cond. 39.8, TDS 0.26.
- (ii) Hirauchi, Yakushima Island, Kagoshima Prefecture. 30°13'42.7" N, 130°30'24.2" E. Gently flowing spring at coast. Sampled 23 March 2003, 12 females, 1 male, pH 8.1, cond. 15, T 18.4 °C, TDS 0.09; 10 June 2003, 36 females, pH 6.62, T 24.6 °C, cond. 30, TDS 0.19; 18 September 2003; 9 females, no water chemistry data; 12 December 2003, 8 females, no water

chemistry data; 8 March 2004, 6 females, pH 6.67, T 13.2 °C, cond. 12.2, TDS 0.08.

- (iii) Ohko no Taki, Yakushima Island, Kagoshima Prefecture. 30°17'41.8" N, 130°24'59.1" E. Springs at base of steep, vegetated slope, water collecting in shallow gutter. Sampled 20 September 2003, 2 females, no water chemistry data; 12 December 2003, 12 females, no water chemistry data; 10 March 2004, 115 females, pH 6.75, T 17.4 °C, cond. 15.7, TDS 0.1; 24 February 2005, 150 females, 1 male, no water chemistry data.
- (iv) Amami-Oshima Island, Kagoshima Prefecture, 28°15'0" N, 129°24'0" E. Gently flowing spring. Sampled 15 July 2003, 13 females, no water chemistry data.
- (v) Shinkai Pond, Kakuma, Kanazawa, Ishikawa Prefecture, 36°32'31.60" N, 136°42'12.39" E. Gently flowing spring above pond. Sampled 9 July 2004, 2 females, pH 6.82, T 20.4 °C, cond. 14.7, TDS 0.1.
- (vi) Noto Peninsular, Ishikawa Prefecture, 37°13'56.3" N, 136°57'38.2" E. Gently flowing spring. Sampled 8 October 2003, 10 females, no water chemistry data.
- (vii) Chichi Jima Island, Ogasawara Islands (Bonin Islands), 27°04'38.5" N, 142°12'53.1" E. Small spring in steep gulley, very shallow water (2 mm) Sampled 31 May 2004, 2 females, no water chemistry data.

Figured material:

All figured material is held at the Lake Biwa Museum, 1091 Oroshimo, Kusatsu, Shiga 525-0001, Japan (Numbers LBM 1430000876–1430000886).

3. SYSTEMATIC DESCRIPTION

Superfamily: Darwinuloidea

Family: Darwinulidae

Genus: *Vestalenula* Rossetti & Martens 1998

Vestalenula cornelia n. sp.

(Figures 1–3.)

(a) Type locality

Coastal area at Yudomari, South Yakushima; 30°13'48.7" N, 130°28'50.5" E. Spring at top of beach with discharge gently flowing over organic, fine grained sediment through area of long grass. Water depth approximately 10 mm.

(b) Type material

Holotype, a female (number LBM 1430000876) from type locality, allotype, a male (number LBM 1430000877) from Hirauchi, paratypes LBM 1430000881 (male) from type locality, LBM 1430000878 (female) from type locality, LBM 1430000879 (female) from type locality, LBM 1430000880 (female) from Hirauchi, LBM 1430000882 (female) from type locality, LBM 1430000883 (female) from type locality, LBM 1430000884 (female) from type locality, LBM 1430000885 (male) from Ohko no Taki (table 1).

(c) Derivation of name

Named after High Vestal Cornelia, the most famous of the Vestal Virgins of the temple of Vesta in Rome. Cornelia was accused of having a secret lover; it is unknown if the accusation was true.

Figure 1. *Vestalenula cornelia* n. sp. (a) Whole female carapace, right lateral view (LBM 1430000884). (b) Whole female carapace, dorsal view, anterior to right (LBM 1430000882). (c) Detail of keel on RV (LBM 1430000883). (d) Female RV, internal view (LBM 1430000883). (e) Female LV, internal view (LBM 1430000883). (f) Male RV, internal view (LBM 1430000881). (g) Male LV, internal view (LBM 1430000881). (h) Female A-1 juvenile RV, internal view (LBM 1430000886, from type locality). (i) Female A-1 juvenile LV, internal view (LBM 1430000886, from type locality). (j) Adductor muscle scars of female RV (LBM 1430000883). (k) Adductor muscle scars of female LV (LBM 1430000883). (l) Adductor muscle scars of male RV (LBM 1430000881). (m) Adductor muscles of male LV (LBM 1430000881). (n) Adductor muscle scars of A-1 juvenile RV (LBM 1430000886). (o) Adductor muscle scars of A-1 juvenile LV (LBM 1430000886). Scale bar, 176 μ m for (a), (b), (d–i); 50 μ m for (c), 60 μ m for (j–o).

(d) *Diagnosis*

Carapace (female) length/height ratio 2.1. Lateral view of carapace with straight hinge, dorsal margin sloping away rapidly anteriorly to tightly curved anterior margin. Ventral margin slightly concave, posterior margin equally rounded. Female keel on postero-ventral margin of RV elongate, approximately 19% of RV length. LV antero-ventral internal tooth elongate. Third segment of An1 without ventro-apical seta. An2 with 3 *Ac* aesthetascs. An2 *G2* and *Gm* claws equal in length and long, approximately 83% length of claw *GM*. *Md z* seta long, last segment with *c* seta. CR with elongate base, length approximately $2.9\times$ base and supporting elongate, robust seta. P-abd present and elongate.

(e) *Description*

Female carapace length 423–449 μ m, height 202–219 μ m. In lateral view with rounded posterior and straight dorsal margin. Anterior margin slopes away from dorsal margin and with apex of anterior curve below mid height. LV overlaps RV. RV with elongated posterior keel. In dorsal view posterior inflated, producing maximum width in posterior third of carapace; anterior pointed. Internally LV with elongate anterior tooth on ventral margin, calcified

inner lamella very narrow. Adductor muscle scars with 7–9 small scars arranged in rosette.

Male carapace length 385–398 μ m, height 181–195 μ m. In lateral view posterior margin less inflated than female, RV lacking keel. Internally, LV with anterior and posterior ventral teeth on ventral margin.

An1 with six segments. First segment rounded, with one short sub-apical dorsal seta. Second segment quadrate with one long and one shorter apical-ventral setae. Third and fourth segments both quadrate with one apical-dorsal seta each (setae *s1* and *s2*). Fifth segment slightly elongate with two long apical-dorsal setae and one long and one short apical-ventral setae. Final segment elongate with one long and one shorter setae and one aesthetasc.

An2 female with five segments. First segment with hook *h* on dorsal edge and one seta on ventral edge. Second segment with three aesthetascs *Ac* and long seta on apical-ventral corner and *exo* on dorsal-apical edge; *exo* consists of base supporting one long seta and one small seta. Third segment with long, thick *s1* seta and aesthetasc *y1* on apical-ventral corner. Fourth segment with *t* seta and aesthetasc *y2* on apical-ventral corner and apically with large claws *G1* and *G3*, slightly smaller claw *G2* and small claw *x*. Final segment with large claw *GM*, slightly smaller claw *Gm* and aesthetasc *y3*.

Figure 2. *Vestalenula cornelia* n. sp. (a) Reconstruction of female with right valve removed (one of each pair of appendages drawn for clarity). (b) Reconstruction of male with right valve removed (one of each pair of appendages drawn for clarity). (c) An1 (LBM 1430000876). (d) Female An2. (e) Female An2 segments (LBM 1430000876). (f) Male An2. (g) Male An2 segments (LBM 1430000877). (h) Md palp (LBM 1430000876). (i) Md coxa (LBM 1430000876). Scale bar, 124 µm for (a) and (b); 50 µm for (c–i).

An2 male with six segments. First three segments similar to those of female. Fourth segment of female divided into two in male (4a and 4b). Seta *t1* transformed to flattened disc-shaped projection. Segment 4a apically with two short, broad setae (*t2* and *t3*) and thin seta (*t4*). Segment 4b with claws *G1*, *G2* and *x* similar to those of

female; claw *G3* of female transformed to flattened, broad and short tear-shaped projection. Final segment similar to that of female.

Md palp large, consisting of three segments. First segment large with eight rake setae and three other setae along inner edge and with branchial plate on outer edge

Figure 3. *Vestalenula cornelia* n. sp. (a) mx (setae on endites not drawn; LBM 1430000876). (b) Female L5 endopodite (LBM 1430000878). (c) Male L5 (LBM 1430000877). (d) Male L5 endopodite (LBM 1430000877). (e) L6 (LBM 1430000876). (f) L7 (LBM 1430000879). (g) Female genital lobe, CR and abdomen (LBM 1430000880). (h) Male Hp (LBM 1430000877). (i) Male Hp (LBM 1430000885).

consisting of five rays. Second segment elongate with large *z* seta, tiny *y* seta and short *w* and *x* setae. Final segment elongate with short sub-apical *b* seta and small *c* seta and five apical claws. Md coxa well developed with numerous endite teeth.

Mx with palp and three endites, all stout. Palp 2-segmented, first segment widens distally, two sub-apical setae and five apical setae, one of which is robust and bent. Final segment small and quadrate, with three stout, apical

setae. Branchial plate with 29 rays, five of which are reflexed.

L5 female, consists of large basis supporting small, three-segmented walking leg, branchial plate with approximately nine setae and endite with numerous apical setae.

L5 male—basis, endite and branchial plate similar to those of female. Palps robust and stout, supporting well-developed grasping hooks terminating in one or two

rounded lobes. Apical-ventral corner with one small, stout, rounded projection and one longer stout seta.

L6 and L7 both five segmented, well-developed legs. Terminal claw of L7 approximately twice as long as that of L6.

CR—female with elongate, slender base supporting one long, robust seta.

CR—male, much smaller than that of female with a proportionally shorter terminal seta.

Females with rounded genital lobe situated between L7 and CR.

Male Hp protruding from posterior of body just anterior of CR. Hp laterally compressed and consisting of elongate basal capsule, terminating in three lobes. Internally Hp with striations (=muscle?) in distal third running oblique to length. Long and slender curved process protrudes from base of Hp and appears to be able to move independently of main body of Hp. Internally process with duct running along length, emerging at base and looping once before entering soft tissue of posterior of body. Directly above Hp and connected to duct, an ovate structure observable (using phase contrast optics) embedded in soft tissue of body.

(i) Comparison with other species

Within the genus *Vestalenula*, there are two lineages distinguished by the length of the postero-ventral, external keel on the RV; short indicates the *boteai* group, long the *danielopoli* group (Rossetti & Martens 1998). The elongated keel in *Vestalenula cornelia* n. sp. indicates that it belongs to the *danielopoli* group, which consists of two other described species; *Vestalenula danielopoli* (Martens *et al.* 1997) and *Vestalenula matildae* (Martens & Rossetti 2002). The lateral outline of *V. cornelia* n. sp. is more elongate than *V. danielopoli* and the keel of *V. danielopoli* is positioned further to the anterior compared with *V. cornelia* n. sp. The internal ventral tooth of the LV is proportionally larger in *V. danielopoli* compared with *V. cornelia* n. sp. *V. matildae* has a more rounded anterior margin and a more elongate lateral outline than *V. cornelia* n. sp. Two other undescribed species of *Vestalenula*, *V. sp. A* and *V. sp. B*, exist (as *Darwinula sp. A* and *D. sp. B*, Danielopol 1980), both of which are much more elongated in lateral view than *V. cornelia* n. sp. Additionally, the keel of *V. sp. A* is more anteriorly situated than that of *V. cornelia* n. sp.

4. DISCUSSION

(a) Morphology of *Vestalenula cornelia* n. sp.

(i) The carapace

Female carapaces of *V. cornelia* n. sp. have the characteristic features of the genus comprising a posterior keel on the RV and an internal anterior-ventral tooth in the LV. The keel acts as a 'doorstop' to prevent the valves from overlapping each other too much ventrally (which could compress the brood space and crush the eggs or juveniles) and is thought to be the remains of an external list (Martens & Rossetti 2002). The males, however, lack the RV keel, but have an additional tooth positioned along the posterior-ventral margin in the LV, and are significantly smaller than females. Male carapace morphology, including the two internal teeth in the LV, lack of keel on the RV,

Table 1. Carapace sizes of type material.

specimen number	sex	length (µm)	height (µm)
LBM 1430000876	female	423	209
LBM 1430000877	male	385	181
LBM 1430000881	male	396	186
LBM 1430000885	male	398	195
LBM 1430000878	female	428	201
LBM 1430000879	female	423	201
LBM 1430000882	female	428	203
LBM 1430000883	female	440	214
LBM 1430000884	female	426	217

Figure 4. Length and height of carapace of *Vestalenula cornelia* n. sp.

shape and size, is similar to that of A-1 juvenile females (figure 4). This raises a problem that was foreshadowed by Griffiths & Horne (1998): it is extremely difficult to distinguish adult males from A-1 female juveniles based on the carapace alone (the adult male carapace is slightly higher, posteriorly, than the A-1) and confirmation can only be achieved by dissection and examination of appendages. It is possible that male carapaces, both modern and fossil, might be distinguished from those of A-1 females by comparison of their carapace pore-patterns (Kamiya & Hazel 1992), although this has yet to be attempted with darwinuloid ostracods.

(ii) The antennae

The sexual dimorphism of the An2 consists of an extra segment in the male formed by the division of the fourth segment. At this boundary, there are three projections, *t1*–*t3* with differing shapes suggesting they are some kind of chemical receptors (aesthetascs). Similar sexual dimorphism of the An2 is known in one other group of ostracods, the Candonidae of the superfamily Cypridoidea. The males of Candonidae also show a division of the fourth segment from which protrudes three, thickened projections, usually of varying shape and size, known as the male sensory bristles (e.g. Meisch 2000) similar to those seen in the males of *V. cornelia* n. sp. Additionally, the G3 claw of female *V. cornelia* n. sp. is transformed into a flattened, broad and short tear-shaped projection in the male.

Within the superfamily Cypridoidea (containing the Candonidae) the female G3 claw is also transformed to a seta in males (e.g. Martens 1987). The carapace shapes, muscle scars and appendages of the Candonidae are generally very different from those of the Darwinuloidea, perhaps suggesting that such sexual dimorphism of the An2 is a plesiomorphy, retained in these two groups.

(iii) *The fifth limbs*

The L5s of the male have well-developed grasping hooks, in contrast to their leg-like form in females. Similar sexual dimorphism of the L5 is present in most podocopan superfamilies (Cypridoidea, Pontocypridoidea, Macrocypridoidea, Sigillioidea, Terrestrialcytheroidea and Cytherelloidea; see e.g. Athersuch *et al.* 1989; Horne *et al.* 2002) and is probably plesiomorphic.

(iv) *The hemipenes, testes and spermatozoa*

The male copulatory appendages or hemipenes (Hp) of *Vestalenula* sp. bear no resemblance to those illustrated for *D. stevensoni* by Brady & Norman (1889), who also did not mention hooks on the L5, or sexually dimorphic An2 in their description of the male, casting further doubt on their supposed male record. The spermiduct lacks complex developments such as the labyrinth of male cypridoidean ostracods; in this and other respects the morphology of the Hp seems most similar to that of the podocopan Order Platycopida (Superfamily Cytherelloidea), hinting at an affinity that has been suggested previously only on the basis of fossil shell morphology, particularly adductor muscle scar patterns (Kristan-Tollman 1977). Whether or not other homologous features of the limbs support such a relationship is currently under investigation.

The testes have not been clearly identified, but since the males do not show any evidence of the testes within the duplicature (such as in the Cypridoidea) we conclude that they must be present within the soft tissue of the posterior section of the body (e.g. as in Bairdioidea and Cytheroidea) and are possibly represented by the ovate structure just dorsal to the base of the Hp. The tube running from the Hp spermiduct loops once and connects to the ovate structure. We considered the possibility that the ovate structure might in fact be a ZO; it certainly appears to differ from the testes of other ostracod groups which typically have three or four lobes (Müller 1894; Hartmann 1968), but it lacks any of the sclerotized structures normally found in a ZO (neither does it resemble the feature in Brady & Norman's (1889) illustration of *D. stevensoni* that Maddocks (1973) identified as a ZO). We do not, therefore, consider it to be a ZO, although the possibility that it represents a precursor to such a structure cannot be ruled out; histological techniques are required to determine accurately the nature and structure of this feature, which will require additional material.

The absence of a ZO and the simple nature of the spermiduct contradict earlier phylogenetic hypotheses (Maddocks 1973; Martens *et al.* 1998) by setting the darwinulocopes apart from the Cypridocopina and Sigilliocopina. We have considered and rejected the possibility that the absence of these features is simply a morphological indication of loss of functionality, since there is no indication of atrophy in any of the dimorphic features described.

No spermatozoa have been observed in males or females (by microscopical observations). Spermatozoa in the Cypridoidea are easily observable in both males and females due to their giant size (e.g. Matzke-Karas 2005), but are rarely reported in other groups due to their much smaller size, requiring specialized techniques for observation. Furthermore, it is possible that female *Vestalenula* do not store spermatozoa (unlike the Cypridoidea) and fertilization may even occur externally of the body and within the brood chamber itself, thus greatly reducing the chance to observe spermatozoa in female specimens. Therefore, at present, no conclusions can be made about the spermatozoa in *V. cornelia* n. sp.

(b) *The occurrence of male Vestalenula cornelia n. sp.*

The collections in March 2003 produced 110 females and two males of *V. cornelia* n. sp.; one male (Number LBM 1430000881) from the type locality at Yudomari and one male at a second locality at Hirauchi (LBM 1430000877). Subsequent collections from Yakushima in June, September and December 2003 and March 2004 yielded an additional 373 females, but no males, but a collection made in February 2005 at Ohko no Taki, Yakushima yielded a third male and 150 females. The first two males were collected within 5 days of each other from two separate populations approximately 2.5 km apart. The third male (LBM 1430000885) was collected almost 2 years later, at a third location approximately 11.4 km west of Hirauchi and 9.5 km west of Yudomari.

Since finding this species in Yakushima, the authors have also found females in Amami-Oshima, Kanazawa and the Noto peninsular (Ishikawa Prefecture) and Chichi Jima (Ogasawara Islands; figure 5) indicating that the species is relatively widespread. The presence or absence of males may be attributed to the following:

(i) *Production of males due to environmental or seasonal factors*

The production of male *V. cornelia* n. sp. may represent a response to certain environmental or seasonal factors, similar to other animal groups. Species that demonstrate environmental sex determination can be found among the rotifers, nematodes, polychaetes, crustaceans, insects, fish and reptiles (Korpelainen 1990). The crustacean *Daphnia magna*, for example, reproduces asexually in favourable environmental conditions, but if exposed to certain stimuli (e.g. shortening of the day light period, decrease in food, lowered oxygen levels) populations begin to produce males and undergo a cycle of sexual reproduction (Herbert 1978). The sex ratio of one ostracod species, the fully sexual *Metacypis cordata* (Brady & Robertson 1870), is known to dramatically vary with the seasons; males are present only for a month or two in the summer, while brooding females are common well into the autumn (Danielopol & Horne 1996; Meisch 2000). The seasonal collections of *V. cornelia* n. sp. made by us every three months for a year may not rule out seasonality as a cause for the appearance of males if such appearances are short-lived. The fact that three males in three different populations were collected during a similar time of year (end of February and end of March), but collections during other months revealed no males, may hint at a seasonal influence.

Figure 5. Known populations of *Vestalenula cornelia* n. sp. 1, Yudomari, Yakushima, Kagoshima; 2, Hirauchi, Yakushima, Kagoshima; 3, Oko no Taki, Yakushima, Kagoshima; 4, Anami-oshima, Kagoshima; 5, Kanazawa, Ishikawa; 6, Noto Peninsular, Ishikawa; 7, Chichi Jima, Ogasawara Islands (Bonin Islands).

(ii) *Rare non-functional relicts*

The three males may represent non-functional relicts that occasionally appear in a population but have no reproductive input. Such rare males have been reported, for example, in parthenogenetic clonal laboratory cultures of the cytheroidean ostracod *L. inopinata* (Geiger *et al.* 1998). The number of males is very low, so that the occasional non-functional male turning up in a population and directly competing with females for food would not have a serious detrimental effect on the long-term survival of the population. However, why three non-functional male relicts would appear in three separate populations within a relatively short period of time (2 years) is puzzling, especially considering the extremely long time period (over 200 Myr) that the family has been hypothesized as being asexual.

(iii) *Rare functional males*

The males of *V. cornelia* n. sp. may be rare, but functional, i.e. contributing to the general genetic diversity of the population. Such a scenario is known in a predominantly asexual parasitoid wasp with rare males. Out of a population of several thousand wasps, only five males were recovered (Belshaw *et al.* 1999). However, molecular data implies that such rare males contributed to the gene pool, leading Belshaw *et al.* (1999) to surmise that rare sex may be more important for the persistence of otherwise asexual lineages than previously appreciated. Therefore, even if very rare, the males of *V. cornelia* n. sp. may be important to the reproductive strategy of the species. Molecular analyses, already planned, may help to resolve the role of males in the reproductive strategy of *V. cornelia* n. sp.

(c) *Implications for the 'ancient asexual' status of Darwinulidae*

The presence of males in one species of darwinulid, even if very rare, may indicate that other species may also produce males. In size and shape, the male of *V. cornelia* n. sp. is similar to an A-1 juvenile female and it is possible that other species may also have small males that have previously been dismissed as juveniles. Such a size difference between males and females is common in entocytherid ostracods, a family consisting of over 170 species; the males are significantly smaller than adult females and of similar size to A-1 female juveniles. The cosmopolitan species *D. stevensoni* has been extensively studied including its life cycle (e.g. McGregor 1969; Sohn 1987; Rossetti & Martens 1996; Van Doninck *et al.* 2003) and no confirmed males have been recorded, strongly indicating that it is a true asexual species. However, males need to be actively sought in populations of other darwinulid species, especially targeting individuals the approximate size of A-1 juveniles.

Little & Hebert (1996) opined that 'most supposed cases of ancient asexuality are fragile, and likely to be shattered by further taxonomic probing'. The confirmed presence of males in one darwinulid species does challenge the view that the family Darwinulidae is one of the oldest asexual lineages in the animal kingdom. The apparent absence of darwinulid males in the fossil record, since the Triassic has been determined by looking for the following characteristics in the preserved carapaces: lack of brood pouch, position of muscle scars (presumed to be more centrally placed in males due to lack of brood chamber) and size dimorphism (Martens *et al.* 2003). However, the

similar size, shape and position of muscle scars of adult male and A-1 juvenile carapaces of *V. cornelia* n. sp. indicate that such criteria may be insufficient to distinguish between adult males and juvenile females. Further work is required, such as detailed shape analysis and carapace pore-pattern distribution analysis, to demonstrate that males are indeed missing in the fossil record and have not just been dismissed as juveniles.

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