Thematic set:
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Cyprideis (Crustacea, Ostracoda) in Australia

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Abstract: The identity of Australian *Cyprideis* has been disputed for several decades. Here, we compare selected aspects of morphology and genetic diversity of two DNA regions (COI and ITS1) between European populations of *C. torosa* and a *Cyprideis* population from southern Western Australia, tentatively assigned to *C.* cf. *australiensis*. We find that the European and Australian specimens belong to two different genetic species according to the 4 theta rule. We also find some differences in morphology between *C. torosa* and *C.* cf. *australiensis* that allow us to differentiate between these two species. Furthermore, we doubt the assumed synonymy between *C. australiensis* and *C. westraliensis*. It would thus seem that at least one, maybe two or even more, species of *Cyprideis* exist in Australasia that are not part of the near-cosmopolitan *C. torosa* cluster. The status of *Cyprideis consobrina* from New Caledonia should also be investigated in light of these new findings.

Keywords: *Cyprideis torosa*; *Cyprideis australiensis*; *Cyprideis westraliensis*; *Cyprideis consobrina*; molecular phylogeny; 4 theta rule; valve morphology; soft part morphology

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Hartmann (1978) described *Cyprideis australiensis* from the estuaries of the Greenough and Chapman Rivers near Geraldton, Western Australia. Shortly after that, in the same month, McKenzie (1978) described *Cyprideis westraliensis*, also from Western Australia, namely from the causeway of Lake Preston, north of Bunbury. One year later, Hartmann (1979) reported *C. australiensis* from a variety of other localities, including the Lechenault Inlet at Bunbury, *c.* 600 km south of Geraldton. Hartmann (1980) found the latter species also from other localities and putatively synonymized *C. westraliensis* with his *C. australiensis*. De Deckker *et al.* (1988) synonymized both Australian species with *C. torosa*. All later records of Australian *Cyprideis*, both fossil and recent, have subsequently been identified as *C. australiensis* (see Wouters (2016) for an exhaustive review of this literature). Wouters (2016) also suggests that *C. australiensis* might actually be a junior synonym of *C. consobrina* (Brady, 1890) originally described from New Caledonia and later reported as *Cyprideis* sp. from the Solomons Islands (Titterton & Whatley 2006).

Here, we test if one Australian species of *Cyprideis* is conspecific with *C. torosa* by comparing the molecular diversity from two DNA regions (the nuclear ITS region and the mitochondrial COI gene) from European (*C. torosa* from Belgium and the Orkney Islands, Scotland) and Australian specimens (*C.* cf. *australiensis* from a stream near Lechenault Inlet in Western Australia, close to where Hartmann (1979) reported his *C. australiensis*). We also compare valve morphology and some soft part features between European and Australian populations. We thus test whether this Australian *Cyprideis* species is part of a single species with a cosmopolitan distribution or if it belongs to an (endemic) Australian species, most likely as the result of vicariant speciation.

Material and methods

Collections

Ostracods were collected from the various sites with a hand net with mesh size of 160 µm in Europe and Australia. We sampled *Cyprideis* populations from Hollandersgatkreek, Belgium, ponds close to the coast on the Orkney Islands, Scotland and a small stream at Leschenault Inlet, Western Australia (see Table 1 for more details). All sampled ostracods were fixed in 95% pure ethanol and stored at 4°C prior to further analyses.

Details of collections:

(1) *Cyprideis torosa*, Hollandersgatkreek (NW Belgium). Approximate coordinates: 51° 16′ 08″ N, 03° 32′ 07″ E; Sint-Laureins, Belgium. Material collected in October 1997 by IS and KM.
(2) *Cyprideis torosa*, near Kirkwall, Orkney Islands (Scotland). Approximate coordinates (of Kirkwall): 58° 58′ 52″ N 2° 57′ 36″ W. Material collected on 8 September 2014 by D.J. Horne and KM.
(3) *Cyprideis* cf. *australiensis*, streamlet at Lechenault Inlet, near Bunbury (Western Australia). Approximate coordinates: 33° 19′ 10.3″ S 115° 41′ 17.3″ E. Material collected on 4 July 2010 by IS and KM. Accompanying ostracod fauna: *Eucypris virens*, *Bennelongia gwelupensis*. Salinity: 2.63 ‰; Electrical conductivity (K25) 3.88 µS cm^{-1}; pH = 7.26.

Morphological analyses

Ostracods were dissected with valves stored dry in micropalaeontological slides and with soft parts used for molecular analyses (see below). Valves were illustrated and measured using scanning electron microscopy (Philips XL30 SEM at RBINS, Brussels).

Molecular analysis

We used the Qiagen Blood and Tissue extraction kit following the manufacturer's protocol to extract DNA of 30 *Cyprideis torosa* specimens from the locations described above and amplified part of the mitochondrial cytochrome c oxidase subunit I (COI) gene and the nuclear Internal transcribed spacer (ITS) region lying between

Table 1. *Overview of samples and genetic data, including Genbank accession numbers*

Sample	Species	Geographical origin	COI (bp)	ITS (bp)	Genbank accession number
Ct202	*Cyprideis torosa*	Belgium, Hollandersgatkreek	462		KY190089
				489	KY190094
Ct205	*C. torosa*	Belgium, Hollandersgatkreek	462	–	KY190088
ORK Ct1	*C. torosa*	Scotland, Orkney Islands	587	–	KY190090
ORK Ct2	*C. torosa*	Scotland, Orkney Islands	587	–	KY190092
ORK Ct3	*C. torosa*	Scotland, Orkney Islands	587		KY190091
				489	KY190095
ORK Ct5	*C. torosa*	Scotland, Orkney Islands	587	–	KY190093
B Ct	*C. torosa*	Belgium, Hollandersgatkreek	–	489	AJ534421
PIK246	*C. australiensis*	WA, Leschenault Estuary	587	–	KY190085
PIK247	*C. australiensis*	WA, Leschenault Estuary	587		KY190087
				487	KY190098
PIK248	*C. australiensis*	WA, Leschenault Estuary	587		KY190083
PIK249	*C. australiensis*	WA, Leschenault Estuary	587		KY190084
				487	KY190096
PIK250	*C. australiensis*	WA, Leschenault Estuary	587		KY190086
				487	KY190097
	Cytherissa lacustris		382	–	JN715749
	C. lacustris		–	410	AJ534422

Sample refers to the abbreviations in Figure 1a and b. bp, lengths of sequence in basepairs; WA, Western Australia.

the ribosomal 18S and 28S region. Both markers, COI and ITS, have been successfully analysed in other ostracods for molecular species delimitations, phylogenetic and phylogeographical analyses (e.g. Schön *et al.* 1998, 2000, 2012, 2014; Martens *et al.* 2005, 2012, 2013; Nunes Brandao *et al.* 2010), and COI is furthermore widely used in DNA barcoding initiatives. For COI, we applied the universal primers of Folmer *et al.* (1994) in PCR volumes of 25 µl with the HotStar Master Mix (Qiagen; 1.5 mM $MgCl_2$, 200 µM dNTP, Tris·Cl, KCl, $(NH_4)_2SO_4$, 1.25 U Taq) and 0.1 µM of each primer. Cycling conditions in a T personal Thermoblock (Biometra) were 15 min at 95°C, followed by 40 cycles with 1 min at 95°C, 1 min at 44°C, 1 min at 72°C, and with a final extension step at 72°C for 10 min. We also amplified part of the nuclear ITS1 and ITS2 regions with the universal primers ITS1 and ITS4 by White *et al.* (1990), using similar PCR conditions as for COI except that the annealing temperature was increased to 50°C. We used agarose gel electrophoresis with subsequent staining of gels with Gelred™ to assess the success of PCR amplifications. PCR products were enzymatically purified with Exo-SAP IT™ (Affymtrix™) and sequenced in both directions with the PCR primers and the Big Dye kit (ABI) on an ABI 3130X following the manufacturer's protocol.

Analyses of sequence data

The forward and reverse sequence of each individual were aligned with ClustalX (Larkin *et al.* 2007), followed by manual checking of the sequence chromatograms being visualized with BioEdit (Hall 2007), correcting any ambiguities and trimming the final alignments to equal length. We confirmed identity of the obtained sequences by BLAST searches (Altschul *et al.* 1990) in Genbank. The optimal 88 models of molecular evolution were assessed with the AICc criterion in jModeltest 2.1.1 (Darriba *et al.* 2012). We reconstructed phylogenies with two different methods, Bayesian Inference (BI) in Mr Bayes 3.2 (Ronquist *et al.* 2011; with 5 million generations, sampling every 100th generation, a burn-in of 25% and the parameters identified by jModeltest for 24 different models) and the Maximum-Likelihood method in PhyML (Guindon & Gascuel 2003; with 1000 bootstrap replicates and the parameters of jModeltest for all 88 models), respectively. All sequences have been submitted to Genbank (accession numbers KY190083–KY190098; see Table 1). As outgroup for both datasets, we used sequences of *Cytherissa lacustris* (see Schön & Martens 2012), which belongs to the same subfamily as *Cyprideis torosa*.

Testing for cryptic diversity

As in other studies on non-marine ostracods with a similar research question, we first identified well-supported phylogenetic sister clades (with bootstraps above 75% or posterior probabilities above 0.85) that could represent different species following the evolutionary genetic species concept (Birky & Barraclough 2009). We then applied MEGA version 6.0 (Tamura *et al.* 2013) to estimate sequence diversities within and between these phylogenetic clades, using the appropriate model with gamma distribution and 1000 replicates. Following Birky *et al.* (2010), we then corrected the obtained estimates of sequence diversities for sample size. According to the 4 theta rule, sequence diversities between two sister clades must be no less than 4–4.3 times larger than within the two clades, depending on the number of sequences per clade (Birky *et al.* 2010). The 4 theta rule has previously been used on bdelloid rotifers (Fontaneto *et al.* 2007, 2009; Birky & Barraclough 2009; Birky *et al.* 2011), sexual and asexual ostracods (Bode *et al.* 2010; Martens *et al.* 2012, 2013; Schön *et al.* 2012; Shearn *et al.* 2012), including specimens from other *Cyprideis* species from Lake Tanganyika, Africa (Schön *et al.* 2014), asexual prokaryotes (Birky *et al.* 2010) and sexual vertebrates and invertebrates (Birky 2013).

Results

Molecular results

Despite successful DNA extractions, we obtained DNA sequence data of COI (of 587 basepairs (bp)) and ITS (495 bp) from only 11 and 7 specimens, respectively. The other sequences were not of ostracod origin as the BLAST searches revealed: the universal COI primers also amplified bacterial DNA while the universal ITS primers also amplified this region from ciliates and plants, probably from material attached to ostracod valves or present in the guts. Amplification success could most likely be increased by developing specific PCR primers for *Cyprideis*, which was unfortunately not possible in the timeframe of the present pilot study. In a molecular study on the *Cyprideis* flock from Lake Tanganyika, universal primers also provided limited success (Schön & Martens 2012). The

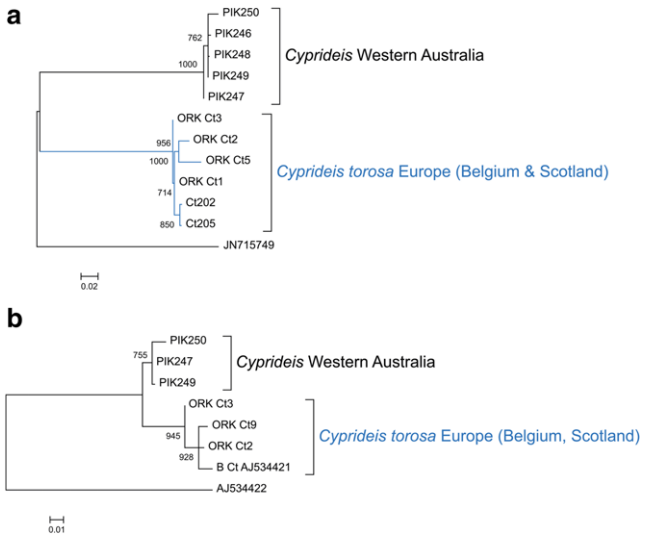

Fig. 1. Phylogenetic trees constructed with (**a**) mitochondrial COI and (**b**) nuclear ITS DNA sequence data. Numbers above branches are bootstraps of a 1000 replicates in Maximum-Likelihood trees, numbers below branches posterior probabilities of Bayesian trees.

Tanganyikan sequences are genetically too distant to serve as a template for developing primers for *Cyprideis senso stricto*. Likewise, the new COI ostracod primer by Nigro *et al.* (2016) was developed for marine, planktonic ostracods, which are evolutionarily too distant to *Cyprideis*.

For the COI data, jModeltest identified the TPM1uf + G model and the following parameters as the optimal model of molecular evolution: freqA = 0.2582, freqC = 0.197, freqG = 0.1991, freqT = 0.3453; [AC] = 1.0000, [AG] = 4.5256, [AT] = 2.3009, [CG] = 2.3009, [CT] = 4.5256, [GT] = 1.0000 and gamma shape = 1.3660. jModeltest also identified the model K80 + G (with kappa = 2.0868, ti/tv = 1.0434 and gamma shape = 1.0670) for ITS.

Phylogenetic trees constructed with both methods and from both COI and ITS (Fig. 1a and b) show a clear separation between Australian and European *Cyprideis* with high statistical support.

Overall, genetic diversity is higher for COI (average 0.9–2.2% among specimens from the same continent) than for ITS (on average 0.8–1.6%; see Table 2). The genetic diversity between European and Australian *Cyprideis* reaches almost 40% for COI, being in the same range as the outgroup, while with 5.4% diversity ITS appears far less variable (Table 2). Genetic variation in ITS when comparing *Cyprideis* from Europe and Australia mainly consists of insertions and deletions (see Fig. 2). When testing for species boundaries according to the evolutionary species concept, only the COI data fulfil the criteria of the 4 theta rule, not ITS (see Table 2).

Morphological results

Here we provide some illustrations and descriptions of valves and some preliminary descriptions of soft part features; a full description of the Australian species will be made elsewhere.

Valves

We have illustrated the valves of specimens of the Australian *Cyprideis* cf. *australiensis* and of Belgian *Cyprideis torosa* (Fig. 3) and also compared these to the excellent illustrations of the European *Cyprideis torosa* in, amongst others, Wouters (2002) and the atlas of Fuhrman (2012, pl. 141, p. 295). There are substantial differences in shape and even structure between the European and Australian specimens:

(1) *C.* cf. *australiensis* is more elongated, especially in the male, with a less pointed posterior side in lateral view;
(2) the male in *C. torosa* is larger and longer than in the female, while in *C.* cf. *australiensis* it is smaller, or maximally of the same length as the female;
(3) in both sexes, the posterior part of the hinge in the right valves of *C.* cf. *australiensis* is less well developed (compare Fig. 3f for *C. torosa* male with Fig. 3n for *C.* cf. *australiensis* male).

Soft parts (not illustrated here)

Some observations were made on two dissections of males of *C.* cf. *australiensis*:

(1) the male right first leg in *C.* cf. *australiensis* has the 'split' setae as illustrated by Wouters (2016: fig. 1a);
(2) the hemipenes of *C.* cf. *australiensis* appear to have the extra lateral (external) whirl next to the (medial?) shield, as illustrated by Hartmann (1978, fig. 155, p. 168).

Discussion

Two genetic species

When applying molecular analyses to European and Australian *Cyprideis*, our pilot study indicates that ostracods from these two continents belong to two genetically different species.

Our COI data provide strong evidence of species-level difference because the obtained genetic distances and phylogenetic groupings

Table 2. *Genetic variability within and between Cyprideis populations from Europe and Western Australia, respectively, calculated with the T3PM model (COI) and the K80 model (ITS)*

Comparison	Marker	*n*	Genetic variability in %	max. θ (within phylogenetic clades)	*D* (between phylogenetic clades)	Ratio D/θ
Within Europe	COI	6	2.24	0.0291	na	
	ITS	4	1.59	0.0218	na	
Within Australia	COI	5	0.89	0.0109	na	
	ITS	3	0.83	0.0127	na	
Among Europe & Australia	COI	5/6	39.50		0.395	**13.58**
	ITS	4/3	5.40		0.054	2.75
Cyprideis – outgroup	COI	11/1	44.10			
	ITS	7/1	30.60			

These data were also used to test with the 4 theta rule if these populations present different genetic species. In order to fulfil the criteria of the 4 theta rule for species status, the ratio of the θ within phylogenetic clades as compared to *D* between phylogenetic sister clades needs to be 4 or more, depending on the number of specimens per clade (Birky *et al.* 2010). Comparisons, for which these criteria are fulfilled, are printed in bold. *n*, number of sequences for each geographical region; θ, population genetic parameter theta, indicating genetic variability within geographical regions corrected for sample size; *D*, genetic distance between phylogenetic sister clades.

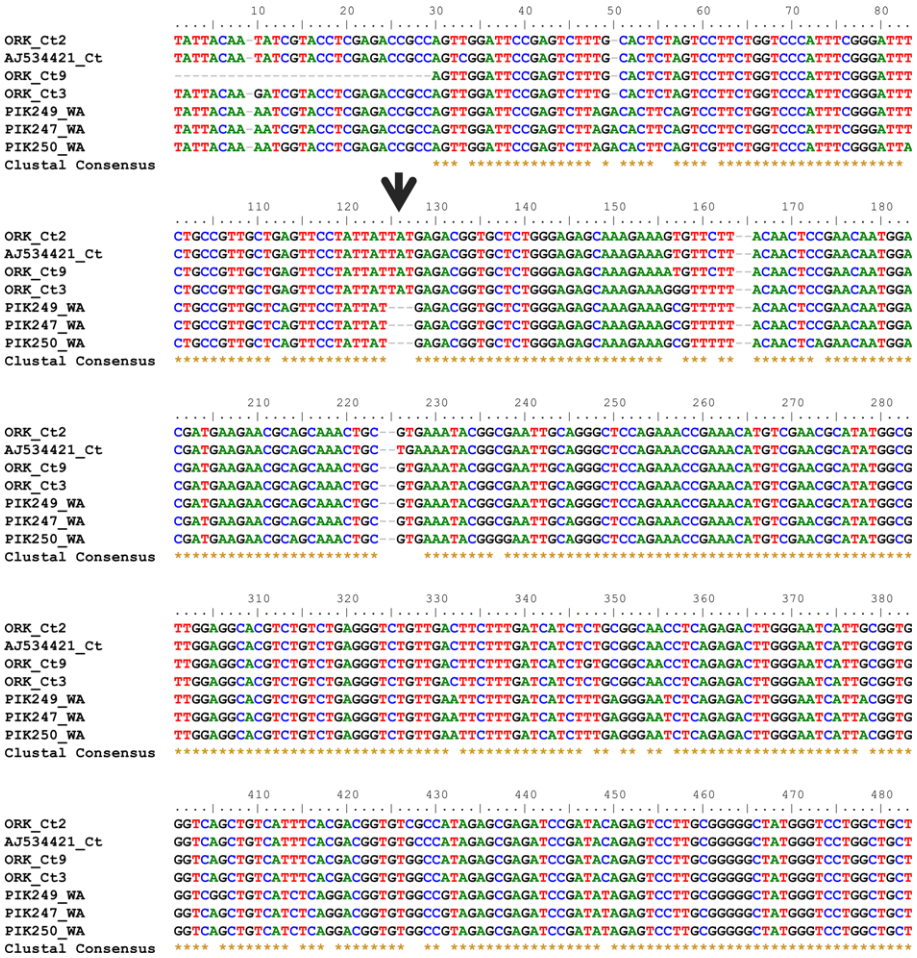

Fig. 2. Alignment of ITS sequences. Identical positions are indicated by asterisk; insertions or deletions by '–'. Note that the sequence of OEK_Ct9 was shorter than the other sequences in the beginning at the 5′ direction. The arrow indicates a unique insertion in ITS of European *Cyprideis* populations.

fulfil the criteria of the 4 theta rule (Birky *et al.* 2010; Table 2). For ITS, our results are less clear. While the criteria of the 4 theta rule are not fulfilled for ITS (Table 2), phylogenetic reconstructions reveal a statistically well-supported separate grouping of *Cyprideis* from Europe and Australia in the ITS trees (Fig. 1b). The major genetic differences between European and Australian *Cyprideis* in our ITS data consist of an insertion of three nucleotides (at position 250; indicated by an arrow in Fig. 2) in the European populations that is absent from the Australian populations. Such insertions or deletions are not taken into account when calculating genetic distances. This can explain, to some extent, why estimates of the genetic ITS distances were lower than those of COI and, consequently, why the ITS data did not fulfil the criteria of the 4 theta rule. Also other authors have observed a much larger genetic variability of the mitochondrial COI than nuclear markers, resulting in lower numbers of genetic species when using ribosomal nuclear DNA sequence data in metazoans (Tang *et al.* 2014), including other non-marine ostracods with less (Schön *et al.* 2000, 2012) or no variability at all (Schön *et al.* 1998, 2010; Koenders *et al.* 2012) in ITS or 28S while COI indicated the existence of different genetic species. Most relevant here is probably the study of Schön *et al.* (2014) where ITS showed no variability at all in two *Romecytheridea* species from the Tanganyikan *Cyprideis* species flock (Schön *et al.* 2014) but where the same specimens fell into two genetically different (cryptic) species according to the variability of their mitochondrial 16S data. Although the molecular evidence for two different *Cyprideis* species in Europe and Australia is stronger for COI, in our opinion the ITS DNA sequence data also suggest these populations are two different evolutionary genetic species. The existence of evolutionary genetic species on different continents is mostly likely owing to vicariant processes and we found similar patterns in darwinulid ostracods from Australia and Europe (Schön *et al.* 2012). The observed large genetic distances in COI, and also differences in ITS, provide strong evidence against a recent introduction of *Cyprideis* ostracods into Australia, in contrast to the situation for the introduced *Eucypris virens* from the same locality in Western Australia (Koenders *et al.* 2012).

Two morphological species

Ostracods have both calcified valves and so-called soft parts comprising limbs and other sclerified body parts. Both are generally used by biologists to characterize and identify species and higher taxa, whereas palaeontologists generally only have valve features to work with. It has been shown in a number of cases that environmental aspects as well as genetic background may have an effect on shape and structure of both valves and soft parts of, for example, the cytherid species *Limnocythere inopinata* (Baird) (Yin *et al.* 1999) and species of the cypridid genera *Mytilocypris* and *Australocypris* (Finston 2004; Halse & McRae 2004). Wouters (2002, 2003) made a qualitative assessment of morphological plasticity in the species *Cyprideis torosa* over wide geographical ranges and postulated that the same might be true for this species. Hence, it is sometimes difficult to assess validity of characters to discriminate at the species level.

In the present species pair, however, there can be little doubt that at least the shape of the valves, and maybe also the structure of the hinge, indicate a specific status for both. Thus, morphology supports genetics in finding that at least the present Australian *Cyprideis* species is not part of a cosmopolitan species (*in casu C. torosa*).

Identity of the Australian species

The Australian species investigated and illustrated here was collected from a small stream which ended in Lechenault Inlet, close to Bunbury,

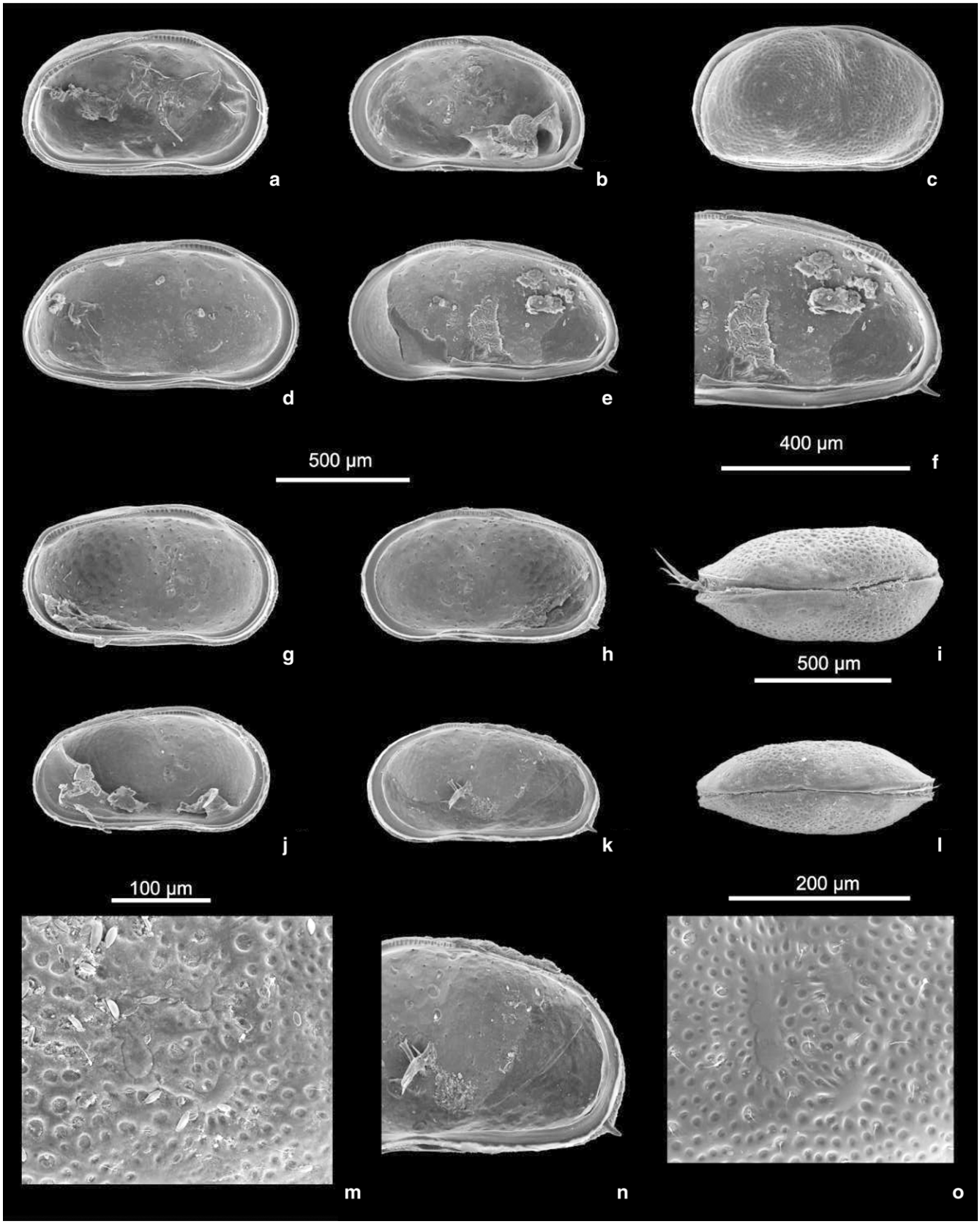

Fig. 3. Scanning electron microscopy illustrations of valves of European *Cyprideis torosa* (**a–f, o** from Hollandersgatkreek, Belgium) and *C.* cf. *australiensis* (**g–n** from streamlet near Leschenault Estuary, Bunbury, Western Australia). Figured specimens deposited in Royal Belgian Institute of Natural Sciences. *Cyprideis torosa*: (**a**) female, LV, internal view; (**b**) female, RV, internal view; (**c**) female, carapace, right lateral view; (**d**) male, LV, internal view; (**e**) male, RV, internal view; (**f**) male, LV, internal view, detail of posterior part; (**o**) female, carapace, lateral view, detail of surface ornamentation. *Cyprideis* cf. *australiensis*: (**g**) female, LV, internal view; (**h**) female, RV, internal view; (**i**) female, carapace, dorsal view; (**j**) male, LV, internal view; (**k**) male, RV, internal view; (**l**) male, carapace, dorsal view; (**m**) male, carapace, lateral view, detail of surface ornamentation; (**n)** male, RV, internal view, detail of posterior part. Scales: a–e, g–l = 500 µm; f, n = 400 µm; m = 100 µm; o = 200 µm.

and this is very close to one of the localities from which Hartmann (1979) reported his species, *C. australiensis.* In all probability, Hartmann did not collect his specimens from the same small streamlet, but from the estuary itself, with a salinity close to that of seawater. A sample in the estuary itself, close to the entrance of the streamlet, taken by KM & IS at the same time as the streamlet was sampled, had a salinity of 10 times that of the streamlet. Moreover, Hartmann, in the original description of *C. australiensis*, reported that the salinity in the Greenough River Estuary (where only dead specimens were collected) was 32.1‰ whereas the living population at the estuary of the Murchison River was found at a salinity of 6.9 (Hartmann 1978, p. 86). He also postulated that it could have been the high salinity at Greenough that caused the death of the *Cyprideis* population. Therefore, it is not impossible that the Leschenault streamlet population, living at low salinity, belongs to Hartmann's species.

Cyprideis australiensis has the split seta on the right first male thoracopod that is also present in our specimens, and which was illustrated as a normal seta (not split) by McKenzie for his species *C. westraliensis* (McKenzie 1978, fig. 24, p. 178). The additional external whirl on the (medial?) shield of the hemipenis, illustrated by Hartmann (1978, fig. 155, p. 168), is also present in our specimens and is also not illustrated by McKenzie for *C. westraliensis* (McKenzie 1978, fig. 26, p. 178). Of course, we cannot tell at this stage if the latter hemipenal feature is a consistent and specific morphological character, or if it is related to other factors, for example it could be that this external feature is only visible in a hemipenis in erection.

Nevertheless, we think that our specimens are not only morphologically (as well as genetically) different from the European *C. torosa* but also sufficiently similar to the Australian species *C. australiensis* to refer to it here as *C.* cf. *australiensis*. In addition, if the above-mentioned illustrations of McKenzie (1978) are correct, *C. australiensis* and *C. westraliensis* are not synonymous and are both different from *C. torosa*. Thus, there is at least one, and maybe two or more, Australian *Cyprideis* species that may have originated through geographical (vicariant) speciation and that do not belong to *C. torosa*.

Outlook

There are at least three further lines of inquiry remaining for future study.

Firstly, the impressive literature overview by Wouters (2016) shows that there might be at least one more Australasian species, namely. *C. consobrina*, reported from New Caledonia by Brady (1890). Wouters (2016) also suggests that *C. australiensis* might be a junior synonym of *C. consobrina.* Resampling habitats on New Caledonia might yield recent material that can be used for molecular and morphological screening, also of hemipenes, and thus could further the present analysis.

Secondly, Hartmann (1979, 1980) reported *C. australiensis* from a variety of other localities along the southwestern coast of Western Australia. A revision of these collections should show if these really all belong to one species, or whether additional species (*C. westraliensis*?) are present.

Finally, the possibility cannot be excluded that still further species of *Cyprideis*, and not belonging to the *C. torosa* clade, might exist in (Western) Australia. Martens *et al.* (2015) summarized the work conducted during a four-year ABRS grant, during which the known number of species of the genus *Bennelongia* in Western Australia was increased from two to 25.

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