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Evolutionary systematics of the Australian Cyzicidae (Crustacea, Branchiopoda, Spinicaudata) with the description of a new genus

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In Australia's arid and semi-arid zone, most aquatic habitats are nonpermanent. Although approximately 70% of its land surface belongs to these zones, very little is known of the iconic 'large branchiopods' that inhabit these important and widespread habitats. In the present study, we investigated 737 Australian specimens of the spinicaudatan taxa *Caenestheria* and *Caenestheriella* with a combination of one mitochondrial (cytochrome oxidase subunit I; COI) and three nuclear (elongation factor 1α, internal transcribed spacer 2, and 28S) markers to assess the diversity of species, their phylogenetic relationships, and phylogeographical history. The initial species delimitation was based on COI employing a combination of phylogenetic analyses and two automated approaches to species delimitation (general mixed Yule coalescent model and Automated Barcode Gap Discovery). The outcome was tested by the nuclear markers and considered under differing species concepts. The number of delineated species ranged from 14–27, in no case being in full agreement with any of the two automated approaches. The lower numbers resulted if inferred reproductive isolation, as required for the biological or Hennigian species concept, was employed. Although nuclear markers did not indicate ongoing reproduction, the lack of sympatric co-occurrences inhibited inferences of definitive reproductive isolation in several instances. If monophyly or an 'independent evolutionary fate' was employed, as required for the phylogenetic or evolutionary species concepts, the species' distribution was of no importance and up to 27 species could be delimitated. Because the Australian representatives of both studied genera could not be clearly separated from each other but constitute a single monophyletic clade separated from all available non-Australian representatives of these genera, we describe a new spinicaudatan genus ***Ozestheria* gen. nov.** to accommodate these species. Populations revealed relatively small levels of genetic differentiation over large areas of central and eastern Australia. By far the most pronounced levels of genetic differentiation were observed towards the north-eastern regions, a pattern possibly explainable by ecological conditions and the movement of nomadic water birds that disperse resting eggs.

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INTRODUCTION

Spinicaudata and other 'large branchiopod' crustaceans (Anostraca, Notostraca, Laevicaudata, and Cyclestheriidae) are an important faunal element of temporary water bodies throughout the world (Dumont & Negrea, 2002). They are perfectly adapted to ephemeral habitats by producing drought-resistant cysts (colloquially termed resting eggs). To induce hatching, a period of desiccation is mandatory, thus precluding most large branchiopods from inhabiting permanent water bodies such as lakes or rivers (Dumont & Negrea, 2002). Therefore, the majority of species occur in regions of increased aridity. In this respect, Australia offers perfect conditions: approximately 70% of the continent's surface area belongs to the arid or

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semi-arid zone with only few permanent water bodies. Temporary water bodies fill after infrequent rain, offering not only huge numbers, but also a great diversity of ephemeral aquatic habitats (Williams, 1981; Kingsford & Porter, 1999). Therefore, it is not surprising that branchiopods are abundant during wet periods, constituting one of the most important and iconic aquatic faunal elements in these regions. Despite their ubiquity, Australian branchiopods were largely ignored during most of the last century, with interest being rekindled by a series of ecological studies of temporary water bodies (Timms & Boulton, 2001; Timms & Richter, 2002; Timms & Sanders, 2002), which highlighted the deficiencies in our current knowledge of their systematics, taxonomy, and general biology (Timms, 2012).

In the present study, we focus on the Australian representatives of the spinicaudatan taxon Cyzicidae, which is one of three families within the Spinicaudata besides Leptestheriidae, and Limnadiidae. The goal is to assess the diversity of the Australia Cyzicidae, their phylogenetic systematics, and their phylogeographical history within an Evolutionary Systematics framework (Glaubrecht, 2007, 2010). Therein, taxonomic diversity, disparity, and genetic variability, as well as the underlying evolutionary causes of speciation, are studied on the basis of phylogenetic systematics. The taxonomy and systematics within the Cyzicidae is under debate. Four genera were proposed by Daday des Deés (1914): *Caenestheria*, *Caenestheriella*, *Cyzicus*, and *Eocyzicus*. These are differentiated by differences in their condyle (a dorsoposterior elongation of the head, which can be elongated and slightly pointed or short and rounded) and the shape of their rostrum. However, some researchers synonomized *Caenestheriella* with *Cyzicus* and *Caenestheria* with *Eocyzicus* (Brtek, 1997), or even all four genera with *Cyzicus* (Williams, 1981). As a result, Williams (1981) referred to all Australian Cyzicidae as *Cyzicus*, whereas Richter & Timms (2005), by accepting Daday's genera, were able to assign the Australian species to all genera except *Cyzicus*. The first molecular phylogenetic analyses resulted in additional complications (Schwentner *et al*., 2009; Weeks *et al*., 2009). Obviously, the only monophyletic cyzicid genus is *Eocyzicus* (*sensu* Daday), albeit it does not constitute a monophyletic clade with the other Cyzicidae but appears to be more closely related to Leptestheriidae or Limnadiidae. The Australian *Eocyzicus* fauna has been studied previously (Schwentner, Timms & Richter, 2014) and is not treated in detail here. The other three cyzicid genera (*Caenestheria*, *Caenestheriella*, and *Cyzicus*) constitute a monophyletic clade, although the genera themselves are each not monophyletic. Instead, they form two monophyletic clades: one featuring all Australian representatives, the other all non-Australian representatives (Schwentner *et al*., 2009; Weeks *et al*., 2009). Such a classification had never been proposed before. One of the goals of the present study is a comprehensive phylogenetic analysis of the Australian Cyzicidae to determine whether they indeed represent a separate, potentially new genus within Cyzicidae and whether the Australian *Caenestheriella* and *Caenestheria* species constitute monophyletic groups each.

The assessment of the species diversity within a given taxon is largely dependent on the types of data available (e.g. molecular genetic markers, morphological characters or ecological parameters) and the applied species concept (Laamanen, Petersen & Meier, 2003; Agapow *et al*., 2004; Tan *et al*., 2008; Schwentner, Timms & Richter, 2011). In the present study, we employ a combination of phylogenetic, genetic distance, and coalescent analyses to identify and delimit putative species. As a first step, 'main lineages' are derived on the basis of the mitochondrial marker cytochrome oxidase subunit I (COI). These main lineages are the first hypothesis of putative species. However, two or more main lineages may together constitute a single species, or two or more species may have erroneously been merged as a single main lineage. Therefore, main lineages need to be corroborated by further data. This will be carried out by the analysis of three nuclear markers [elongation factor 1α (EF1α), internal transcribed spacer 2 (ITS2) and 28S RNA]. The overall results are then considered in the light of different species concepts. Because each species concept focuses on certain properties of the species' biology and their evolutionary history, main lineages may be differently translated into species under each concept (Agapow *et al*., 2004).

We employed the biological species concept (BSC; Mayr, 1942) and Hennigian species concept (HSC; Meier & Willmann, 2000), which require reproductive isolation among species; the phylogenetic species concept (PSC; Mishler & Theriot, 2000a), which defines species as 'the smallest monophyletic groups worthy of formal recognition' and which requires a phylogenetic analysis prior to species delimitation; and the evolutionary species concept (ESC; Wiley & Mayden, 2000a), which defines species as 'an entity [. . .] that maintains its identity from other such entities through time and over space and that has its own independent evolutionary fate and historical tendencies'. Under the PSC and ESC, species are not necessarily reproductively isolated (Mishler & Theriot, 2000b; Wiley & Mayden, 2000b); however, monophyly and an independent evolutionary fate would most certainly not be achieved if putative species were freely reproducing. Thus, indications of reproduction among main lineages (e.g. shared nuclear haplotypes) would question their distinctiveness as species also under the

PSC and ESC. To confirm that genetic differentiation is not a consequence of current geographical separation of intraspecific populations, reproductive isolation can only be assessed for main lineage pairs that occur in the same pool (syntopic) or at least in the same area (sympatric) where gene flow can be reasonably assumed. In another spinicaudatan taxa, modified scales on male claspers, which are used to hold on to the female during mating, appeared to be a reliable character to morphologically differentiate reproductively isolated species (Schwentner *et al*., 2011). It was assumed that the scales could be part of a mate recognition system, in which case observable differences in these scales could be used to delimit species under the BSC and HSC as well. Therefore, these scales were also studied herein for a subset of main lineages.

MATERIAL AND METHODS

Collection details

Adult specimens were collected between 1999 and 2011 (Fig. 1; for collection and specimen details, see also Supporting information, Tables S1 and S2) and fixed and stored in absolute ethanol. In addition, several specimens were reared from sediment samples collected from dried out pools. Details on rearing conditions are provided in Schwentner, Timms & Richter (2012b). All specimens in the present study were

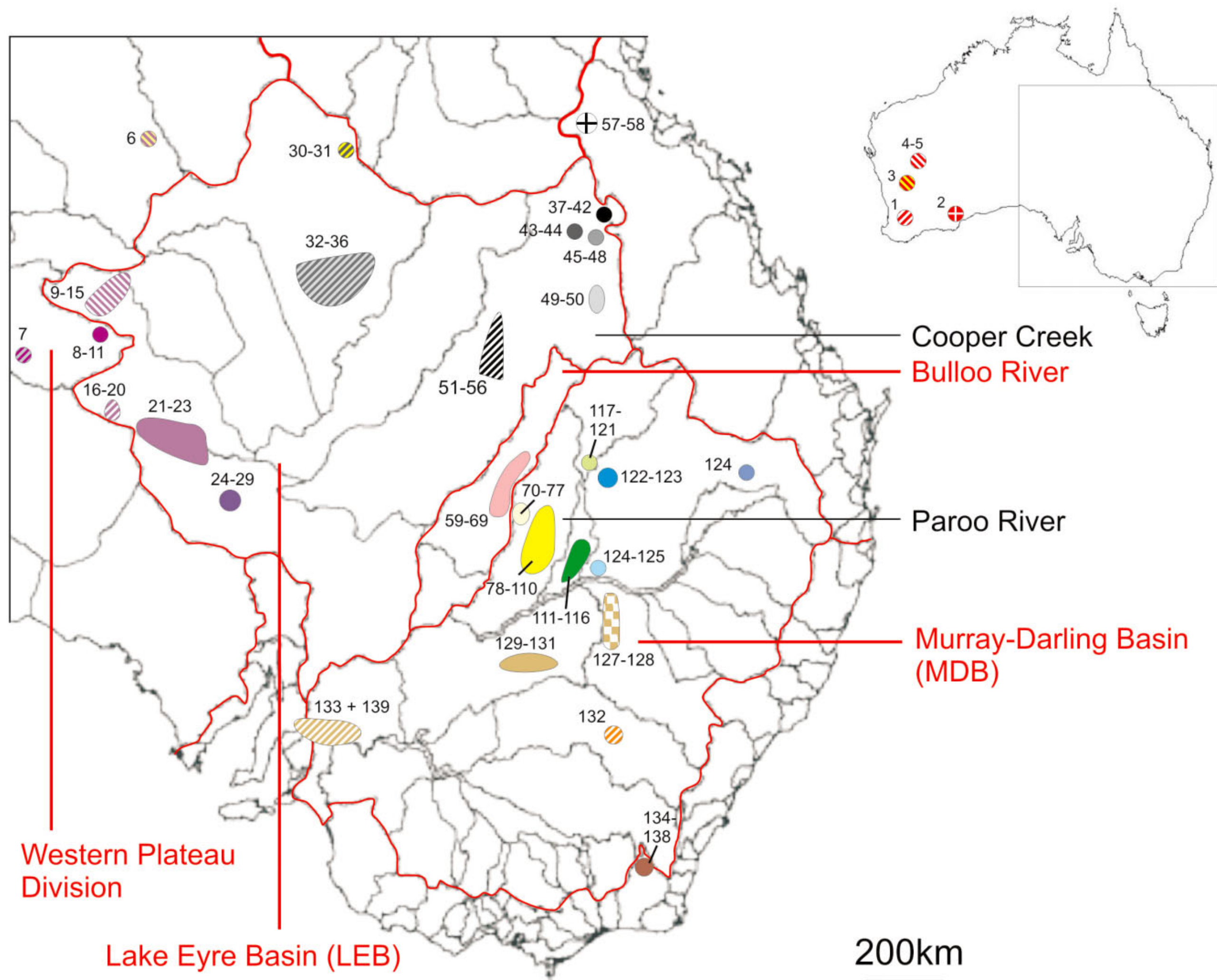

Figure 1. Map showing the sampled localities used in the present study. The larger map depicts the main drainage systems (red lines) and the catchments of individual rivers (black lines). Geographically closely associated localities were grouped together, and the colour coding corresponds to the networks shown in Figs 3, 5. The numbers correspond to the locality numbers in the Supporting information (Table S1).

Table 1. List of primer used in the present study

Gene	Primer	Sequenze (5′- to 3′)	Reference
COI	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	Folmer *et al*. (1994)
	LCO2	TCN ACH AAY CAT AAA GAY ATT GGA AC	Primer designed by L. Krebes and R. Bastrop
	LCO3	TCN ACH AAY CAT AAA GAY ATT GGT AC	Krebes *et al*. (2010)
	HCOoutout	GTA AAT ATA TGN TGN GCT C	Folmer *et al*. (1994)
	HCO-MZ1-rev	CTT TVA TDC CNG TVG GSA CWG CRA TAA TYA T	Krebes *et al*. (2010)
	HCO709	AAT NAG AAT NTA NAC TTC NGG GTG	Blank *et al*. (2008)
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer *et al*. (1994)
EF1α	HaF2For1	GGG YAA AGG WTC CTT CAA RTA TGC	Richter, Olesen & Wheeler (2007)
	2R53ST	CAG GAA ACA GCT ATG ACG CGA ACT TGC AAG CAA TGT GAG C	Richter *et al*. (2007)
ITS2	ITS3	GCA TCG ATG AAG AAC GCA GC	White *et al*. (1990)
	ITS28	CGC CGT TAC TAG GGG AAT CCT TGT AAG	Wagstaff & Garnock-Jones (1998)
28S rRNA	D1,D2 fw1	AGC GGA GGA AAA GAA ACT A	Sonnenberg, Nolte & Tautz (2007)
	D1,D2 rev2	ACG ATC GAT TTG CAC GTC AG	Sonnenberg *et al*. (2007)

For cytochrome oxidase subunit I (COI), always one LCO and one HCO primer were combined. The most successful combinations were: LCO2/HCOoutout, LCO1490/HCO2198, and LCO3/HCO709. EF1α, elongation factor 1α; ITS2, internal transcribed spacer 2.

registered individually at the Australian Museum Sydney (see Supporting information, Table S2).

DNA extraction, PCR amplification, sequencing, and alignments

DNA was extracted from muscular tissue following the HotSHOT method of Montero-Pau, Gómez & Muñoz (2008). The tissue was incubated for 30 min at 95 °C in 40 μL of the alkaline lysis buffer (NaOH 25 mM, disodium ethylenediaminetetracetici acid 0.2 mM, pH 8.0) and then mixed with 20 μL of neutralizing solution (Tris-HCl 40 mM, pH 5.0). Polymerase chain reaction (PCR) reactions were performed with a total volume of 30 μL consisting of 3 μL of each primer (100 mM each; for a list of all primers, see Table 1), 3 μL of dNTPs (2 mM; Fermentas), 0.15 μL of MolTaq polymerase (Molzym), 3 μL of 10 × buffer, 0.8–1.3 μL of $MgCl_2$ (50 mM) and 4.5 μL of DNA extract topped up with H_2O. PCR protocols had an initial denaturation step of 94 °C for 1 min, followed by 38 amplification cycles of 1 min at 94 °C, 30 s at 44–51 °C, and 1 min at 72 °C, and a final elongation step of 5 min at 72 °C. Success of PCR amplification was assessed by gel electrophoresis on 1.5% agarose/TAE gel containing 0.01% ethidiumbromide. PCR products were purified using paramagnetic beads (Agencourt AMPure XP, Beckman Coulter) in accordance with the manufacturer's instructions with final elution in 30 μL of H_2O. Sequencing was performed with the PCR primers using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI 3110 xl (Applied Biosystems). The resulting electropherograms were analyzed using SEQUENCHER, version 4.1.4 (Gene Codes). All sequences were submitted to GenBank (see Supporting information, Table S2). The sequences of the COI, EF1α, and 28S gene fragments were aligned using the CLUSTALW (Thompson, Higgins & Gibson, 1994) algorithm implemented in BIOEDIT, version 7.0.9.0 (Hall, 1999). The ITS2 sequences featured a large number of indels, which greatly compromised the quality of the alignment. Also using MUSCLE (Edgar, 2004) did not improve the alignment of the full dataset. Sequences were subdivided into two groups of relatively closely-related individuals or lineages (based on the combined analysis; see Phylogenetic relationships among main lineages). Alignments for each group of sequences were performed independently. Lineage C did not align well with any of the other two groups and was treated separately.

Initial identification of main lineages

To obtain a first estimate of potential species boundaries, main lineages were derived from the COI dataset. Two computational approaches were employed: the Automated Barcode Gap Discovery (ABGD; Puillandre *et al*., 2012) and the general mixed Yule coalescent model

(GMYC; Pons *et al*., 2006). To gain a conservative estimate of species boundaries, we treated the most inclusive groups of specimens/sequences derived by these methods as main lineages, and thus as the first approximation for species delineation. Several of the main lineages were further subdivided in one or both approaches (e.g. for lower assumed barcode gaps in the ABGD). These less inclusive groups indicate cases where several species may have been erroneously merged into a single main lineage. In the present study, we report such less inclusive groups as 'sublineages' within main lineages.

In the ABGD analysis, barcode gaps are derived from the dataset and recursively reapplied to partition the dataset into groups of sequences (Puillandre *et al*., 2012). Genetic distances between these groups are always larger than the respective barcode gap, whereas, within each group, each sequence is connected to at least one other sequence with genetic distances below the respective gap. The software provides partitions for a range of genetic distances as assumed barcode gaps. To gain a detailed resolution of barcode gaps and the resulting groupings, the number of 'steps' was set to 300; otherwise, standard settings were kept. This allowed the identification of those genetic distances that represent upper or lower bounds of barcode gaps, which all result in a particular grouping of sequences. The analysis was run on the web-based version of the software. We used uncorrected *p*-distances, which were calculated using MEGA5 (Tamura *et al*., 2011) and included all available sequences. The most inclusive grouping derived here corresponds to the main lineages reported further on; all other (less inclusive) groupings are treated as sublineages. The GMYC (Pons *et al*., 2006) analysis was performed with the respective packages in R, version 2.12.0 (R Development Core Team, 2010). Based on the branching pattern in a specified ultrametric tree, species-level and population-level (intraspecific) evolutionary processes are differentiated. The ultrametric tree was obtained with BEAST, version 1.7.5 (Drummond *et al*., 2012), employing a Yule speciation prior and a strict molecular clock. The analysis was run for 2×10^7 generations, saving every 2000th generation and discarding the first 10% as burn-in. The tree was annotated with TREEANNOTATOR, version 1.7.5 (Drummond *et al*., 2012) and sufficiently large effective sampling sizes (> 200) were verified with TRACER, version 1.5 (Rambaut & Drummond, 2007). The single-threshold GMYC model was then fitted to the obtained maximum clade credibility tree.

Monophyly of main lineages was assessed by phylogenetic analyses. To reduce computation time sequences were collapsed into haplotypes and each haplotype was included only once in the analyses. As an outgroup, we chose the North American species *Cyzicus gynecia*. The analysis of Bayesian inference was performed with MrBayes, version 3.2 (Ronquist & Huelsenbeck, 2003). Four chains were run for 6×10^6 generations, with sampling every 1200th generation, and discarding the first 10% as burn-in. The best fitting model (HKY+I + G) was chosen using MRMODELTEST (Posada & Crandall, 2001). Maximum Parsimony analysis was performed using WINCLADA (Nixon, 1999) implementing NONA (Goloboff, 1999) with 100 replications, 1000 starting trees, and 1000 trees to keep. Bootstrap support for each node was assessed with 1000 replications.

Differentiation of main lineages in EF1α and ITS2

To infer whether main lineages are also differentiated from each other in the nuclear encoded gene fragments of EF1α and ITS2 similar phylogenetic analyses were performed as with COI. The settings of the Bayesian and Maximum Parsimony analyses were identical. However, all available sequences were included in the phylogenetic analyses instead of using haplotypes. In addition network analyses were run with NETWORK, version 4.6.1.1 (Fluxus Technology Ltd). Such networks may help to identify closely-related groups of sequences that may not have been monophyletic in the phylogenetic analysis.

Phylogenetic relationships among main lineages

The phylogenetic relationships among the identified main lineages within Australia and their relationships to non-Australian cyzicids were assessed by combined analyses of COI, EF1α, and 28S. The 28S fragment was not employed for inferences of reproductive isolation because the differentiation between closely-related spinicaudatan species is often too low (Schwentner *et al*., 2014). It is well suited, however, to resolve the phylogenetic relationships between Australian and non-Australian species (Schwentner *et al*., 2009). We did not include ITS2 in the combined analysis because not all sequences could be fit into a single alignment (see DNA extraction, PCR amplification, sequencing, and alignments). The phylogenetic analyses were performed as described for COI. The Bayesian analysis was partitioned according to the three gene fragments and all parameters were unlinked among partitions.

Images of the claspers of 15 individuals of eight Australian main lineages, as well as of *Cyzicus californicus* and *Cyzicus tetracerus* were taken by scanning electron microscopy at the Elektronenmikroskopischen Zentrum Rostock. Focus was set on scales or other structures at the tip of the movable finger as these were

useful characters to distinguish among closely-related species of the spinicaudatan genus *Limnadopsis* (Schwentner *et al*., 2011). Claspers were dissected off, cleaned in an ultrasonic bath for 2–3 s, transferred into 100% acetone, critically point dried (Emitech, K850), and sputter coated with gold. They were mounted on the specimen holder described by Pohl (2010). Images were taken with a DSM 906a (Zeiss). A few claspers were scanned at the Australian Museum Sydney on an EVO LS15 (Zeiss), where claspers were mounted on scanning electron microscopy stubs.

Distribution, dispersal, and phylogeographical history of main lineages

To obtain an estimate of the genetic diversity and differentiation within main and sublineages, haplotype (h) and nucleotide (π) diversities were calculated with ARLEQUIN, version 3.5 (Excoffier & Lischer, 2010). Haplotype diversity is a measure of the genetic diversity in terms of the relative abundance and frequency of haplotypes (the probability that two randomly chosen haplotypes are different), nucleotide diversity is a measure of the differentiation among haplotypes (the probability that two randomly chosen homologous nucleotide sites are different) (Excoffier & Lischer, 2010). To visualize the genetic diversity and its geographical distribution within all main lineages, haplotype networks for the COI dataset were calculated with NETWORK, version 4.6.1.1 (Fluxus Technology Ltd). The pronounced genetic differentiation among main lineages led to complex reticulations among main lineages and compromised the intralineage resolution. Therefore, networks were calculated for each main lineage separately.

Population differentiation within main lineages was assessed by pairwise Φ_{ST} estimates and SAMOVA analyses (spatial analysis of molecular variance; Dupanloup, Schneider & Excoffier, 2002). In both cases, only populations for which with at least four specimens for the respective main lineage were available were included. Populations with no or nonsignificant differentiation are assumed to have been in recent contact by dispersal with gene flow. However, it is not possible to distinguish regular, ongoing gene flow from recently founded populations that did not yet result differentiation of the populations. Φ_{ST} estimates were calculated with ARLEQUIN, version 3.5. Significance was assessed at the 0.05 level by 110 permutations. SAMOVA combines the genetic and geographical data to assign population to groups in such a way that the differentiation among groups is maximized. Accordingly, the analysis has to be repeated with different predefined numbers of groups. The resulting grouping that resulted in the highest F_{CT} (variation among groups) should mirror the geographical subdivision of populations of the respective main lineage.

Two different neutrality tests were performed, Fu's F_S (Fu, 1997) and Tajima's D (Tajima, 1989), to identify main and sublineages that deviate from neutrality. For both tests, all specimens of the respective lineages were included (tests were not calculated if fewer than five specimens were available). Stable populations are typically characterized by positive values for both tests. Significant negative values are indicative of deviations from mutation–drift equilibrium. This can be caused by an excess of rare haplotypes after recent expansion events. During expansion events, 'new' haplotypes can accumulate as genetic drift is reduced (e.g. extinction of haplotypes), whereas the mutation rate should remain constant.

RESULTS

Alignments

The alignment of all 737 COI sequences had a total length of 569 bp, of which 230 bp were variable and 220 bp were parsimony informative. The alignment contained no indels. The deduced amino acid sequences had a length of 189 amino acids, of which 18 were variable and contained no stop codons. The alignment of all 64 EF1α sequences had a length of 711 bp, of which 128 bp were variable and 89 bp were parsimony informative; no indels were present. The deduced amino acid sequences contained 237 amino acids, of which only seven were variable and featured no stop codons. The 28S alignment featured 724 bp. If all available Cyzicidae were included 102 bp were variable and 102 bp were parsimony informative; if only Australian representatives are considered 48 bp were variable and 35 were parsimony informative. Because of rampant indels the ITS2 sequences were not combined in a single alignment (see DNA extraction, PCR amplification, sequencing, and alignments). The alignment featuring all preventatives of lineages A, B, G, I, K, L, O, P, Q, R, and S had a length of 587 bp, of which 131 bp were variable and 95 were parsimony informative. The alignment including all representatives of lineages D, E, F, M, and N had a length of 661 bp, of which 183 bp were variable and 171 bp were parsimony informative. In addition, both ITS2 alignments contained numerous indels.

Initial identification of main lineages

The GMYC analysis resulted in 29 lineages and the ABGD analysis of the COI dataset retrieved 21 lineages (no additional lineage was present in the ABGD analysis; rather, several lineages were collapsed), when barcode thresholds of 5.4–8.7% were employed (Table 2). Because the latter is the most conservative initial

Table 2. Number of main and sublineages derived by GMYC and ABGD analyses

	Percentage	Number of retrieved lineages	Sublineages
GMYC	–	29	D1, 2, D3, N1, N2, H1, H2, Q1, Q2, 5, Q3, Q4
ABGD	0.09–1.39	29	D1, 2, D3, N1, N2, H1, H2, Q1, Q2, 5, Q3, Q4
	1.40–1.79	28	D1+2, D3, N1, N2, H1, H2, Q1, Q2, 5, Q3, Q4
	1.82–2.08	27	D1+2, D3, N1, N2, H1, H2, Q1, Q2+5, Q3, Q4
	2.11–2.30	26	D1+2, D3, N1, N2, H1, H2, Q1+2 + 5, Q3, Q4
	2.32–3.96	23	H1, H2, N1, N2
	4.02–5.36	22	H1, H2
	5.43–8.80	21*	
	> 8.9	1**	

The percentages correspond to the respective genetic distance threshold value applied to partition the dataset into lineages in the Automated Barcode Gap Discovery (ABGD) analysis. In the present study, we used the most conservative partition (= 21 lineages) to identify putative species (= main lineages); further lineages that were retrieved by lower threshold values or the general mixed Yule coalescent model (GMYC) are reported as sublineages.
*Corresponds to all 21 main lineages; **if a threshold value > 8.9% is applied, all sequences collapse into a single group.

estimate of putative species within our dataset, we treat these 21 lineages as main lineages. Main lineages were labelled A–W. Higher threshold values in the ABGD collapsed all lineages and smaller thresholds successively yielded additional lineages (Table 2), which we treat as sublineages. Sublineages were retrieved for main lineages D (up to three sublineages: D1–D3), H (up to two sublineages: H1 and H2), N (up to two sublineages: N1 and N2), and Q (up to five sublineages: Q1–Q5) depending on the respective threshold values (Table 2). The highest number of lineages (main lineages plus all sublineages) was 29 for threshold values of 0.9–1.39% in the ABGD. Smaller thresholds resulted in an arbitrary number of additional lineages, which are not reported here. These 29 lineages correspond to the 29 lineages of the GMYC analysis.

Pairwise genetic distances (all as uncorrected p-distances) between main lineages ranged from 8.1% to 21.2% (Table 3). The lowest pairwise distances was observed between main lineages U and Q (8.1–11.1%), whereas all other pairwise distances exceeded 11.1%. Within main lineages, the largest distances were 2.6% if they did not feature any sublineages. Otherwise, the maximum intralineage distance reached up to 7.1% among sublineages (Table 3). However, the smallest intralineage distance that connects all sublineages within each main lineage is lower: 5.4% for D, 6.0% for H, 4.0% for N, and 5.2% for Q (Table 4; see also Supporting information, Table S3). These smallest distances are those distances that are relevant for the ABGD.

The phylogenetic analysis of COI recovered all main lineages and most of the sublineages as monophyletic with very high support (at least if more than one haplotype was available; Fig. 2). Only sublineage Q5 was not monophyletic but still clearly differentiated from all other sublineages within Q.

Many of the 21 main lineages occur in sympatry or syntopy. A pairwise comparison of main lineages revealed that 64 pairs of main lineages occur in sympatry (of 210 pairwise comparisons), 41 of these even in syntopy (Table 3; sympatry is indicated by identical colours in Figs 3, 6). The highest number of sympatric co-occurrences was recorded in the central Paroo catchment (10 main lineages; yellow in Figs 1, 3, 6). It is noteworthy that most sublineages do not occur in sympatry and are often well separated geographically. The only exceptions are Q3 and Q5, which co-occur in central and eastern Australia and Q4 and Q5, which co-occur in Western Australia.

Inference of reproductive isolation among main lineages

All 21 main lineages are clearly differentiated from each other in the analyses of the ITS2 and EF1α datasets (Fig. 3; see also Supporting information, Fig. S1). In the phylogenetic analysis of EF1α, a few main lineages were paraphyletic (H with respect to I; M with respect to N; Q with respect to P; and S with respect to O; see Supporting information, Fig. S1); however, in no case were individuals randomly distributed among lineages and the network analysis clearly support the differentiation of S and O and M and N (Fig. 3). The paraphyly of H and Q is caused by the strong differentiation among their sublineages. Of the five sublineages of Q, only Q4 and Q5 showed evidence of recent reproduction because the only individual of Q5 from Western Australia (yellow-red striped in Figs 1, 3) features an EF1α sequence related to Q4. Conversely, Q3 and Q5, which occur commonly sympatric, are

Table 3. COI genetic distances among and sympatric and syntopic occurrences of main lineages

	A	B	C	D	E	F	G	H	I	K	L
A	0.0–1.9	11.1–12.2	15.1–16.3	15.1–18.3	14.9–17.3	17.1–17.9	18.6–19.5	14.1–15.8	15.4–16.2	18.2–19.3	17.9–18.6
B		0.0–2.1	12.3–13.7	14.5–17.5	12.8–14.8	17.6–18.5	17.5–18.5	15.6–17.4	16.0–17.9	17.9–19.5	15.9–16.6
C			0.0–2.6	12.9–16.1	12.7–15.1	16.3–17.4	17.7–18.7	13.0–15.3	14.6–15.8	15.1–16.7	17.2–18.1
D				0.0–7.1*	16.3–17.9	19.1–21.0	18.1–21.0	15.1–17.9	16.8–19.0	17.6–21.0	18.9–21.0
E					0.0–2.1	15.8–17.1	20.4–21.2	13.5–16.0	15.1–17.1	16.9–19.3	16.0–17.0
F						0.0	18.6–18.8	18.1–2.0	17.0–17.4	18.5–19.5	20.2
G							0.0–0.4	17.3–20.4	18.1–19.0	13.6–14.8	17.2
H								0.0–6.3*	14.3–16.0	15.3–16.9	15.9–16.6
I									0.0–0.6	14.6–16.0	17.0–17.8
K										0.0–1.6	13.3–14.4
L											–
M											
N											
O											
P											
Q											
R											
S											
T											
U											
W											

	M	N	O	*P*	Q	R	S	T	U	W
A	16.6–17.6	17.2–19.6	16.2–18.8	18.1–18.8	16.1–18.6	16.0–16.7	16.8–19.4	17.1–18.1	17.8–18.3	15.2–16.0
B	16.8–18.1	14.4–17.1	16.2–18.2	16.4–18.5	15.7–18.8	16.1–17.2	14.9–17.1	15.4–16.3	16.9–17.0	15.2–16.0
C	15.8–17.4	15.5–17.8	15.0 –16.5	12.1–13.5	13.7–17.1	14.8–16.2	15.0–16.9	14.5–15.8	15.5–16.2	14.1–15.6
D	15.8–19.9	17.4–20.8	17.4–21.5	16.2–18.5	15.9–20.0	15.6–18.9	16.2–20.6	15.6–16.8	16.6–19.1	14.3–17.0
E	14.5–15.5	13.2–15.0	16.3–18.1	14.2–16.9	13.4–16.0	16.5–17.5	15.1–16.9	15.1–15.9	16.0–16.6	15.2–16.7
F	19.4 –19–9	17.9–18.6	18.8–19.5	18.6–19.0	17.6–19.7	17.4–18.1	18.3–19.7	18.7	18.5	17.4
G	18.6–19.2	19.9–20.8	17.6–19.3	16.9–17.3	18.4–20.8	17.8–18.5	17.6–19.6	19.7	19.2–19.3	16.9–17.1
H	16.1–18.5	16.9–19.0	14.1–15.5	14.9–17.6	14.1–16.0	14.2–16.3	13.3–15.5	16.1–17.3	16.5–18.6	13.0–14.8
I	16.4–17.2	16.2–19.0	15.7–16.5	16.3–17.2	14.6–16.8	15.1–16.0	15.3–17.0	17.9–18.7	17.0–17.8	10.8–11.1
K	18.4–19.9	18.3–20.0	16.4–17.9	15.8–16.7	16.5–19.5	17.5–19.5	16.3–18.3	17.8–18.6	19.0–19.7	15.4–16.0
L	18.9–19.3	18.5–20.0	17.6–18.6	20.2–20.4	17.2–18.3	20.0–20.2	16.8–17.4	18.0	20.0	16.3
M	0.0–1.1	14.4–16.5	18.1–20.0	17.5–18.5	16.3–18.7	15.5–16.9	17.2–18.8	17.3–17.6	18.3–19.2	18.0–18.4
N		0.0–4.9*	17.5–20.0	17.0–17.6	14.4–16.9	17.0–18.5	16.3–20.0	15.2–16.5	16.6–17.2	15.2–16.1
O			0.0–0.9	17.7–18.6	15.6–18.5	16.5–17.6	10.5–12.4	17.6–18.0	18.3–19.2	16.1–16.9
P				0.0–0.2	11.3–12.8	13.7–14.2	16.7–17.9	17.6–17.8	12.9–13.2	16.5–16.7
Q					0.0–6.8*	14.1–16.2	15.6–19.7	15.4–17.1	8.1–11.1	14.3–16.9
R						0.0–1.8	14.9–16.3	15.8	13.7–13.9	15.2–15.6
S							0.0–3.2	16.3–17.6	17.3–19.2	15.2–16.0
T								–	17.3	15.8
U									–	16.7
W										–

All genetic distances are pairwise uncorrected *p*-distances as a percentage summarized for all individuals of the respective main lineages. Main lineages pairs that occur syntopically (e.g in the same water body) are indicated in dark grey, and pairs that occur sympatrically (areas with the same colours in Figs 1, 3, 5) are indicated in light grey.
*Features sublineages, for genetic distances within and among sublineages, see the Supporting information (Table S3).

Table 4. Scales and setae at the tip of the movable finger of the male clasper

Main lineage	Individual	Clasper	Number of scales	Shape
B	AM P.80862	1R	5	Slender, claw-like scales
		2R	5	Slender, claw-like scales
C	AM P.82576	1R	9	Slender, claw-like scales
		2R	8	Slender, claw-like scales
K	AM P.91559	1R	4	Slender, claw-like scales
	AM P.82540	1R	5	Slender, claw-like scales
		2R	5	Slender, claw-like scales
M	AM P.82574	1R	5	Slender, claw-like scales
		2R	6	Slender, claw-like scales
N	AM P.80859	1R	4	Slender, claw-like scales
	AM P.82534	1R	6	Slender, claw-like scales
	AM P.82401	1R	6	Slender, claw-like scales
		2R	6	Slender, claw-like scales
	AM P.91182	1R	5	Slender, claw-like scales
		2R	5	Slender, claw-like scales
	AM P.82578	1R	5	Slender, claw-like scales
		2R	5	Slender, claw-like scales
O	AM P.80861	1R	5	Slender, claw-like scales
	AM P.91628	1R	7	Slender, claw-like scales
		2R	6	Slender, claw-like scales
R	AM P.82538	1R	6	Slender, claw-like scales
		2R	6	Slender, claw-like scales
S	AM P.80858	1R	5	Slender, claw-like scales
		2R	4	Slender, claw-like scales
	AM P.82536	1R	4	Slender, claw-like scales
		2R	4	Slender, claw-like scales
Cyzicus californicus		1R	~50	Field of hair-like setae
		2R	~50	Field of hair-like setae
Cyzicus tetracerus	Specimen 1	1R	0	No scale or setae
		2R	0	No scale or setae
	Specimen 2	1R	0	No scale or setae
		2R	0	No scale or setae
		1L	0	No scale or setae
		2L	0	No scale or setae

For details of the described structures, see Fig. 5. 1R, 2R, 1L, and 2L refer to the first right, second right, first left and second left clasper of the individuals, respectively.

clearly differentiated in ITS2 and EF1α. Sublineages H1 and H2, as well as D1–D3, are clearly differentiated in ITS2 and EF1α, whereas N1 and N2 are differentiated in ITS2 only, with an identical sequence in EF1α.

Phylogenetic relationships among main lineages

The phylogenetic relationships among all main lineages differed in the analyses of the single gene fragments. Here, we focus on the results of the most inclusive analysis: the combined analysis of COI, EF1α, and 28S. A clade comprising all Australian lineages is well supported as monophyletic, as is a separate clade of all studied non-Australian *Caenestheriella*, and *Cyzicus* specimens (Fig. 4); it should be stressed that only few non-Australian representatives were available. Within the Australian clade, all Australian *Caenestheria* main lineages form a well supported monophylum, whereas the Australian *Caenestheriella* are paraphyletic with respect to the Australian *Caenestheria* clade. It is noteworthy that all main lineages comprising several sublineages are monophyletic as well.

The claspers of all studied Australian representatives featured four to nine long, slender, claw-like scales at the tip of the movable finger (Fig. 5; Table 4). A differentiation among main lineages is apparently not possible because the number of scales varies within main lineages and often even between claspers of single individuals (Table 4). However, the difference towards non-Australian species is striking: although *C. californicus* has a field of hair-like setae, *C. tetracerus*

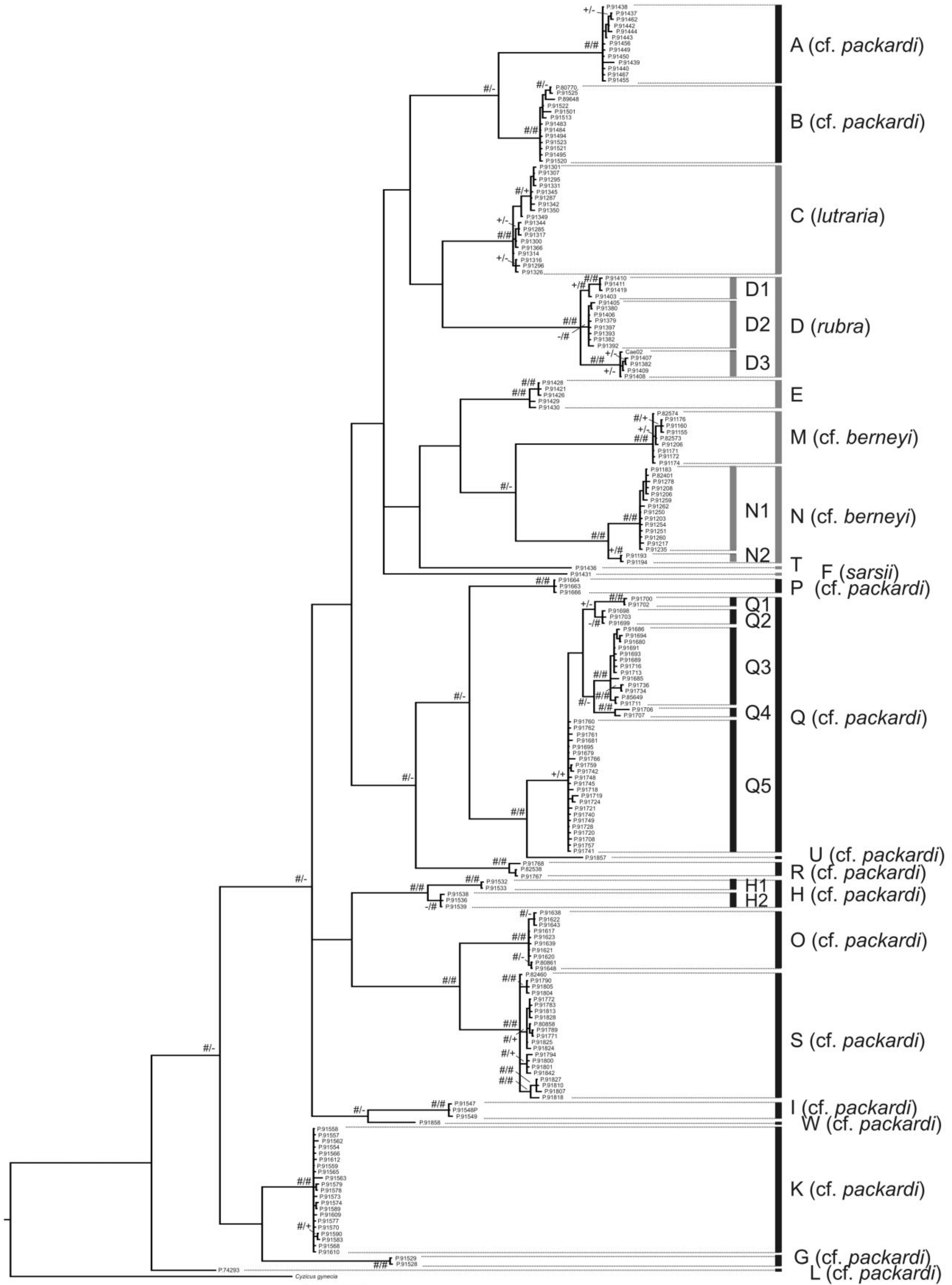

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Figure 2. Bayesian inference majority rule tree based on analysis of cytochrome oxidase subunit I (COI). Each retrieved haplotype was included only once. All derived main and sublineages are depicted. Black vertical bars indicate those lineages whose specimens feature an elongated condyle (originally classified as *Caenestheriella*), whereas grey vertical bars indicate those with a short and rounded condyle (originally classified as *Caenestheria*). For each branch, posterior probabilities and bootstrap support of the Maximum Parsimony analyses are given. #, For support values ≥ 0.95 or ≥ 95; +, for support values ≥ 0.90 or 90, respectively (– indicates support < 0.9 or < 90, if both are lower, no support is stated).

did not feature any outgrowth on any of its four claspers (Fig. 5). Also, *Eocyzicus* has different outgrowths. In this taxon, they are also relatively long and slender but with a fringe along its edge and they appear softer (L. Tippelt & M. Schwentner, unpubl. data). The claw-like scales may constitute a morphological characteristic of the Australian Cyzicidae. Given the well supported monophyly of the Australian Cyzicidae, the non-monophyly of traditional cyzicid genera, and the claw-like scales, we propose *Ozestheria* gen. nov. as a new genus summarizing all Australian representatives formerly recognized as *Caenestheria* or *Caenestheriella*.

SYSTEMATICS

BRANCHIOPODA LATREILLE, 1817

DIPLOSTRACA GERSTAECKER, 1866–1879

ONYCHOCAUDATA OLESEN & RICHTER, 2013

SPINICAUDATA LINDER, 1945

CYZICIDAE STEBBING, 1910

OZESTHERIA GEN. NOV. SCHWENTNER & RICHTER

Etymology: The name is derived from Oz, a colloquial abbreviation for Australia (the only continent the genus is known from) and Estheria, a former name for the Cyzicidae, which was established by Rüppel (1837) and is still part of several generic names within the Spinicaudata.

Diagnosis: Overall morphology resembles that of other Cyzicidae; carapace with umbo and with or without sculpturing between growth lines; condyle either short and rounded or elongated and pointed; male and female rostrum without posterior margin (in contrast to *Eocyzicus* and *Cyzicus*); posterior trunk segments with several dorsal spines (in contrast to *Eocyzicus*, which features only one spine per segment); telson with numerous, irregular spines; scales at the tip of the movable finger of the male claspers always slender and claw-like.

Type species: Ozestheria lutraria (Brady, 1886).

Further species: The two Australian species formerly assigned to *Caenestheriella* are transferred to *Ozestheria* gen. nov., namely *Ozestheria mariae* (Olesen & Timms, 2005) and *Ozestheria packardi* (Brady, 1886) including all three varieties of the latter *Ozestheria packardi* var. *typical* (Spencer & Hall, 1896), *O. packardi* var. *cancellata* (Spencer & Hall, 1896), and *Ozestheria packardi* var. *minor* (Spencer & Hall, 1896). Furthermore, all species formerly assigned to *Caenestheria* are now transferred to *Ozestheria* gen. nov., namely *Ozestheria berneyi* (Gurney, 1927), *Ozestheria dictyon* (Spencer & Hall, 1896), *Ozestheria elliptica* (Sars, 1897), *A. lutraria* (Brady, 1886), *Ozestheria rubra* (Henry, 1924), *Ozestheria rufa* (Dakin, 1914), and *Ozestheria sarsii* (Sayce, 1903) (for more details, see Taxonomic implications). The assignment of *O. rufa*, *O. dictyon*, and *O. elliptica* to *Ozestheria* gen. nov. is somehow ambiguous because no specimens of these species were available in the present study; however, our results strongly suggest that all Australian species share a common ancestry.

Distribution: The genus is only known from Australia.

DISTRIBUTION, DISPERSAL, AND PHYLOGEOGRAPHICAL HISTORY OF MAIN LINEAGES

The geographical distribution of the *Ozestheria* gen. nov. main and sublineages reveals some conspicuous patterns. None of the main lineages occurs exclusively in central Australia; rather, each is present in eastern Australian localities as well. Only two sublineages, D1 and N2, are restricted to central Australia. Conversely, several main and sublineages are restricted to eastern Australia (D1, D2, E, F, H, I, K, N1, O, P, R, U, and W), several of which are restricted either to the Paroo River catchment or the north-eastern Cooper Creek catchment. Furthermore, geographical 'outlier' populations of eastern Australia featured endemic lineages each; for example, localities 57–58 (H1), 133 (F and U), 134–139 (P), and all lineages occurring in Western Australia (G, L, Q2, Q4, and T) are endemic therein, with Q5 being the only exception. It is noteworthy that most pairs of main or sublineages that are in a sister groups relationship in the combined phylogenetic analysis do not occur in the same region but are often geographically clearly separated (e.g. F

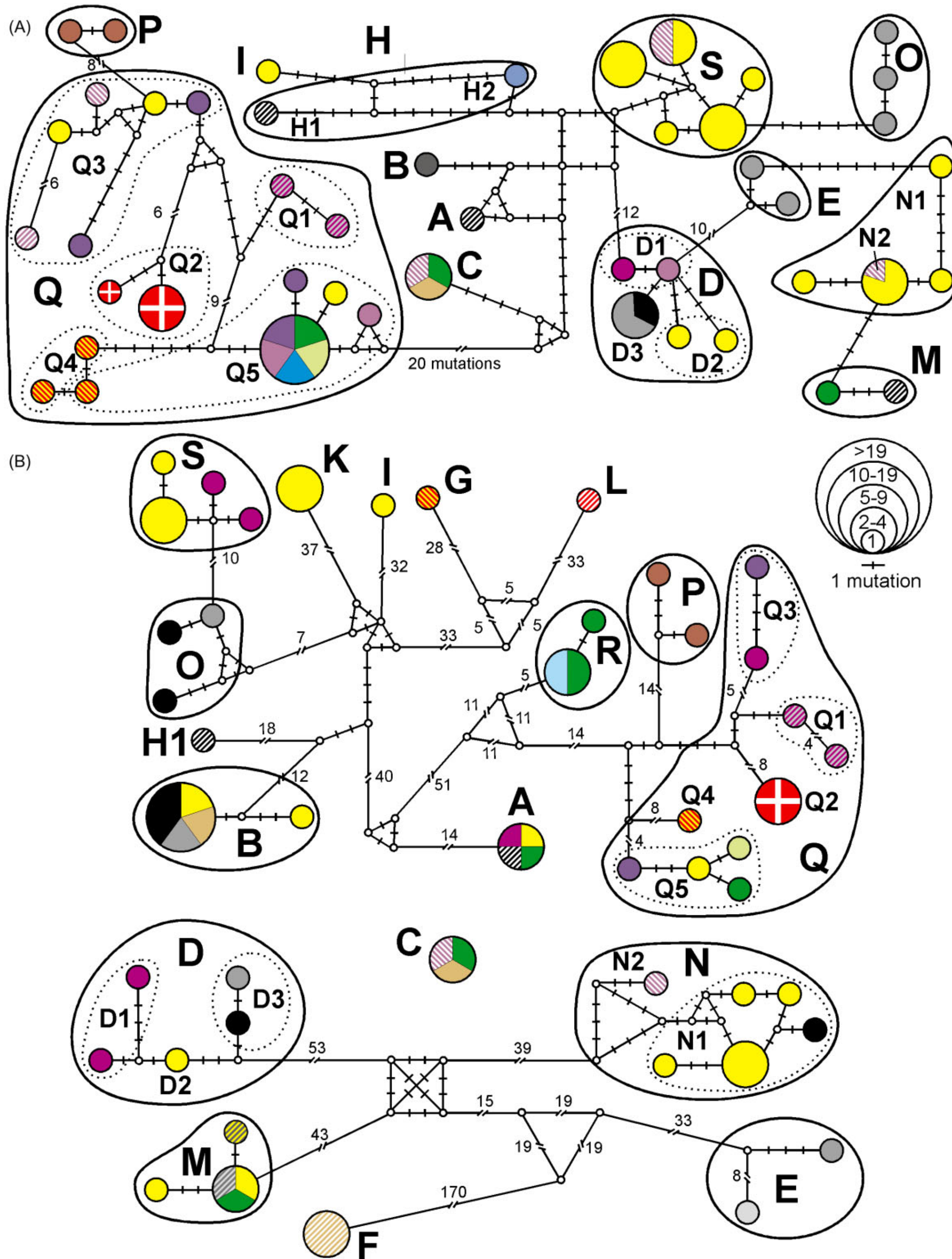

Figure 3. Median-joining networks of (A) elongation factor 1α (EF1α) and (B) internal transcribed spacer 2 (ITS2). For ITS2, three separate networks, each featuring closely-related species, were calculated because an alignment including all specimens was largely ambiguous. Main lineages are inidcated by continuous lines, and possible sublineages by dotted lines. Affiliation of specimens to respective main and sublineages based on prior analyses of COI (Fig. 2). All available sequences were included and each circle represents a specific sequence, with the size corresponding to its frequency (see scale). The colours code for the localities from which specimens were collected and correspond to Fig. 1. The vertical marks or numbers on the connecting lines represent the respective mutational steps.

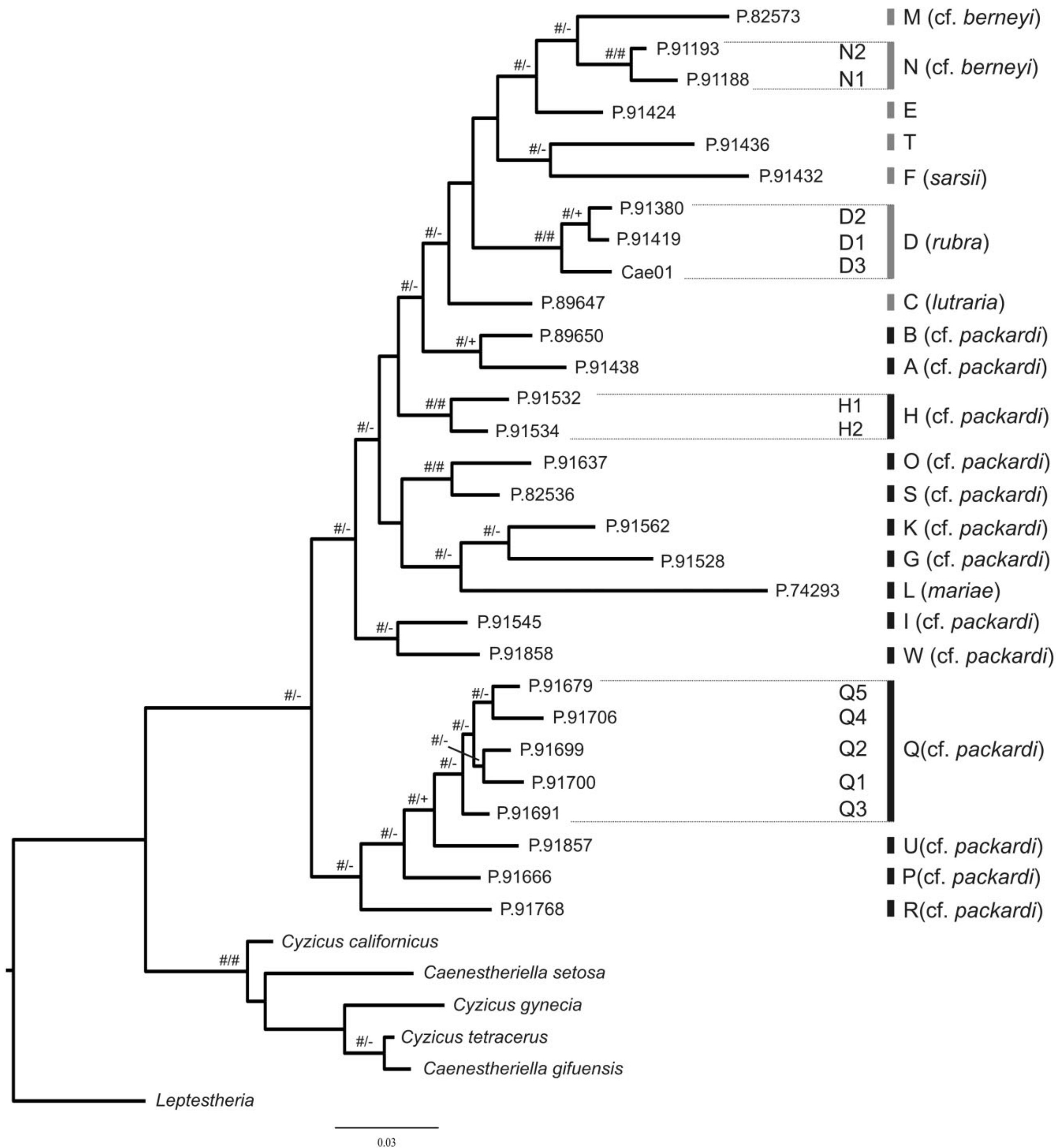

Figure 4. Bayesian inference majority rule tree based on a combined analysis of cytochrome oxidase subunit I (COI), elongation factor 1α (EF1α) and 28S. Each main and sublineage was included only once. Black vertical bars indicate those lineages whose specimens feature an elongated condyle (originally classified as *Caenestheriella*), whereas grey vertical bars indicate those with a short and rounded condyle (originally classified as *Caenestheria*). For each branch, posterior probabilities and bootstrap support of the Maximum Parsimony analyses are given. #, For support values ≥ 0.95 or ≥ 95; +, for support values ≥ 0.90 or 90, respectively (– indicates support < 0.9 or < 90, if both are lower, no support is stated).

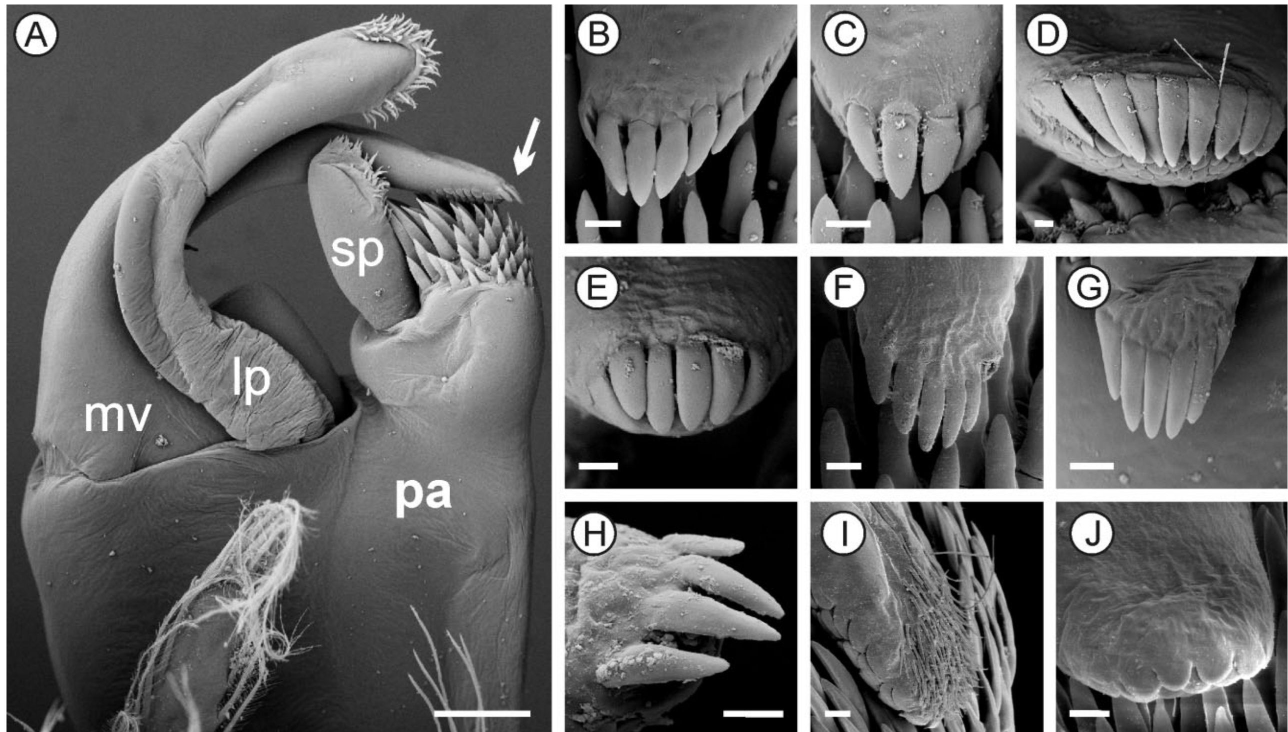

Figure 5. Scales on the tip of the movable finger of male clasper. A, overview of typical spinicaudatan male clasper (lineage N [AM P.82401] first right clasper, posterior view), the arrow indicates the relevant scales at the tip of the movable finger. B–J, details of the scales of various cyzicid species (mostly in frontal view); B, lineage N (AM P.82401), second right clasper; C, lineage N (AM P.80859), first right clasper; D, lineage C (AM P.82576), first right clasper; E, lineage K (AM P.82540), second right clasper; F, lineage O (AM P.91628), second right clasper; G, lineage R (AM P.82538), second right clasper; H, lineage S (AM P.82536), first right clasper; I, *Cyzicus californicus* (Richter, private collection), first right clasper; J, *Cyzicus tetracerus* (Richter, private collection), first right clasper. AM numbers correspond to registration numbers of specimens in the collection of the Australian Museum (for details, see Table S2). Scale bars: A, 100 μm, B–J, 10 μm. lp, large palpus; mf, movable finger; pa, palm; sp, small palpus.

& T; O & S; G & K; D1, D2 & D3; N1 & N2; H1 and H2; I & W; U & Q) (Figs 4, 6). Only A & B, M & N, and Q3 & Q5 are lineage pairs in potential sister group relationships that occur sympatrically, Q3 & Q5 and M & N occur even syntopically.

The large number of delimited main lineages compromised a comprehensive phylogeographical analysis because, for many lineages, too few specimens were available. This was furthered by the rather high degree of syntopic occurrences (Table 3), which reduced the number of specimens per lineage retrieved from many sites. Only few main and sublineages studied deviated significantly from neutrality (e.g. mutation–drift equilibrium) in one or both applied tests (Table 5): A, D2, D3, K, N, and Q5. The haplotypes of these lineages are mostly closely related (also evident in the often low haplotype diversity; Table 5) and show a star-like pattern in the respective haplotype networks (Fig. 6). This is typical for species with recent expansion, possibly preceded by a bottleneck event that reduced its prior genetic diversity. Also, several other lineages feature star-like patterns within their haplotype networks but with additional genetically more divergent haplotypes as well (e.g. B, M, N1, Q3, and S; Fig. 6). Here, recent expansion may have occurred as well, although the genetic diversity appears to not have been compromised by a recent bottleneck event, or at least a greater portion of its genetic diversity prevailed. Another indication of recent expansion is the presence of geographically widespread haplotypes in several lineages (A, B, C, K, M, N, Q5, and S; Fig. 6), which is often the central haplotype of a star-like pattern.

The SAMOVA revealed only for main lineage S any grouping of populations that correlated to geographical regions (see Supporting information, Table S4): central Australian (populations 6, 8, 31; Fig. 1) versus eastern Australian populations (62, 69, 77, 96, 100, 101, 102, 103, 104, 107, 110; Fig. 1). In all other instances, either no grouping was supported or the

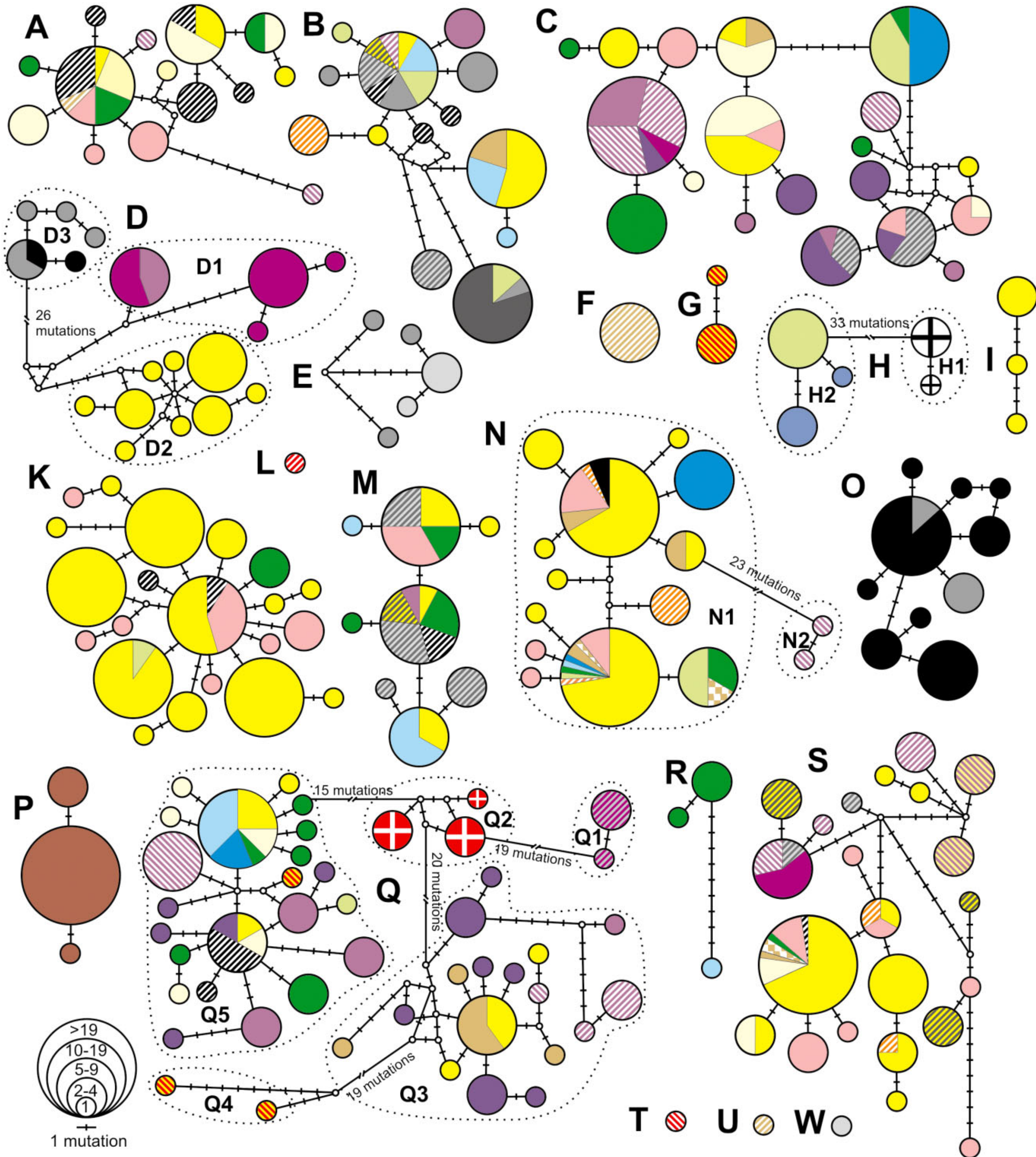

Figure 6. Median-joining haplotype networks of cytochrome oxidase subunit I (COI). Networks were calculated for each respective main lineage separately ('numbering' by letters corresponds to main lineages). Possible sublineages are indicated by dotted lines. All available sequences were included and each circle represents a specific sequence (= haplotype), with the size corresponding to its frequency (see scale). The colours code for the localities from which specimens were collected and correspond to Fig. 1. The vertical marks or numbers on the lines connecting haplotypes represent the respective mutational steps between haplotypes.

Table 5. Properties of all main and sublineages based on COI

	Number of individuals	Number of populations	Number of haplotypes	H (mean ± SD)	π (mean ± SD)	Fu's F_S (P-value)	Tajima's D (P-value)
A	37	12	14	0.85 ± 0.05	0.0046 ± 0.0028	−5.263 (0.014)*	−1.706 (0.020)*
B	57	19	13	0.88 ± 0.03	0.0093 ± 0.0052	0.587 (0.603)	0.842 (0.824)
C	99	27	19	0.90 ± 0.02	0.0116 ± 0.0061	0.076 (0.523)	1.077 (0.905)
D	43	11	18	0.94 ± 0.02	0.0298 ± 0.0152	0.809 (0.683)	0.911 (0.856)
D1	19	4	4	0.74 ± 0.08	0.0084 ± 0.0049	2.841 (0.903)	1.412 (0.930)
D2	17	5	9	0.96 ± 0.04	0.0035 ± 0.0026	−4.271 (0.000)*	−1.234 (0.109)
D3	7	2	5	1.00 ± 0.08	0.0035 ± 0.0027	−2.019 (0.037)*	−0.040 (0.455)
E	11	4	5	0.82 ± 0.12	0.0100 ± 0.0061	0.147 (0.519)	−0.468 (0.338)
F	5	1	1	1.00	0.00	–	
G	5	1	2	0.40 ± 0.24	0.0014 ± 0.0014	1.040 (0.613)	−0.973 (0.172)
H	13	4	5	0.79 ± 0.08	0.0230 ± 0.0125	6.322 (0.987)	0.725 (0.817)
H1	3	2	2	0.67 ± 0.31	0.0012 ± 0.0015	0.201 (0.399)	0.00 (0.985)
H2	10	2	3	0.64 ± 0.10	0.0024 ± 0.0018	0.949 (0.701)	0.700 (0.775)
I	5	2	3	0.80 ± 0.16	0.0028 ± 0.0024	0.061 (0.318)	−0.175 (0.501)
K	69	10	22	0.93 ± 0.02	0.0043 ± 0.0026	−13.515 (0.00)*	−1.942 (0.00)*
L	1	1	1	–	–	–	–
M	37	13	8	0.87 ± 0.04	0.0037 ± 0.0024	−1.011 (0.327)	−0.297 (0.454)
N	101	36	16	0.81 ± 0.03	0.0053 ± 0.0031	−2.723 (0.198)	−1.978 (0.008)*
N1	99	35	14	0.80 ± 0.03	0.0037 ± 0.0023	−3.526 (0.074)	−1.254 (0.091)
N2	2	1	2	1.00 ± 0.50	0.0018 ± 0.0025	–	–
O	35	7	10	0.83 ± 0.05	0.0039 ± 0.0025	−0.143 (0.530)	−0.206 (0.454)
P	27	6	3	0.27 ± 0.10	0.0005 ± 0.0006	−1.088 (0.142)	−0.978 (0.187)
Q	88	26	46	0.98 ± 0.01	0.0265 ± 0.0133	−5.603 (0.081)*	−0.430 (0.382)
Q1	3	1	2	0.67 ± 0.31	0.0012 ± 0.0015	–	–
Q2	5	1	3	0.80 ± 0.16	0.0039 ± 0.0030	0.804 (0.628)	0.957 (0.784)
Q3	26	9	17	0.95 ± 0.03	0.0148 ± 0.0080	−2.486 (0.172)	−0.990 (0.153)
Q4	2	1	2	1.00 ± 0.50	0.0216 ± 0.0225	–	–
Q5	51	14	22	0.95 ± 0.02	0.0051 ± 0.0031	−6.756 (0.004)*	−1.567 (0.037)*
R	4	2	3	0.83 ± 0.22	0.0088 ± 0.0064	1.506 (0.718)	−0.834 (0.129)
S	91	28	22	0.80 ± 0.04	0.0100 ± 0.0054	−2.046 (0.268)	−0.946 (0.188)
T	1	1	1	–	–	–	–
U	1	1	1	–	–	–	–
W	1	1	1	–	–	–	–

For each main lineage and their respective sublineages, the number of individuals, number of populations, number of haplotypes, the haplotype diversity (H), nucleotide diversity (π), Fu's F_S and Tajima's D are given. For lineages with less than five individuals, the latter two are not reported. *Significant at $P < 0.05$.

respective groups did not reflect any geographical correlation (e.g. lineages A and M; see Supporting information, Table S4). Population differentiation estimated by Φ_{ST} was very heterogeneous (see Supporting information, Table S5). Among all studied regions of central (western Lake Eyre Basin and eastern Western Plateau Division) and eastern Australia (eastern Lake Eyre Basin and Murray-Darling Basin) and at all geographical scales therein, populations with low and nonsignificant as well as high and significant estimates were revealed (see Supporting information, Table S5), often within single lineages. However, certain tendencies are observable (see Supporting information, Table S5): greater genetic differentiation among central and eastern Australian populations, than within these regions (C, D, Q5, and S); among central Australian populations, the genetic differentiation is often larger than among eastern Australian populations, despite similar geographical distances among populations (C, D, Q5, and S); within eastern Australia, the most northern populations of the Murray-Darling Basin are often less differentiated towards populations further north (from the north-eastern Cooper) than to other populations of the Murray-Darling Basin (e.g. B, C, M, and N); otherwise, the differentiation within the Murray-Darling Basin is often rather low (D, K, M, N, Q5, and S).

DISCUSSION

From lineages to species

The perception and delimitation of species is heavily dependent on the researcher's notion of species and thus the underlying species concept (Laamanen *et al*., 2003; Agapow *et al*., 2004; Tan *et al*., 2008; Schwentner *et al*., 2011). Our initial partition into 21 main lineages and eight additional sublineages was independent of any particular species concept; rather, it was based on computational approaches employing genetic distances or coalescence theory. However, the goal should not be the identification or delimitation of lineages, if taxonomic conclusions are to be drawn, but of species and here the interpretation may differ for each species concept.

If monophyly of species is used as the defining criterion because, in the PSC of Mishler & Theriot (2000a), all 21 main lineages can be assumed to represent species. Each is well supported as monophyletic, even though some were paraphyletic in the phylogenetic analysis of EF1α. However, monophyly as a species and not monophyly in single gene trees is decisive and there is little doubt concerning the monophyly of all 21 main lineages. Also, most of the sublineages are monophyletic and thus potential 'phylogenetic species'. Exceptions are sublineage pairs Q4 + Q5 and N1 + N2, whose reciprocal monophyly may have been broken up by relatively recent reproduction among their members (see below). All other sublineages (D1, D2, D3, H1, H2, Q1, Q2, and Q3) as well as the pair Q4 + Q5 are likely to represent monophyletic species under the PSC, which would raise the number of 'phylogenetic species' for *Ozestheria* gen. nov. to 27.

Similarly, these 27 main and sublineages are likely to represent 'evolutionary species' under the ESC (Wiley & Mayden, 2000a). All of them can be assumed to have their 'own independent evolutionary fate and historical tendencies' (Wiley & Mayden, 2000a). As neither monophyly, nor reproductive isolation is required (Mishler & Theriot, 2000b; Wiley & Mayden, 2000b), sublineages N1, N2, Q4, and Q5 could also each represent an 'evolutionary species', potentially raising the number of 'evolutionary species' to 29.

Reproductive isolation, as required for the BSC (Mayr, 1942) and the HSC (Meier & Willmann, 2000), cannot be unambiguously inferred in many instances. Congruence between mitochondrial and nuclear genes implies the absence of gene flow and thus the absence of current reproduction. Such congruence can be observed for the 27 main and sublineages differentiated under the PSC. However, the absence of gene flow and thus of current reproduction could be a result of geographical separation rather than reproductive isolation in several instances. Only lineage pairs that occur in the same water body (syntopic) or at least in the same area (sympatric) had the chance of reproduction and only in these cases can reproductive isolation be assumed. Otherwise, reproduction could commence whenever geographical isolation is overcome.

Forty-one pairs of main lineages occurred in sympatry (Table 3) and all of these main lineage pairs can be assumed to be reproductively isolated and thus represent 'biological species'. These include ten main lineages (A, B, C, D, I, K, M, N, Q, and S), which co-occur in the central Paroo catchment (yellow; Fig. 1), eight main lineages (A, C, K, M, N, R, Q, and S) from the southern Warrego River catchment (dark green; Fig. 1), six main lineages (B, C, H, K, N, and Q) from the northern Warrego River catchment (light green; Fig. 1), six main lineages (A, B, K, M, Q, and S) from the central Cooper Creek catchment (striped black and white; Fig. 1), and six main lineages (B, D, E, N, O, and W) from the northern Cooper Creek catchment (variants of black and grey but not striped; Fig. 1). No inference of reproductive isolation is possible for geographically isolated main or sublineages. These main lineages are predominantly endemic in Western Australia (G, L, and T), southern South Australia (F and U) or mountainous habitats in south eastern Australia (P) and do not occur in sympatry with most other

main lineages. Similarly, most sublineages do not occur sympatrically with other closely related sublineages, which impedes inference of their potential for reproduction.

Given the considerable genetic differentiation among main lineages in COI (> 8.1% and in most cases even exceeding 11%) and both nuclear markers, it is probable that most main lineages are indeed reproductively isolated from each other. A comparable degree of genetic differentiation was also observed between other reproductively isolated spinicaudatan species (Schwentner *et al*., 2011, 2014; Schwentner, Timms & Richter, 2012a), as well as other large branchiopod species (Murugan *et al*., 2002; Adamowicz, Hebert & Marinone, 2004; Penton, Hebert & Crease, 2004; Ketmaier *et al*., 2012; Pinceel *et al*., 2013; Schwentner *et al*., 2013). However, there is no imperative relationship between genetic differentiation and the potential for reproduction.

Here, the sublineages of Q offer an interesting example where the degree of genetic differentiation does not correlate to the potential for reproduction. Sublineages Q3 and Q5 have genetic distances in COI of 4.4–6.0% (see Supporting information, Table S3). They occur widely sympatric in eastern and central Australia (shared colours in Figs 3, 6) and, because of their clear differentiation in EF1α and ITS2, can be assumed to represent two separate, reproductively isolated 'biological species'. Furthermore, Q5 is separated from Q4 by a slightly larger genetic distance in COI (4.5–6.8%; Fig. 3), although Q4 and Q5 are not reproductively isolated. Q4 has been documented only from Western Australia, a locality from which a single individual of Q5 was recovered. This individual features an EF1α sequence very similar to those of Q4 and well differentiated from all other Q5 specimens (ITS2 was not available from this individual). This is a great example, where, after the geographical separation of two populations was overcome (possibly by long-distance dispersal by Q5 to Western Australia), reproduction was still possible, despite considerable genetic differentiation. Such examples highlight the problem associated with delimitating 'biological species' based solely on patterns of genetic differentiation of mitochondrial markers as proposed by DNA barcoding (Hebert *et al*., 2003), or approaches as ABGD (Puillandre *et al*., 2012) or GMYC (Pons *et al*., 2006), because these were unable to delimit these lineages correctly. For biodiversity assessments, where exact species affiliations are not essential, such approaches may be sufficient because inaccuracies as a result of wrongly lumping or splitting species will even out (Balke *et al*., 2013; Modica *et al*., 2014). However, if the focus is on evolutionary phenomena such as speciation and if detailed taxonomic conclusions are to be drawn, inaccuracies or ambiguities need to be identified and resolved as best as possible (Schwentner *et al*., 2011; Adams *et al*., 2014). As a consequence, the species status of all other geographically isolated main and sublineages remains ambiguous for now. Only the sublineages of N show indications of ongoing reproduction because they share an identical EF1α sequence. However, they are differentiated in COI and ITS2. These patterns may either be the result of an ancestral polymorphism in EF1α or reproduction; here, we follow the more conservative line of evidence and treat N as a single 'biological species'.

In summary, we think that 14 main and sublineages (A, B, C, D, E, H, I, K, M, N, Q3, Q5, R, and S) can be unambiguously delimitated as 'biological species'. It should be emphasized that, for all other main lineages and most sublineages, no evidence points against their reproductive isolation. Their delimitation as 'biological species' remains ambiguous only because of their allopatric distribution. By uncovering additional sympatric occurrences in the future or by mating experiments, it appears likely that several (probably most) of these lineages will prove to be reproductively isolated as well.

The application of the various species concepts resulted in 14 to 27 delimitated species. It is not surprising that fewer species were delimitated based on the BSC because it is known to be more restrictive (Agapow *et al*., 2004). However, in our opinion, reproductive isolation among species remains the single best criterion. Indeed, this criterion is indirectly applied in the other species concepts as well. Freely reproducing populations would hardly be assumed to be monophyletic or to have independent evolutionary fates (here sublineage pairs Q4 + Q5 and N1 + N2); conversely, populations that are reproductively isolated would hardly be denied their delimitation by other concepts (e.g. sublineages Q3 and Q5). Therefore, the discrepancy among species concepts is largely based on different interpretations of genetically differentiated lineages that do not occur in sympatry, and for which the test of reproductive isolation has not yet taken place in nature. Because further studies may corroborate reproductive isolation between other lineages as well, the discrepancy of delimited *Ozestheria* gen. nov. species by the different species concepts may close eventually.

TAXONOMIC IMPLICATIONS

Previous studies indicated that the Cyzicidae are split into three well differentiated monophyla: *Eocyzicus*, all other Australian Cyzicidae, and all other non-Australian Cyzicidae (Schwentner *et al*., 2009; Weeks *et al*., 2009; apparently, Weeks *et al*., 2009 summarized all *Caenestheria* and *Caenestheriella* as *Cyzicus*). The latter two are sister groups, whereas the phylogenetic

position of *Eocyzicus* was ambiguous (Schwentner *et al.*, 2009). Because the Australian representatives of *Eocyzicus* were studied in detail previously (Schwentner *et al.*, 2014), we only focus on the remaining Cyzicidae here. Our analyses included a much larger number of Australian representatives and clearly support the differentiation into an Australian and a non-Australian monophyletic group, which is in conflict with the traditional classification of cyzicid genera. Here, we introduce the new genus *Ozestheria* gen. nov. for all Australian Cyzicidae that were previously classified as *Caenestheria* and *Caenestheriella*. Within *Ozestheria* gen. nov., the species with a short condyle (previously classified as *Caenestheria*) constitute a monophyletic group in the phylogenetic analysis of all genes (not in the separate analyses of COI and EF1α), whereas the group with an elongated condyle (previously classified as *Caenestheriella*) is paraphyletic. Thus, the condyle length is rather stable and changed only once within *Ozestheria* from elongated to short (or twice if the single genes analyses are correct), although it is not a valid character to differentiate cyzicid genera. Although the scales at the tip of the movable finger of the male clasper proved useless for the delimitation of *Ozestheria* gen. nov. species, they provided the first potentially diagnostic character for this taxon. Such slender and claw-like scales were not observed in any other spinicaudatan taxon (Schwentner *et al.*, 2011; L. Tippelt & M. Schwentner, unpubl. data). We hesitate to suggest any name for the non-Australian Cyzicidae because a comprehensive revision including more non-Australian representatives is required.

Currently, nine species of Cyzcidae (not counting species of *Eocyzicus*) are recognized for Australia (Richter & Timms, 2005). Of these, five could be assigned to species delimitated here (all now transferred to *Ozestheria* gen. nov.): *O. lutraria* (lineage C), *O. mariae* (lineage L), *O. rubra* (lineage D), and *O. sarsii* (lineage F). All but *O. mariae* were previously classified as *Caenestheria*. In addition, lineages M and N both correspond morphologically to *O. berneyi*, albeit which of the two is a new species and which is the 'true' *O. berneyi* (Gurney, 1927) could not be determined. Three previously described species, *Caenestheria rufa*, *Caenestheria dictyon*, and *Caenestheria elliptica*, could not be assigned to any of the delimited species, although the overall results suggest that they should also be transferred to the newly erected genus *Ozestheria* gen. nov. These three species may represent further species in addition to the 14 to 27 species delimited herein (see above). The species *O. packardi* (previously classified as *Caenestheriella*) could not be assigned to any single species delimited herein, rather all species with a long condyle (indicated by black vertical bars in Figs 2, 4, apart from *O. mariae* = lineage L) were identified as *O. packardi* in previous studies (Sars, 1896; Spencer & Hall, 1896; Timms & Richter, 2002; Timms, 2009a). Indeed, Spencer & Hall (1896) already described three varieties: *O. packardi* var. *typical*, *O. p.* var. *cancellata*, and *O. p.* var. *minor*. They remarked on the great morphological variability and that the three varieties only represent the observed extreme forms, although intermediate forms were present as well. For this reason, Spencer & Hall (1896) refrained from raising the varieties to species. Given the large number of lineages and species delimited in the present study, even for central and southern Australia where *O. packardi* and its varieties were described from (Brady, 1886; Spencer & Hall, 1896), difficulties in morphologically separating species are easily conceivable, especially because many of the species occur sympatrically or even syntopically. Whether the three varieties indeed correspond to one distinct species each cannot be answered in the present study.

The present study may serve as the basis for a thorough morphological revision of the species of this taxon and may help to differentiate intraspecific variability from interspecific variation more clearly. Obviously five to 21 *Ozestheria* species (if *C. rufa*, *C. dictyon*, and *C. elliptica* were indeed not among the species delimited herein) await their description, and further morphological data may help to solve some of the current ambiguities. In the case of the spinicaudatan *Limnadopsis tatei*, it was shown that a good portion of the assumed intraspecific variability could be allocated to interspecific variation of two genetically differentiated species (Schwentner *et al.*, 2012a), a finding typical for branchiopods (Murugan *et al.*, 2009; Korn *et al.*, 2010). Thus, the large morphologic intraspecific variation observed in many spinicaudatans and other branchiopods (Longhurst, 1955; Timms & Richter, 2009; Timms, 2009b) may partly be attributable to unrecognized cryptic diversity and incorrectly drawn species boundaries.

Distribution, dispersal, and phylogeographical history

The geographical distribution of species and of their intraspecific genetic diversity depends on a variety of different factors, such as not only ecological conditions and competition or the species' ability for dispersal, but also geological and climatic changes. In Australia, the latter is characterized by an overall trend towards aridification, in particular in the last approximately 15 Myr (Martin, 2006; Byrne *et al.*, 2008). During glacial cycles of the Pleistocene, the climate alternated between dry/cold glacials, and wet/warm interglacials (Martin, 2006; Byrne *et al.*, 2008). Although we are currently in an interglacial period, Australia's climate has been arid since the mid Holocene approximately 5000 years ago (Marx, McGowan & Kamber, 2009; Quigley

et al., 2010). The cyclic climatic changes of the Pleistocene greatly affected the distribution of species within the arid-zone, restricting species to refugial areas during climatic adverse conditions. Today, many terrestrial species feature either strong geographical structure of their genetic variation (Chapple & Keogh, 2004; Shoo *et al.*, 2008; Kearns *et al.*, 2009; Pepper *et al.*, 2011a, b; Sistrom, Donnellan & Hutchinson, 2013) or hardly any genetic variation at all (Joseph & Wilke, 2007; Kuch *et al.*, 2005; Strasburg *et al.*, 2007; Byrne *et al.*, 2008 and Byrne, 2008). The former indicates historic structure, potentially reflecting multiple historic refugial areas without subsequent dispersal. The latter was attributed to the species' greater vagility (Byrne *et al.*, 2008) but may rather be the result of recent expansion from single refugial sites. The absence of additional refugia and thus of competitive exclusion through priority effects of resident populations (Waters, 2011) may have made these expansions possible.

Many species of *Ozestheria* gen. nov. and of other spinicaudatans are widespread in central and south-eastern Australia. In most instances, numerous unique and divergent haplotypes are present in central Australia (e.g. eastern Western Plateau and western Lake Eyre Basin) or central parts of the Murray-Darling Basin (mainly the Paroo and neighbouring Warrego River catchments). This suggests potential refugial sites in these two areas during periods of adverse climatic conditions (Schwentner *et al.*, 2012b, 2014). However, the differentiation is not as pronounced as in most other arid-zone species (Chapple & Keogh, 2004; Shoo *et al.*, 2008; Kearns *et al.*, 2009; Pepper *et al.*, 2011a, b) with numerous closely-related haplotypes occurring in both areas. Thus, dispersal and gene flow must have (or may still) occurred among these regions, despite the presence of local populations. Apparently dispersal is sufficiently extensive, at least at times, to overcome gene flow limiting priority effects, although these are considered to be particularly high for taxa with resting eggs such as branchiopods (De Meester *et al.*, 2002). Dispersal is most likely mediated by migratory water birds, which are abundant in inland Australia during wet seasons (Kingsford, Curtin & Porter, 1999; Kingsford & Porter, 1999; Green *et al.*, 2008; Reid, Kingsford & Jaensch, 2009). As a consequence, drainage system borders are no barrier to dispersal and gene flow as they are for fish, crayfish or mussels (Carini & Hughes, 2004; Hughes *et al.*, 2004; Faulks, Gilligan & Beheregaray, 2010). In Australia, genetic differentiation for such animals is often correlated with drainage system borders; in particular, populations of the Lake Eyre Basin and Bulloo River are genetically differentiated from those of the Murray-Darling Basin. By contrast, populations of *Ozestheria* gen. nov. or other spinicaudatan species or other branchiopods (Schwentner *et al.*, 2012b, 2013, 2014) inhabiting the Bulloo and the Murray-Darling Basin are hardly differentiated from each other and share many haplotypes.

In the Bulloo River catchment (pink in Figs 1, 3, 6), the central Cooper Creek catchment (striped black and white in Figs 1, 3, 6), and the central Georgina River catchment (striped light and dark grey in Figs 1, 3, 6), most of the retrieved genetic diversity is found in other areas as well, especially in the central Murray-Darling Basin. This suggests recent expansion, which is in accordance with patterns of population differentiation and signs of expansion for several *Ozestheria* gen. nov. species by neutrality tests. Apparently, arid conditions, which have been dominating Australia's climate for the last approximately 5000 years (Marx *et al.*, 2009; Quigley *et al.*, 2010), are favourable for spinicaudatans, which may appear surprising for aquatic animals. However, spinicaudatans are restricted to temporary water bodies and require their habitats to regularly dry out to induce hatching of their resting eggs in a subsequent wet period (Dumont & Negrea, 2002). Wetter climates may increase the permanency of water bodies, which reduces the number of suitable habitats for spinicaudatans. Although precipitation is low, the frequency of extensive flooding increased since the mid Holocene, suggesting overall low, although sometimes extreme, rainfalls (Quigley *et al.*, 2010), comprising ideal conditions for species relying on temporary water bodies.

The most striking break in the distribution and intraspecific differentiation of *Ozestheria* gen. nov. and other spinicaudatan species is observable towards north-eastern Australia. Populations from north-eastern Australia (north-eastern Cooper Creek in the north eastern Lake Eyre Basin, populations 37–50; variants of black and grey, but not striped in Figs 1, 3, 6) are set apart from those of all other studied areas in central and eastern Australia, even those further south within the Cooper Creek catchment. Most otherwise widely distributed species do not occur there; rather, the majority of species are endemic (e.g. *Ozestheria* gen. nov. species E, O, and W, as well as *Eocyzicus* sp. W; Schwentner *et al.*, 2014), or their populations are genetically well differentiated from those of all other areas (e.g. D3 or *Limnadopsis birchii* and *Limnadopsis parvispinus*; Schwentner *et al.*, 2012b). This has been explained by the availability of temporary water bodies during wet seasons and the resulting migratory behaviour of water birds (Schwentner *et al.*, 2012b, 2014). Computer models suggest that, in central and eastern Australia, these habitats are 'interconnected' for migratory water birds. Step-by-step, they are all within the reach of a day's flight, except those in north-eastern Australia (Roshier *et al.*, 2001; Roshier, Klomp & Asmus, 2006). Thus, dispersal to and from the latter area could be less common. In addition, north-eastern Australia belongs to a different ecoregion with

tropical or subtropical vegetation, whereas most other studied populations are in regions with temperate or xeric vegetation (Interim Biogeographic Regionalisation for Australia, Version 7). Differences in submerged and riparian vegetation could restrict the distribution of spinicaudatans (Timms & Boulton, 2001; Nhiwatiwa *et al*., 2011). Furthermore, the presence of locally adapted species or intraspecific populations may prevent the successful establishment of migrants even if dispersal occurred (Boileau, Hebert & Schwartz, 1992; De Meester *et al*., 2002; Waters, 2011). This may further the division between the north-east and the rest of Australia. Nevertheless, recent effective dispersal between north-eastern Australia and a population further south was recently shown for the branchiopod *Cyclestheria hislopi* (Schwentner *et al*., 2013) and is also obvious for *Ozestheria* gen. nov. B and N because these featured several identical haplotypes among north-eastern and other populations.

Rogers (2014) suggested that stochastic long-distance dispersal to regions previously uninhabited by the same species may be the underlying cause of speciation in branchiopods. Subsequent gene flow between the original and the newly-founded populations would be rare at most, especially if no further suitable habitats are available in-between and if local adaptation has commenced. This could explain why most putative sister species pairs of *Ozestheria* gen. nov. are geographically widely separated. Often, one species occurs in a geographical 'outlier' locality, such as north-eastern or Western Australia.

In summary, at least three regions can be identified as historic refugial areas in eastern and central Australia: north-eastern Australia (numerous endemic species and genetically divergent intraspecific lineages), Murray-Darling Basin (possibly the Paroo River catchment and adjacent river catchments; numerous endemic species and divergent intraspecific lineages), and central Australia (numerous genetically divergent intraspecific lineages). In addition, in Western Australia, one or several additional refugia must have existed due to the large number of endemic species. Spinicaudata were possibly restricted to these refugia during periods of frequent precipitation, expanding when the climate became more arid. Thus, they would have benefitted greatly from the progressing aridification of Australia, which may explain the great diversity of species observed there.

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REFERENCES

Adamowicz SJ, Hebert PDN, Marinone MC. 2004. Species diversity and endemism in the *Daphnia* of Argentina: a genetic investigation. *Zoological Journal of the Linnean Society* **140:** 171–205.

Adams M, Raadik TA, Burridge CP, Georges A. 2014. Global biodiversity assessment and hyper-cryptic species complexes: mare than one species of elephant in the room? *Systematic Biology* **63:** 518–533.

Agapow PM, Bininda-Emonds ORP, Crandall KA, Gittleman JL, Mace GM, Marshall JC, Purvis A. 2004. The impact of species concept on biodiversity studies. *Quarterly Review of Biology* **79:** 161–179.

Balke M, Hendrich L, Toussaint EFA, Zhou X, von Rintelen T, De Bruyn M. 2013. Suggestions for a molecular biodiversity assessment of South East Asian freshwater invertebrates. Lessons from the megadiverse beetle (Coleoptera). *Journal of Limnology* **72:** 61–68.

Blank M, Laine AO, Juerss K, Bastrop R. 2008. Molecular identification key based on PCR/RFLP for three polychaete sibling species of the genus *Marenzelleria*, and the species' current distribution in the Baltic Sea. *Helgoland Marine Research* **62:** 129–141.

Boileau MG, Hebert PDN, Schwartz SS. 1992. Nonequilibrium gene-frequency divergence – persistent founder effects in natural-populations. *Journal of Evolutionary Biology* **5:** 25–39.

Brady GS. 1886. Notes on freshwater Entomostraca from South Australia. *Proceedings of the Zoological Society of London* **54:** 82–93.

Brtek J. 1997. Checklist of the valid and invalid names of the 'large branchiopods' (Anostraca, Notostraca, Spinicaudata and Laevicaudata), with a survey of the taxonomy of all Branchiopoda. *Zborník Slovenského Národného Múzea* **43:** 3–66.

Byrne M. 2008. Evidence for multiple refugia at different time scales during Pleistocene climatic oscillations in southern Australia inferred from phylogeography. *Quaternary Science Reviews* **27:** 2576–2585.

Byrne M, Yeates DK, Joseph L, Kearney M, Bowler J, Williams MAJ, Cooper S, Donnellan SC, Keogh JS,

Leys R, Melville J, Murphy DJ, Porch N, Wyrwoll KH. 2008. Birth of a biome: insights into the assembly and maintenance of the Australian arid zone biota. *Molecular Ecology* **17:** 4398–4417.

Carini G, Hughes JM. 2004. Population structure of *Macrobrachium australiense* (Decapoda : Palaemonidae) in Western Queensland, Australia: the role of contemporary and historical processes. *Heredity* **93:** 350–363.

Chapple DG, Keogh JS. 2004. Parallel adaptive radiations in arid and temperate Australia: molecular phylogeography and systematics of the *Egernia whitii* (Lacertilia : Scincidae) species group. *Biological Journal of the Linnean Society* **83:** 157–173.

Daday des Deés E. 1914. Monographie Systématique des Phyllopodes Conchostracés. *Annales des Sciences Naturelles* **10:** 39–339.

Dakin WJ. 1914. Fauna of Western Australia. II The Phyllopoda of Western Australia. *Proceedings of the Zoological Society of London* **1914:** 293–305.

De Meester L, Gomez A, Okamura B, Schwenk K. 2002. The monopolization hypothesis and the dispersal–gene flow paradox in aquatic organisms. *Acta Oecologica-International Journal of Ecology* **23:** 121–135.

Drummond AJ, Suchard MA, Xie D, Rambaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* **29:** 1969–1973.

Dumont HJ, Negrea SV. 2002. *Guides to the identification of the microinvertebrates of the continental waters of the world. Branchiopoda*. Leiden, The Netherlands: Backhuys Publishers.

Dupanloup I, Schneider S, Excoffier L. 2002. A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology* **11:** 2571–2581.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32:** 1792–1797.

Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10:** 564–567.

Faulks LK, Gilligan DM, Beheregaray LB. 2010. Clarifying an ambiguous evolutionary history: range-wide phylogeography of an Australian freshwater fish, the golden perch (*Macquaria ambigua*). *Journal of Biogeography* **37:** 1329–1340.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3:** 294–299.

Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147:** 915–925.

Gerstaecker CEA. 1866–1879. In: Bronns HG, ed. *Klassen und Ordnungen des Tierreichs (Band 5, 1. Abteilung)* 5 (5) (no exact year is available for the establishment of the Diplostraca).

Glaubrecht JM. 2007. Die Ordnung des Lebendigen. Zur Geschichte und Zukunft der Systematik in Deutschland. In: Wägele JW, ed. *Höhepunkte der zoologischen Forschung im deutschen Sprachraum. Festschrift zur 100. Jahresversammlung der Deutschen Zoologischen Gesellschaft in Köln*. Marburg: Basiliken Press, 59–110.

Glaubrecht JM. 2010. Evolutionssystematik limnischer Gatropoden. Habilitationsschrift zur Erlangung der Lehrbefähigung für das Fach Zoologie, Humboldt-Universität zu Berlin.

Goloboff P. 1999. *NONA (No Name)*, Version 2. Published by the Author, Tucuman, Argentina.

Green AJ, Jenkins KM, Bell D, Morris PJ, Kingsford RT. 2008. The potential role of waterbirds in dispersing invertebrates and plants in arid Australia. *Freshwater Biology* **53:** 380–392.

Gurney R. 1927. Some Australian freshwater Entomostraca reared from dried mud. *Proceedings of the General Meetings for Scientific Business of the Zoological Society of London* **1927:** 59–79.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41:** 95–98.

Hebert PDN, Cywinska A, Ball SL, Dewaard JR. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B, Biological Sciences* **270:** 313–321.

Henry M. 1924. A monograph of the freshwater Entomostraca of New South Wales. IV. Phyllopoda. *Proceedings of the Linnean Society of New South Wales* **49:** 120–137.

Hughes JM, Baker AM, Bartlett C, Bunn S, Goudkamp K, Somerville J. 2004. Past and present patterns of connectivity among populations of four cryptic species of freshwater mussels *Velesunio* spp. (Hyriidae) in central Australia. *Molecular Ecology* **13:** 3197–3212.

Joseph L, Wilke T. 2007. Lack of phylogeographic structure in three widespread Australian birds reinforces emerging challenges in Australian historical biogeography. *Journal of Biogeography* **34:** 612–624.

Kearns AM, Joseph L, Edwards SV, Double MC. 2009. Inferring the phylogeography and evolutionary history of the splendid fairy-wren *Malurus splendens* from mitochondrial DNA and spectrophotometry. *Journal of Avian Biology* **40:** 7–17.

Ketmaier V, Marrone F, Alfonso G, Paulus K, Wiemann A, Tiedemann R, Mura G. 2012. Mitochondrial DNA reginoalism and historical demography in the extant populations of *Chirocephalus kerkyrensis* (Branchiopoda: Anostraca). *PLoS ONE* **7:** e30082.

Kingsford RT, Curtin AL, Porter J. 1999. Water flows on Cooper Creek in arid Australia determine 'boom' and 'bust' periods for waterbirds. *Biological Conservation* **88:** 231–248.

Kingsford RT, Porter J. 1999. Wetlands and waterbirds of the Paroo and Warrago rivers. In: Kingsford RT, ed. *A free-flowing river: the ecology of the Paroo River*. Hurstville: NSW National Parks and Wildlife Service, 23–50.

Korn M, Green AJ, Machado M, Garcia-de-Lomas J, Cristo M, da Fonseca LC, Frisch D, Perez-Bote JL,

Hundsdoerfer AK. 2010. Phylogeny, molecular ecology and taxonomy of southern Iberian lineages of *Triops mauritanicus* (Crustacea: Notostraca). *Organisms Diversity & Evolution* **10:** 409–440.

Krebes L, Blank M, Jurss K, Zettler ML, Bastrop R. 2010. Glacial-driven vicariance in the amphipod *Gammarus duebeni*. *Molecular Phylogenetics and Evolution* **54:** 372–385.

Kuch U, Keogh JS, Weigel J, Smith LA, Mebs D. 2005. Phylogeography of Australia's king brown snake (*Pseudechis australis*) reveals Pliocene divergence and Pleistocene dispersal of a top predator. *Die Naturwissenschaften* **92:** 121–127.

Laamanen TR, Petersen FT, Meier R. 2003. Kelp flies and species concepts – the case of *Coelopa frigida* (Fabricius, 1805) and *C. nebularum* Aldrich, 1929 (Diptera : Coelopidae). *Journal of Zoological Systematics and Evolutionary Research* **41:** 127–136.

Latreille PA. 1817. Les crustacés, les arachnides et les insectes. In: Cuvier G, ed. *Lerègne animal distribué d'après son organisation, pour servir de base à l'histoire naturelle des animaux et d'introduction à l'anatomie coparée*, Vol. III. Paris: Déterville, 1–653.

Linder F. 1945. Affinities within the Branchiopoda with notes on some dubious fossils. *Arkiv för zoologi* **37:** 1–28.

Longhurst AR. 1955. A review of the Notostraca. *Bulletin of the British Museum of Natural History (Zoology)* **3:** 3–57.

Martin HA. 2006. Cenozoic climatic change and the development of the arid vegetation in Australia. *Journal of Arid Environments* **66:** 533–563.

Marx SK, McGowan HA, Kamber BS. 2009. Long-range dust transport from eastern Australia: a proxy for Holocene aridity and ENSO-type climate variability. *Earth and Planetary Science Letters* **282:** 167–177.

Mayr E. 1942. *Systematics and the origin of species from the viewpoint of a zoologist*. New York, NY: Columbia University Press.

Meier R, Willmann R. 2000. The Hennigian species concept. In: Wheeler QD, Meier R, eds. *Species concepts and phylogenetic theory*. New York, NY: Columbia University Press, 30–43.

Mishler BD, Theriot EC. 2000a. A defense of the phylogenetic species concept (*sensu* Mishler and Theriot): monophyly, apomorphy and phylogenetic species concepts. In: Wheeler QD, Meier R, eds. *Species concepts and phylogenetic theory*. New York, NY: Columbia University Press, 179–184.

Mishler BD, Theriot EC. 2000b. The phylogenetic species concept (*sensu* Mishler and Theriot): monophyly, apomorphy and phylogentic species concepts. In: Wheeler QD, Meier R, eds. *Species concepts and phylogenetic theory*. New York, NY: Columbia University Press, 44–54.

Modica MV, Puillandre N, Castelin M, Zhang Y, Holford M. 2014. A good compromise: rapid and robust species proxies for inventorying biodiversity hotspots using the Terebridae (Gastropoda: Conoidea). *PLoS ONE* **9:** e102160.

Montero-Pau J, Gómez A, Muñoz J. 2008. Application of an inexpensive and high-throughput genomic DNA extraction method for the molecular ecology of zooplanktonic diapausing eggs. *Limnology and Oceanography – Methods* **6:** 218–222.

Murugan G, Maeda-Martínez AM, Obregon-Barboza H, Hernandez-Saavedra NY. 2002. Molecular characterization of the tadpole shrimp *Triops* (Branchiopoda : Notostraca) from the Baja California Peninsula, Mexico: new insights on species diversity and phylogeny of the genus. *Hydrobiologia* **486:** 101–113.

Murugan G, Obregon-Barboza H, Maeda-Martínez AM, Timms BV. 2009. Co-occurrence of two tadpole shrimp, *Triops* cf. *australiensis* (Branchiopoda : Notostraca), lineages in middle Paroo, north-western New South Wales, with the first record of *Triops* hermaphrodites for the Australian continent. *Australian Journal of Zoology* **57:** 77–84.

Nhiwatiwa T, Brendonck L, Waterkeyn A, Vanschoenwinkel B. 2011. The importance of landscape and habitat properties in explaining instantaneous and long-term distributions of large branchiopods in subtropical temporary pans. *Freshwater Biology* **56:** 1992–2008.

Nixon KC. 1999. *WinClada*, Version 1.00.08. Published by the Author, Ithaca, USA.

Olesen J, Richter S. 2013. Onychocaudata (Branchiopoda: Diplostraca), a new high-level taxon in branchiopod systematics. *Journal of Crustacean Biology* **33:** 62–65.

Olesen J, Timms BV. 2005. Caenestheriella mariae n.sp. (Crustacea:Branchiopoda: Spinicaudata: Cyzicidae): a new clam shrimp from Western Australia. *Zootaxa* **824:** 1–8.

Penton EH, Hebert PDN, Crease TJ. 2004. Mitochondrial DNA variation in North American populations of *Daphnia obtusa*: continentalism or cryptic endemism? *Molecular Ecology* **13:** 97–107.

Pepper M, Doughty P, Hutchinson MN, Keogh JS. 2011a. Ancient drainages divide cryptic species in Australia's arid zone: morphological and multi-gene evidence for four new species of beaked geckos (Rhynchoedura). *Molecular Phylogenetics and Evolution* **61:** 810–822.

Pepper M, Ho SYW, Fujita MK, Keogh JS. 2011b. The genetic legacy of aridification: climate cycling fostered lizard diversification in Australian montane refugia and left low-lying deserts genetically depauperate. *Molecular Phylogenetics and Evolution* **61:** 750–759.

Pinceel T, Vanschoenwinkel B, Waterkeyn A, Vanhove MPM, Pinder A, Timms BV, Brendonck L. 2013. Fairy shrimps in distress: a molecular taxonomic review of the diverse fairy shrimp genus *Branchinella* (Anostraca: Thamnocephalidae) in Australia in the light of ongoing environmental change. *Hydrobiologia* **700:** 313–327.

Pohl H. 2010. A scanning electron microscopy specimen holder for viewing different angles of a single specimen. *Microscopy Research and Technique* **73:** 1073–1076.

Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S, Kamoun S, Sumlin WD, Vogler AP. 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology* **55:** 595–609.

Posada D, Crandall KA. 2001. Selecting the best-fit model of nucleotide substitution. *Systematic Biology* **50:** 580–601.

Puillandre N, Lambert A, Brouillet S, Achaz G. 2012. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* **21:** 1864–1877.

Quigley MC, Horton T, Hellstrom JC, Cupper ML, Sandiford M. 2010. Holocene climate change in arid Australia from speleothem and alluvial records. *Holocene* **20:** 1093–1104.

R Development Core Team. 2010. *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Rambaut A, Drummond AJ. 2007. *Tracer*, Version 1.4, Available at: http://beast.bio.ed.ac.uk/Tracer

Reid JRW, Kingsford RT, Jaensch RP. 2009. Waterbird surveys in the channel country floodplain wetlands, Autumn 2009. Report by Australian National University, Canberra, University of New South Wales, Sydney, and Wetlands International – Oceania, Brisbane, for the Australian Government Department of Environment, Water, Heritage and the Arts.

Richter S, Olesen J, Wheeler WC. 2007. Phylogeny of Branchiopoda (Crustacea) based on a combined analysis of morphological data and six molecular loci. *Cladistics* **23:** 301–336.

Richter S, Timms BV. 2005. A list of the recent clam shrimps (Crustacea : Laevicaudata, Spinicaudata, Cyclestherida) of Australia, including a description of a new species of *Eocyzicus*. *Records of the Australian Museum* **57:** 341–354.

Rogers DC. 2014. Larger hatching fraction in avian dispersed anostracan eggs (Branchiopoda). *Journal of Crustacean Biology* **32:** 135–143.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Roshier DA, Klomp NI, Asmus M. 2006. Movements of a nomadic waterfowl, grey teal *Anas gracilis*, across inland Australia – results from satellite telemetry spanning fifteen months. *Ardea* **94:** 461–475.

Roshier DA, Robertson AI, Kingsford RT, Green DG. 2001. Continental-scale interactions with temporary resources may explain the paradox of large populations of desert waterbirds in Australia. *Landscape Ecology* **16:** 547–556.

Rüppel E. 1837. Über *Estheria dahalacensis* Rüppel neue Gattung auis der Familie der Daphniden. *Abhandlungen aus dem Senckenberg Museum* **2:** 117–128.

Sars GO. 1896. Development of *Estheria packardi* Brady, as shown by artificial hatching from dried mud. *Archiv for Mathematik og Naturvidenskab* **18:** 1–27.

Sars GO. 1897. On some West-Australian Entomostraca raised from dried sand. *Archiv for Mathematik og Naturvidenskab* **19:** 1–35.

Sayce OA. 1903. The Phyllopoda of Australia, including descriptions of some new genera and species. *Proceedings of the Royal Society of Victoria* **15:** 224–261.

Schwentner M, Clavier S, Fritsch M, Olesen J, Padhye S, Timms BV, Richter S. 2013. *Cyclestheria hislopi* (Crustacea: Branchiopoda): a group of morphologically cryptic species with origins in the Cretaceous. *Molecular Phylogenetics and Evolution* **66:** 800–810.

Schwentner M, Timms BV, Bastrop R, Richter S. 2009. Phylogeny of Spinicaudata (Branchiopoda, Crustacea) based on three molecular markers – an Australian origin for *Limnadopsis*. *Molecular Phylogenetics and Evolution* **53:** 716–725.

Schwentner M, Timms BV, Richter S. 2011. An integrative approach to species delineation incorporating different species concepts: a case study of *Limnadopsis* (Branchiopoda: Spinicaudata). *Biological Journal of the Linnean Society* **104:** 575–599.

Schwentner M, Timms BV, Richter S. 2012a. Description of four new species of *Limnadopsis* from Australia (Crustacea: Branchiopoda: Spinicaudata). *Zootaxa* **3315:** 42–64.

Schwentner M, Timms BV, Richter S. 2012b. Flying with the birds? Recent large-area dispersal of four Australian *Limnadopsis* species (Crustacea: Branchiopoda: Spinicaudata). *Ecology and Evolution* **2:** 1605–1626.

Schwentner M, Timms BV, Richter S. 2014. Evolutionary systematics of the Australian *Eocyzicus* fauna (crustacea: Branchiopoda: Spinicaudata) reveals hidden diversity and phylogeographic structure. *Journal of Zoological Systematics and Evolutionary Research* **52:** 15–31.

Shoo LP, Rose R, Doughty P, Austin JJ, Melville J. 2008. Diversification patterns of pebble-mimic dragons are consistent with historical disruption of important habitat corridors in and Australia. *Molecular Phylogenetics and Evolution* **48:** 528–542.

Sistrom M, Donnellan SC, Hutchinson MN. 2013. Delimiting species in recent radiations with low levels of morphological divergence: a case study in Australian *Gehyra* geckos. *Molecular Phylogenetics and Evolution* **68:** 135–143.

Sonnenberg R, Nolte AW, Tautz D. 2007. An evaluation of LSU rDNA D1-D2 sequences for their use in species identification. *Frontiers in Zoology* **4:** 6.

Spencer B, Hall TS. 1896. Crustacea. *Report on the Work of the Horn Expedition to Central Australia. II. Zoology* **8:** 227–248.

Stebbing TR. 1910. General catalogue of South-African carustacea. *Annals of the South African Museum* **6:** 281–599.

Strasburg JL, Kearney M, Moritz C, Templeton AR. 2007. Combining phylogeography with distribution modeling: multiple Pleistocene range expansions in a parthenogenetic gecko from the Australian arid zone. *PLoS ONE* **2: DOI: 10.1371/journal.pone.0000760**.

Tajima F. 1989. Statistical-method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123:** 585–595.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28:** 2731–2739.

Tan DSH, Ali F, Kutty SN, Meier R. 2008. The need for specifying species concepts: how many species of silvered langurs (*Trachypithecus cristatus* group) should be recognized? *Molecular Phylogenetics and Evolution* **49:** 688–689.

Thompson JD, Higgins DG, Gibson TJ. 1994. Clustal-W – improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673–4680.

Timms BV. 2009a. A revision of the Australian endemic clam shrimp *Limnadopsis* Spencer & Hall (Crustacea: Branchiopoda: Spinicaudata: Limnadiidae). *Records of the Australian Museum* **61:** 49–72.

Timms BV. 2009b. Biodiversity of large branchiopods of Australian saline lakes. *Current Science* **96:** 74–80.

Timms BV. 2012. An appraisal of the diversity and distribution of large branchiopods (Branchiopoda: Anostraca, Laevicaudata, Spinicaudata, Cyclestheriida, Notostraca) in Australia. *Journal of Crustacean Biology* **32:** 615–623.

Timms BV, Boulton AJ. 2001. Typology of arid-zone floodplain wetlands of the Paroo River (inland Australia) and the influence of water regime, turbidity, and salinity on their aquatic invertebrate assemblages. *Archiv fur Hydrobiologie* **153:** 1–27.

Timms BV, Richter S. 2002. A preliminary analysis of the conchostracans (Crustacea : Spinicaudata and Laevicaudata) of the middle Paroo catchment of the Australian arid-zone. *Hydrobiologia* **486:** 239–247.

Timms BV, Richter S. 2009. The clam shrimp *Eocyzicus* (Branchiopoda: Spinicaudata: Cyzicidae) in Australia. *Journal of Crustacean Biology* **29:** 245–253.

Timms BV, Sanders PR. 2002. Biogeography and ecology of Anostraca (Crustacea) in middle Paroo catchment of the Australian arid-zone. *Hydrobiologia* **486:** 225–238.

Wagstaff SJ, Garnock-Jones PJ. 1998. Evolution and biogeography of the Hebe complex (Scrophulariaceae) inferred from ITS sequences. *New Zealand Journal of Botany* **36:** 425–437.

Waters JM. 2011. Competitive exclusion: phylogeography's 'elephant in the room'? *Molecular Ecology* **20:** 4388–4394.

Weeks SC, Chapman EG, Rogers DC, Senyo DM, Hoeh WR. 2009. Evolutionary transitions among dioecy, androdioecy and hermaphroditism in limnadiid clam shrimp (Branchiopoda: Spinicaudata). *Journal of Evolutionary Biology* **22:** 1781–1799.

White T, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 322.

Wiley EO, Mayden RL. 2000a. A defense of the evolutionary species concept. In: Wheeler QD, Meier R, eds. *Species concepts and phylogenetic theory*. New York, NY: Columbia University Press, 198–208.

Wiley EO, Mayden RL. 2000b. The evolutionary species concept. In: Wheeler QD, Meier R, eds. *Species concepts and phylogenetic theory*. New York, NY: Columbia University Press, 70–92.

Williams WD. 1981. Inland aquatic systems, an overview. In: Keast A, ed. *Ecological biogeography of Australia*. The Hague: Dr W. Junk, 1079–1099.

SUPPORTING INFORMATION

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

Figure S1. Bayesian inference majority rule tree based on analysis of elongation factor 1α (EF1α). All available sequences were included. Affiliation of specimens to respective main and sublineages based on prior analyses of cytochrome oxidase subunit I (COI) (Fig. 2). Black vertical bars indicate those lineages whose specimens feature an elongated condyle (originally classified as *Caenestheriella*), whereas grey vertical bars indicate those with a short and rounded condyle (originally classified as *Caenestheria*). For each branch, posterior probabilities and bootstrap support of the Maximum Parsimony analyses are given. #, For support values ≥ 0.95 or ≥ 95; +, for support values ≥ 0.90 or 90, respectively (– indicates support < 0.9 or < 90, if both are lower, no support is stated).

Table S1. Details of collection events and localities. For each locality, the respective main lineages are given. The localities are arranged according to main drainage systems (Divisions) and catchments of individual rivers (River basins). The locality numbers correspond to Fig. 1 and Table S5. BS, Bloodwood Station; CNP, Currawinya National Park; MS, Muella Station; RS, Rockwell Station; NSW, New South Wales; NT, Northern Territory; QLD; Queensland; SA, South Australia; ST, Sumana Station; WA, Western Australia; YS, Yarromere Station.

Table S2. Details of all studied specimens. For each studied specimen, the respective main lineage, locality (numbers correspond to Fig. 1 and Table S1), Australian Museum registration number, and all GenBank accession numbers are given.

Table S3. Genetic distances of cytochrome oxidase subunit I (COI) within and among sublineages. All genetic distances are pairwise uncorrected *p*-distances as a percentage, summarized for all individuals of the respective sublineages. Sublineages pairs that occur syntopically (e.g. in the same water body) are indicated in dark grey.

Table S4. Population subdivisions obtained by SAMOVA (spatial analysis of molecular variance). Only populations with four or more individuals of the respective lineage were included in the analysis. The grouping that resulted in the highest F_{CT} (variation among groups) should mirror the geographical subdivision of populations of the respective main lineage. In several cases, however, no maximum was reached. Here, no population subdivision can be deduced (indicated by '–'). If a maximum was reached, the corresponding F_{CT} value, the number of inferred groups and the populations of each group are given. Population numbers correspond to those in Fig. 1 and Table S1.

Table S5. Pairwise Φ_{ST} values for COI among populations of several main lineages. Letters correspond to main or sublineages. Only populations with four or more individuals of the respective lineage were included in the analysis. Locality numbers correspond to Fig. 1 and Table S1. For each locality, the corresponding River basin is given (see also Table S1).