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Evolutionary systematics of the Australian *Eocyzicus* fauna (Crustacea: Branchiopoda: Spinicaudata) reveals hidden diversity and phylogeographic structure

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Abstract

Although 'large branchiopods' are an important faunal element of the temporary water bodies in Australia's vast (semi)arid regions, knowledge of their diversity, distribution and ecology is still poor. Here, on the basis of one mitochondrial [cytochrome oxidase subunit I (COI)] and three nuclear (EF1α, ITS2 and 28S) markers, we present new data relating to the diversity and phylogeography of eastern and central Australian *Eocyzicus* (Spinicaudata) fauna. Using a combination of phylogenetic, haplotype network and DNA barcoding analyses of COI, 312 individuals were grouped into eleven main lineages. To infer whether these lineages are reproductively isolated from each other (the prerequisite for species delineation according to the Biological or Hennigian Species Concepts), separate analyses of each nuclear marker were performed on a subset of specimens. Although some lineages are non-monophyletic in the analysis of one nuclear marker, this is mostly attributed to processes such as incomplete lineage sorting rather than ongoing reproduction. The eleven lineages translate into at least seven species whose reproductive isolation is additionally indicated by sympatry, including both Australian *Eocyzicus* species previously described. Another three lineages may constitute further species, but their clear allopatric distribution rendered the test for reproductive isolation inapplicable. One lineage appears not to be reproductively isolated and is therefore considered a genetically distinct lineage within one of the other species, and one divergent lineage within *E. argillaquus* may constitute an additional species. Although sympatry is very common – six species occur in the central Paroo River catchment in eastern Australia, for instance – syntopic occurrence is rare. It is possible that a combination of differing habitat preferences and priority effects inhibits the presence of more than one *Eocyzicus* species per water body. There is little to no genetic differentiation between certain populations of the species found in eastern and central Australia (e.g. the Murray–Darling Basin, the Bulloo River catchment and the eastern and northern Lake Eyre Basin; LEB), suggesting high dispersal rates within this large area. Between the central Australian populations themselves, however (e.g. those inhabiting the central and western LEB), genetic differentiation is pronounced, probably as a result of the lack of abundance of important dispersal vectors (aquatic birds) and the lower diversity and density of suitable habitats in the area. The most prominent biogeographical break exists towards north-eastern Australia (north-east LEB), which does not share species with any other region studied.

Key words: cryptic species - diversity – DNA barcoding – niche differentiation – species concepts

Introduction

Historically, the taxonomy of a given taxon was dependent on the taxonomist's notion of the morphological variation required or sufficient for species delimitation. As a consequence, the number of recognized species depended heavily on the taxonomist's interpretation of the observable variation. For taxa such as branchiopods, where intraspecific variability appears to be high and interspecific variation low, a vast number of species have been described over time, only to have been synonymized in subsequent investigations. Nearly 70 notostracan species were synonymized to only nine by Longhurst (1955), for example, while all extant Cyclestheriidae were synonymized with *Cyclestheria hislopi* (Olesen et al. 1996) and 22 of the 59 spinicaudatan species described by Daday de Deés have since been synonymized with other species (see Adamovicz and Purvis 2005). Molecular genetic techniques offer an additional tool in this field of research, and their increasing use has led to the identification of several new species or the renewed delineation of several previously synonymized species [e.g. Adamowicz et al. 2004 (*Daphnia*); King and Hanner 1998 (*Lepidurus*); Korn and Hundsdoerfer 2006 (*Triops*); Korn et al. 2010 (*Triops*); Kotov et al. 2006 (*Daphnia*); Schwentner et al. 2012b (*Limnadopsis*); Schwentner et al. 2013 (*Cyclestheria*)]. By 2005, Adamovicz and Purvis counted 20 branchiopod species that had been formally described after first being identified genetically and the number continues to increase. Species with what was assumed to be great morphological plasticity have been recognized as species complexes and delimited into multiple species with a much lower level of intraspecific variability (e.g. Murugan et al. 2009; Korn et al. 2010; Schwentner et al. 2012a). Nevertheless, for many morphological characters, intraspecific variability overlaps with interspecific variation among closely related species (e.g. Schwentner et al. 2012a), which explains the difficulty of purely morphological species delimitation. This emphasizes the need for an integrative taxonomy approach within a framework of evolutionary systematics. Evolutionary systematics comprises the study of taxonomic diversity, disparity and genetic variability and of the underlying evolutionary causes of speciation on the basis of phylogenetic systematics (Glaubrecht 2007, 2010). Only by combining different kinds of data, it will be possible to accurately assess branchiopod species richness and disentangle their complex and enduringly confusing taxonomy (Schwentner et al. 2011). The persistent lack of a sound branchiopod taxonomy is not a problem for systematists or taxonomists alone. Branchiopods are important components of many aquatic ecosystems: cladocera play a key part in the zooplankton communities of many lakes, and 'large branchiopods' are an important feature of the invertebrate fauna of temporary water bodies, with both playing an important role in the respective food webs (Bunn and Davies 1999). Deficiencies in the branchiopod taxonomy, then, impede studies into ecosystem functioning, monitoring programs and conservation efforts as well.

Two thirds of Australia's land surface is dominated by arid or semi-arid climates (Martin 2006) with very few permanent water

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bodies; however, our knowledge of the diversity and phylogeographical history of species inhabiting temporary water bodies in inland Australia is very limited. Non-permanent water bodies that fill after erratic rainfall and persist for weeks or months are common and widespread in inland Australia and constitute the typical freshwater habitat (Williams 1981). However, most of our knowledge of the phylogeographical histories of Australian freshwater species is based on species that inhabit permanent water bodies such as rivers or permanent water holes (e.g. Carini and Hughes 2004; Hughes et al. 2004; Faulks et al. 2010). These studies into molluscs, fish and crayfish have revealed strong genetic differentiation between the various main drainage systems of eastern and central Australia, for example, the Murray–Darling Basin (MDB), the Bulloo River catchment and the Lake Eyre Basin (LEB; Hughes et al. 2009; see Fig. 1). The dependence of the species in question on permanent water prevents them from crossing drainage system borders and has restricted gene flow between drainage systems for the last few hundred thousand or million years (Carini and Hughes 2004; Hughes and Hillyer 2006). By contrast, the unique fauna of temporary water bodies is made up of species either able to migrate across land (e.g. aquatic insects, which may also inhabit permanent water bodies) and/or able to survive the long drought periods (e.g. 'large branchiopods', which are restricted to temporary habitats). Large branchiopods lay resting eggs that are drought-resistant and passively dispersed via wind or animal vectors (Dumont and Negrea 2002). The first studies into Australian 'large branchiopods', namely *Cyclestheria hislopi* and several *Limnadopsis* species, showed that dispersal and gene flow are not restricted by drainage system borders (Schwentner et al. 2012b, 2013). In the case of *Limnadopsis*, this was explained by the presence and movement of migratory waterfowl – the most probable dispersal vector. During wet seasons, migratory waterfowl exhibit high abundances in areas where no genetic differentiation among *Limnadopsis* populations was observed (Kingsford and Porter 1999; Kingsford et al. 1999). Interestingly, *Limnadopsis* populations from north-eastern Australia (termed 'Buchanan' due its proximity to Lake Buchanan) were genetically differentiated from south-eastern and central Australian populations (Schwentner et al. 2012b), which may indicate the existence of a barrier to dispersal.

In this study, we study the diversity and phylogeography of the Australian 'large branchiopods' of the taxon *Eocyzicus* Daday (1914). The taxon *Eocyzicus* is one of 18 currently recognized extant genera within the 'clam shrimp' taxon Spinicaudata, four of which belong to the Cyzicidae. The taxonomy of the Cyzicidae is disputed, and its genera *Eocyzicus*, *Cyzicus*, *Caenestheria* and *Caenestheriella* have been synonymized by some (for a summary, see Richter and Timms 2005). Molecular phylogenetic analyses, however, have clearly supported the monophyly of *Eocyzicus* as a clade distinct from all other cyzicid taxa (Schwentner et al. 2009). *Eocyzicus* was first recognized in Australia in K. C. Richardson's M.Sc. thesis in 1929. Richardson's manuscript was never published, however, leaving the first published report of *Eocyzicus* in Australia to be made by two of the authors of the present study in 2002 (Timms and Richter 2002). Only two *Eocyzicus* species are described for this continent as yet. Both are endemic and were first described from the catchment of the Paroo River in eastern Australia: *E. parooensis* Richter and Timms (2005) and *E. argillaquus* Timms and Richter (2009).

To assess the diversity of the Australian *Eocyzicus* fauna, we first identified 'main lineages' on the basis of the analyses of cytochrome oxidase subunit I (COI). COI exhibits the greatest diversity of the markers used herein and has proved useful in a range of studies (e.g. Hebert et al. 2003; Adamowicz et al. 2004; Murugan et al. 2009; Schwentner et al. 2011; Vanschoenwinkel et al. 2012). Main lineages are determined by a combination of genetic distance and phylogenetic analyses and the Automated Barcode Gap Discovery (ABGD) method (Puillandre et al. 2012). Main lineages correspond to the concept of molecular operational taxonomic units (MOTUs; Floyd et al. 2002) as they are not necessarily identical to species. In a second step, we anal-

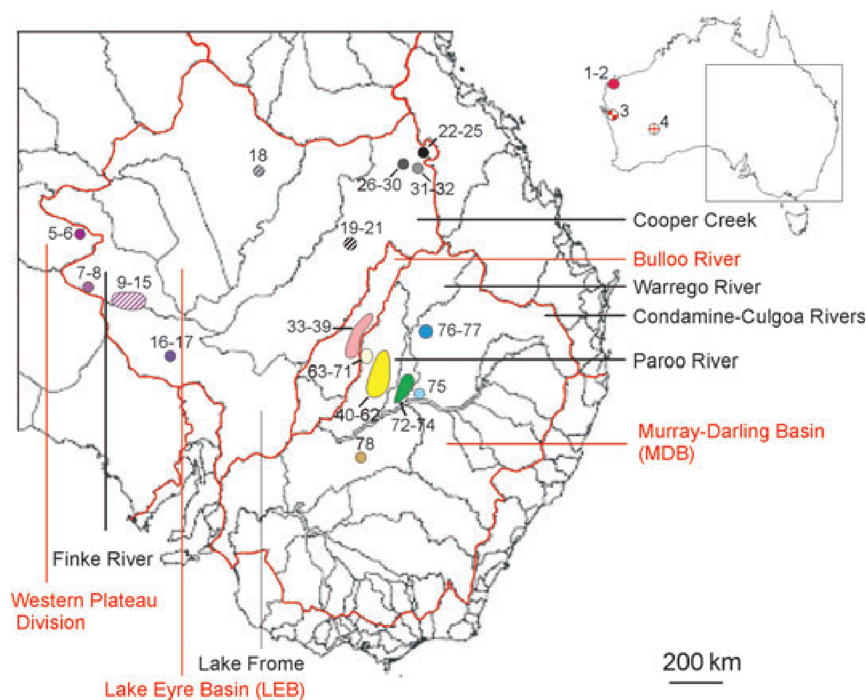

Fig. 1. Map showing the sampling localities used in this study. The larger map depicts the main drainage systems (red lines) and the catchments of individual rivers (black lines). Closely associated water bodies were grouped together, and their colour coding corresponds to the networks shown in Figs 2 and 4. The numbers correspond to the locality numbers in Table 1

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ysed three nuclear gene fragments in a subset of specimens from each main lineage: elongation factor 1 α (EF1α), internal transcribed spacer 2 (ITS2) and 28S rRNA. The translation of main lineages into discrete species depends on the methodology followed and the species concept used, as each concept requires different criteria (Laamanen et al. 2003; Tan et al. 2008; Schwentner et al. 2011). In the Biological Species Concept (BSC; Mayr 1942) and the Hennigian Species Concept (HSC) (Meier and Willmann 2000), reproductive isolation between species is the defining criterion. In our study, a clear differentiation (e.g. no shared sequences) between the nuclear genes of any two main lineages was an indication of reproductive isolation between them. Although consistent differentiation in the mitochondrial and nuclear genomes demonstrates the absence of current gene flow, it does not necessarily permit conclusions to be drawn about the potential for reproduction. If the main lineages are geographically separated (allopatric), gene flow may be restricted simply by geographical distance rather than mechanisms of reproductive isolation (Dobzhansky 1937), and reproduction might occur as soon as they came into contact again. Only when main lineages occur in the same region (sympatric) or even the same temporary pool (syntopic) is the absence of current gene flow a sufficient indicator of reproductive isolation, as in these cases mating would have been possible. Of course, other concepts are based on different criteria or definitions of species. The Evolutionary Species Concept (ESC; e.g. Wiley and Mayden 2000a) and the Phylogenetic Species Concept (PSC; e.g. Mishler and Theriot 2000a), for example, define a 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' (Wiley and Mayden 2000a) and as 'the smallest monophyletic groups worthy of formal recognition' (Mishler and Theriot 2000a). Species do not need to be reproductively isolated in either concept (Mishler and Theriot 2000b; Wiley and Mayden 2000b; see also Mayden 1999), meaning that geographical distribution is not an impediment to species recognition. Indeed, allopatric distribution might in the ESC even be an argument for independent evolutionary fate. Under the ESC and the PSC, then, monophyly and genetic differentiation (e.g. genetic distance) may suffice to infer that main lineages do actually represent distinct species. This comes close to the notion of species delineation using DNA barcoding in which species are delimited on the basis of a barcoding gap separating lower intraspecific from greater interspecific genetic distances (Hebert et al. 2003).

We hypothesize that additional, as yet undescribed, *Eocyzicus* species exist in Australia. Continuing on this assumption, if several species are found to occur sympatrically but not syntopically, we propose that they have different habitat preferences and/or are too competitive for syntopic occurrence. If syntopic occurrence is found to be common, we argue for niche differentiation within habitats. Expecting similar dispersal mechanisms to those found in *Limnadopsis*, we hypothesize that the level of intraspecific genetic differentiation over most of eastern and central Australia will be low, if single species are indeed distributed that far.

Materials and Methods

Collection details

Most specimens were collected as adults using hand nets (see Table 1 for collection details). In addition, surface sediment samples were collected from several sites, and specimens were reared later in the laboratory (see Schwentner et al. 2011 for details on hatching and rearing conditions). Specimens were fixed and stored in 100% ethanol or RNAlater (Qiagen, Hilden, Germany) and were deposited at the Australian Museum in Sydney (for details of specimens and their respective collection and GenBank registration numbers, see Table S1). The ecological parameters of the sampled water bodies were not consistently recorded, so habitats are only classified according to easily observable characteristics (e.g. turbid versus clear, fresh versus hyposaline).

DNA extraction, amplification, sequencing and alignment

Genomic DNA extraction followed the HOTSHOT protocol put forward by Montero-Pau et al. (2008) with a final volume of 60 μl. PCRs were carried out using a total of 30 μl consisting of 0.15 μl MolTaq (Molzym, Bremen, Germany), 3 μl of each primer (10 mM each; see Table 2), 3 μl 10 × buffer (Molzym), 0.8 μl $MgCl_2$ (50 mM), 3 μl dNTPs (200 μm each), 4.5 μl template DNA and purified water. To PCR amplify the COI gene fragment, a range of LCO and HCO primers were used in varying combinations. The most successful combinations were LCO2/HCOoutout and LCO3/HCO709. PCR programs for COI and EF1α consisted of an initial denaturation step at 94°C for 1 min, 38 amplification cycles (94°C for 1 min, 46°C for 30 s for COI and 51°C for EF1α, 72°C for 1 min) and a final extension of 72°C for 5 min. The 40 PCR amplification cycles for 28S were carried out at 94°C for 20 s, 52.5°C for 20 s and 72°C for 1.5 min (Sonnenberg et al. 2007), and the 31 amplification cycles for ITS2 were carried out at 94°C for 30 s, 50°C for 30 s and 72°C for 2 min. All PCRs were run on a TGradient thermocycler (Eppendorf, Hamburg, Germany), and PCR products were visualized on 1.5% agarose/TAE gel stained with 0.01% ethidium bromide. PCR products were purified with AMPure magnetic beads (Beckman Coulter, Krefeld, Germany) following the manufacturer's instructions. The purified PCR products were sequenced using the PCR primers. Sequencing was carried out either using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) on an ABI 3110 XL (Applied Biosystems) or by Qiagen. The resulting electropherograms were analysed using SEQUENCHER 4.1.4 (Gene Codes, Ann Arbor, MI, USA). All sequences obtained in the present study are deposited at GenBank under the following accession numbers: KC583545 – KC584007.

There are various copies of ITS2 and EF1α within the genome. Several of the ITS2 and EF1α sequences contained multiple ambiguous nucleotide positions, indicating the presence of more than one allele. If these nucleotide positions coincided with positions that varied between genetic lineages, the respective PCR product was cloned to identify potentially shared haplotypes/alleles. Cloning was performed using a T4 DNA ligase kit with blue/white selection (Promega, Mannheim, Germany) following manufacturer's instructions and using the same cleaned up PCR product as used for the sequencing reaction. Clones containing inserts were transferred into 50 μl H_2O and lysed at 95°C for 10 min. Cloned PCR fragments were re-amplified using the same primers as before, and those of the same length as the original PCR product (estimated on a 1.5% agarose/TAE gel) were cleaned up and sequenced (procedures as described above). The number of sequenced clones varied and was increased until all relevant ambiguous positions were accounted for.

Sequences were aligned using CLUSTALW (Thompson et al. 1994) in Bioedit 7.0.9.0 (Hall 1999). The number of variable and parsimony-informative sites was determined using MEGA5 (Tamura et al. 2011). The amino acid composition of the protein-coding genes was deduced from the nucleotide sequences using MEGA5 set to the 'invertebrate mitochondrial' code for COI and 'standard' for EF1α. Potential stop codons indicative of pseudogenes were identified on the basis of the translated amino acid sequences.

Identification of 'main lineages'

Genetic lineages were initially identified on the basis of analyses of the COI data set. These 'main lineages' were identified using a combination of phylogenetic analyses, haplotype networks, genetic distances and automated detection of the potential barcoding gap.

Bayesian phylogenetic analysis (four runs of six chains of $6*10^6$ generations each) was carried out in MRBAYES 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Every 1200th generation was sampled, and the first 10% of the sampled generations discarded as burn-in. The GTR + I + G substitution model was identified by MEGA5 as the best fitting model under the AIC criterion. Each haplotype was included only once in the Bayesian analysis.

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Table 1. Details of collection events and localities

Drainage system	Locality	Lineages	Locality description	Coordinates
Onslow Coast	1	U, Z	Samphire swamp a few km south Onslow, WA, 14.03.09	* 21°43′S, *115°05′E
	2	Z	Deep samphire swamp near Onslow, WA, 12.03.2009	21°43′12.3″S, 115°05′56.5″E
	3	Q	Muggon claypan, via Carnarvon, WA, 08.07.2011	26°46′54″S, 115°40′53″E
Salt Lake (WPD)	4	R	Lake Carey, WA, 08.03.2011	*29°10″S, *122°20″E
Sandy Desert (WPD)	5	Z	Island hyposaline lake 60 km North Kulgera, NT, 10.03.2011	25°19′23.2″S, 133°12′41.7″E
	6	Z	Lake 20 km West Erldunda, NT, 10.03.2011	25°14′36.5″S, 132°59′40.3″E
Finke River (LEB)	7	S	Large claypan 40 km north Marla, SA, 10.03.2011	26°59′48.9″S, 133°24′55.2″E
	8	Argill	Vegetated stony dugout 34 km north Marla, SA, 10.03.2011	27°05′26.8″S, 133°28′16.2″E
	9	Argill, Y	Old small dugout 105 km east Marla, SA, 11.03.2011	27°10′00.2″S, 134°33′07.2″E
	10	Argill	Daisy claypan 106 km east Marla, SA, 11.03.2011	27°10′02.2″S, 134°33′30.7″E
	11	X	Cane grass swamp 44 km west of Oodnadatta, SA, 11.03.2011	27°20′07.1″S, 135°07′47.7″E
	12	X	Claypan 44 km west Oodnadatta, SA, 11.03.2011	27°20′20.6″S, 135°08′00.3″E
	13	Y	Vegetated clear water swamp 43 km west Oodnadatta, SA, 11.03.2011	27°20′38.9″S, 135°08′40.3″E
	14	X	Cane grass swamp 26 km north Oodnadatta, SA, 11.03.2011	27°24′18.0″S, 135°21′00.1″E
	15	Argill	Dam 60 km north Oodnadatta, SA, 11.03.2011	27°03′13.4″S, 135°15′04.4″E
Lake Frome (LEB)	16	X	Stony claypan 20 km north Williams Creek, SA, 12.03.2011	28°51′30.1″S, 136°09′49.1″E
	17	Argill	Deepened claypan 19 km south William Creek, SA, 12.03.2011	29°04′55.0″S, 136°31′59.5″E
Georgina River (LEB)	18	U	Old borrow pit 8 km east of Boulia, QLD, 04.03.2011	22°55′44.6″S, 139°58′23.7″E
Cooper Creek (LEB)	19	Argill	Gidgee claypan 9 km on Tenham Station, QLD, 28.02.2011	25°41′02.4″S, 143°00′59.4″E
	20	Argill	Horse paddock claypan on Springfield Station, QLD, 01.03.2011	25°49′29.6″S, 143°04′07.9″E
	21	U	Thunda Lake, QLD, 08.04.2009; raised from sediment	25°25′46.0″S, 143°08′13.8″E
	22	W	Small lake behind major beach of Lake Buchanan (Y1), YS, QLD, 24.02.2008	S21°30′0.78″S, 145°48′52.6″E
	23	W	Creek pool south Lake Constant, YS QLD, 24.02.2008	S21°33′46.2″S, 145°47′39.2″E
	24	W	Small pool (Y8), YS, QLD, 04.04.2009	21°33′29.5″S, 145°47′06.5″E
	25	W	Morra Creek (M1), YS, QLD, 03.04.2009	21°28′51.9″S, 145°49′34.0″E
	26	W	Small pool (H1), ST, QLD, 02.04.2009	22°18′38.8″S, 145°22′57.0″E
	27	W	Small pool (H2), ST, QLD, 02.04.2009	22°18′29.6″S, 145°21′56.7″E
	28	W	Small pool (H4), ST, QLD, 02.04.2009; raised from sediment	22°17′47.1″S, 145°21′21.3″E
	29	W	Small pool (H5), ST, QLD, 02.04.2009	22°17′41.6″S, 145°21′21.9″E
	30	W	Small pool (H8), ST, QLD, 03.04.2009; raised from sediment	22°18′29.5″S, 145°23′00.3″E
	31	W	Lake Galilee, QLD, 15.02.2010	22°25′37.3″S, 145°42′13.4″E
	32	W	Artificial pool south Lake Dunn, QLD, 14.02.2010	22°39′44.8″S, 145°44′40.2″E
Bulloo River catchment	33	Argill	Yapunyah pool, QLD, 28.02.2011	27°49′09.6″S, 144°09′26.5″E
	34	U	Flood out of dam, 84 km south Thargomindah, QLD, 26.02.2011	28°39′46.7″S, 143°48′40.8″E
	35	Y	Swamp near Thargomindah Station, QLD, 26.02.2011	28°03′12.5″S, 143°47′11.5″E
	36	Argill	Toebiter claypan, QLD, 27.02.2011	28°02′30.8″S, 144°17′50.7″E
	37	T	Roadside dugout with yellow lilies, QLD, 27.02.2011	27°58′26.8″S, 144°18′34.9″E
	38	T	Coolibah swamp 16 km from highway, QLD, 27.02.2011	27°57′34.8″S, 144°18′22.4″E
	39	Argill	Claypan 45 km east of Thargomindah, QLD, 27.02.2011	28°05′15.0″S, 144°15′47.0″E

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Table 1. (continued)

Drainage system	Locality	Lineages	Locality description	Coordinates
Paroo River catchment (MDB)	40	X	Big Kangaroo Pan, RS, QLD, 1999	28° 57′S, 144° 58′E
	41	Z	Lake Bulla, RS, QLD, 09.06.2007	28°54′S, 144°55′E
	42	Paroo	Gidgee Lake, BS, NSW, 19.02.2010 (*locus typicus* of *Eocyzicus parooensis*)	29°33′10.4″S, 144°50′12.7″E
	43	Paroo	Woolshed Salt lake, BS, NSW, 19.02.2010	29°31′44.3″S, 144°51′11.1″E
	44	Paroo	Horseshoe Lake, BS, NSW, 19.02.2010	29°31′32.2″S, 144°45′57.8″E
	45	Z	Roskos Paleolake, BS, NSW, 19.02.2010	29°27′42.9″S, 144°48′12.5″E
	46	U, Z	Freshwater Lake, BS, NSW, 19.02.2010	29°29′14.7″S, 144°49′59.0″E
	47	Y	Beverley’s Pool, BS, NSW, 19.02.2010	29°32′12.0″S, 144°51′16.1″E
	48	X	Island claypan 500 m east of Freshwater Lake, BS, NSW, 28.09.2010	*29° 29′S, *144° 50′E
	49	U	Sues Pan, BS, NSW, 21.2.2011	29°29′05.6″S, 144°48′38.0″E
	50	U	Upper Crescent Pool, BS, NSW, 19.01.2010	29°32′33.6″S, 144°52′16.5″E
	51	U	Lower Crescent Pool, BS, NSW, 19.01.2010	29°32′34.5″S, 144°51′31.6″E
	52	U	Vosper Pool, BS, NSW, 19.01.2010	29°32′03.9″S, 144°50′37.7″E
	53	U	Lismore Bore, MS, NSW, 19.01.2010	29°31′50.7″S, 144°59′28.1″E
	54	Y	Small lake on East Boundary, north side of road, MS, NSW, 20.02.2010	29°31′38.5″S, 145°00′43.7″E
	55	Y	Small lake on East Boundary, south side of road, MS, NSW, 20.02.2010	29°31′54.1″S, 145°01′02.4″E
	56	U	Muella vegetated pool 1, MS, NSW, 31.03.2009	29°31′10.3″S, 144°56′21.8″E
	57	U	Muella vegetated pool 2, MS, NSW, 31.03.2009	29°31′00.3″S, 144°56′22.7″E
	58	U	Pool on Yungerina, MS, NSW, 20.01.2010	29°28′14.8″S, 145°06′27.2″E
	59	Argill, X	Lower Lake Eliza, MS, NSW, 20.02.2010	29°25′28.9″S, 145°03′41.8″E
	60	Y	Grassy pool north of Yantabulla, NSW, 20.01.2010	29°19′04.8″S, 145°00′31.5″E
	61	U	Yantabulla black box swamp, NSW, 31.03.2009	29°20′18.0″S, 145°00′12.1″E
	62	U	Black box swamp near Cumeroo, NSW, 20.01.2010	29°15′41.2″S, 145°09′29.0″E
	63	Argill	Big Darko claypan, CNP, QLD, 25.2.2011 (*locus typicus* of *E. argillaquus*)	28°52′19.1″S, 144°17′34.5″E
	64	X	Turbid claypan south of North Kaponyee, CNP, QLD, 24.2.2011	28°49′27.4″S, 144°19′44.5″E
	65	Argill	Claypan north of windmill, CNP, QLD, 24.2.2011	28°48′28.8″S, 144°18′09.1″E
	66	Argill	Well-vegetated claypan, CNP, QLD, 24.2.2011	28°47′19.4″S, 144°17′43.3″E
	67	X	Triops claypan, CNP, QLD, 24.2.2011	28°47′14.9″S, 144°17′49.1″E
	68	X	Vegetated island claypan, CNP, QLD, 24.2.2011	28°47′14.0″S, 144°17′45.7″E
	69	X	Claypan at old Wyara Junction, CNP, QLD, 24.2.2011	28°47′49.4″S, 144°17′55.6″E
	70	Argill	Vegetated claypan near Bilby enclosure, CNP, QLD, 24.2.2011	28°52′16.2″S, 144°24′37.3″E
	71	Argill, X	Claypan halfway up northern fence of Bilby enclosure, CNP, QLD, 25.2.2011	28°52′12.8″S, 144°21′52.1″E
Warrego River (MDB)	72	U	East of Lake Lauradale, NSW, 29.03.2009	29°51′ 22′S, 145°38′49″E
	73	U	Gerara composite swamp, 21.01.2010NSW,	29°11′47.0″S, 146°17′03.0″E
	74	U	Claypan-like pool west of Engonia, NSW, 21.01.2010	29°18′32.8″S, 145°44′06.9″E
Condamine-Culgoa Rivers (MDB)	75	U	Turbid pool, NSW, 21.01.2010	29°32′29.3″S, 146°24′50.1″E
	76	U	Cane grass swamp 75 km east Wyandra, QLD, 17.02.2010	27°23′03.5″S, 146°36′33.7″E
	77	U	Grassy turbid swamp, QLD, 18.02.2010	27°41′52.4″S, 146°45′44.7″E
Darling River (MDB)	78	X	Barnato Lake, NSW, 22.01.10; raised from sediment	31°36′45.2″S, 144°59′20.0″E

Argill, *E. argillaquus*; BS, Bloodwood Station; LEB, Lake Eyre Basin; MDB, Murray–Darling Basin; MS, Muella Station; NSW, New South Wales; NT, Northern Territory; QLD, Queensland; RS, Rockwell Station; Paroo, *E. parooensis*; SA, South Australia; ST, Sumana Station; WA, Western Australia; WPD, Western Plateau Division; YS, Yarromere Station; *, coordinates estimated.

The locality numbers correspond to Fig. 1. In addition to the description of each locality, the drainage system, coordinates and the main cytochrome oxidase subunit I lineages obtained are given.

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Table 2. All primers used in this study

Gene	Primer	Primer sequence 5′-3′	Authors
COI	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG	Folmer et al. (1994)
	LCO2	TCN ACH AAY CAT AAA GAY ATT GGA AC	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)
ITS2	ITS3	GCA TCG ATG AAG AAC GCA GC	White et al. (1990)
	ITS28	CGC CGT TAC TAG GGG AAT CCT TGT AAG	Wagstaff and Garnock-Jones (1998)
EF1α	HaF2For1	GGG YAA AGG WTC CTT CAA RTA TGC	Richter et al. (2007)
	2R53ST	CAG GAA ACA GCT ATG ACG CGA ACT TGC AAG CAA TGT GAG C	Richter et al. (2007)
28S	D1, D2 fw1	AGC GGA GGA AAA GAA ACT A	Sonnenberg et al. (2007)
	D1, D2 rev2	ACG ATC GAT TTG CAC GTC AG	Sonnenberg et al. (2007)

Haplotype networks were calculated using the median joining algorithm in NETWORK 4.6.1.0 (Fluxus Technology, Suffolk, England). To start with, one network containing all COI sequences was calculated. Separate networks were then calculated for each main cluster of sequences also identifiable in the Bayesian analysis. Because haplotype networks are better suited to intraspecific analyses, the relationships within these clusters may have been distorted in the more inclusive network. The clusters ultimately corresponded to the main genetic lineages. To assess their genetic differentiation, pairwise uncorrected *p*-distances were calculated in MEGA5 within and between these clusters. The uncorrected *p*-distance corresponds to the genetic distance as observed in per cent without applying an evolutionary substitution model. To objectively test the separation of ‘main genetic lineages’ from less inclusive lineages, we used the ABGD method set out by Puillandre et al. (2012), in which specific barcoding gaps derived from the actual data are used to partition the sequences into lineages. Genetic distances between sequences of different lineages are always larger than the inferred upper bound of the barcoding gap, while each sequence within the lineages is connected to at least one other sequence of the same lineage by a genetic distance below the lower bound of the barcoding gap. The maximum genetic distance within a given lineage may therefore exceed the inferred barcoding gap. The web-based version of ABGD was run using the *p*-distance matrix obtained with MEGA5 and standard settings (Pmin = 0.001, Pmax = 0.1).

Genetic diversity and genetic differentiation within each of the lineages were assessed by calculating haplotype (h) and nucleotide (π) diversities using ARLEQUIN 3.5 (Excoffier and Lischer 2010; the haplotypes occurring in each pool are listed in Table S2). Haplotype diversity estimates genetic diversity on the basis of the relative number of different haplotypes, while nucleotide diversity is a measure of the genetic differentiation between haplotypes. Population differentiation was assessed by calculating pairwise Φ_{ST} between populations. Each single sampled water body was treated as a separate population, although only those for which at least four sequences were available were included in the Φ_{ST} calculation. Significance was assessed at the 0.05 level. In addition, significance was also assessed after Bonferroni correction (where the aspired significance level is divided by the number of comparisons) to avoid erroneous significance assumptions caused by multiple pairwise comparisons. We used this method conservatively, taking the total number of pairwise comparisons across species and not of each species separately. The significance level after Bonferroni correction was 0.0037.

To assess whether the other three markers resulted in identical clusters of specimens (the same main lineages), phylogenetic analyses and network and genetic distance calculations were carried out for each marker as described above. It should be noted for EF1α and ITS2 that several sequences derived from cloning were available, meaning that sequences of a single individual may appear in several positions within the networks/phylogenetic trees. Only a single network was calculated for each marker. To calculate uncorrected *p*-distances, sequences were assigned to the same main lineage as identified using COI beforehand. Gaps are not included in distance calculations, which means that the genetic distances for ITS2 are slightly underestimated as certain nucleotide changes (e.g. the emergence of indels) are not accounted for. The ITS2 sequences could not be meaningfully aligned to non-*Eocyzicus* sequences (e.g. *Caenestheria* or *Leptestheria*) due to the vast number of indels, so the ITS2 phylogenetic analysis remains unrooted. Because 28S rDNA exhibited virtually no genetic diversity, only the network was constructed for this marker but no phylogenetic tree.

Results

Alignments

The alignment of the partial COI sequences contained 312 sequences of 537 bp, of which 206 bp were variable and 188 bp parsimony informative (not including the outgroup). The alignment contained no indels, and most variation was present at the third codon position. The deduced amino acid sequence consisted of 179 amino acids and no stop codons. Only 20 were variable and nine parsimony informative. The ITS2 sequence alignment was 665 bp in length and consisted of 100 sequences of 55 individuals. All in all, 122 positions were variable, 59 of which were parsimony informative (not counting indels). The alignment included a number of short (≤10 bp) indels, usually one to three nucleotides in length. The EF1α alignment consisted of 40 sequences of 681 bp in length from 29 individuals (not including sequences from GenBank). Fifty-seven base pairs were variable and 30 parsimony informative. The derived amino acid alignment only featured ten variables and one parsimony-informative position at a total length of 227 bp and no stop codons. The alignment of the 18 28S rDNA sequences was 755 bases long; of these only five were variable and three parsimony informative (not counting indels).

Identification of ‘main genetic lineages’

The phylogenetic and network analyses of COI consistently resulted in eleven ‘main lineages’, which were also recovered in the ABGD analyses: *Eocyzicus parooensis*, *E. argillaquus* and nine lineages not attributable to any described species (referred to hereinafter as lineages Q – Z; Table 3). Assigning *E. parooensis* and *E. argillaquus* to their respective lineages was unproblematic as samples from the species’ *locus typicus* were included and both localities only yielded a single lineage each. All lineages with more than one specimen available were reciprocally monophyletic (Figs 2 and 3) and supported by posterior probabilities ≥0.99. All eleven main lineages exhibited a clear gap between intra- and interlineage genetic distance (Table 6): the intralineage uncorrected *p*-distances did not exceed 3.8%, while interlineage distances were at least 7.1% (between *E. parooensis* and lineage R) and exceeded 9.7% in all other instances. The ABGD analyses suggested a barcoding gap with a lower bound of 2.5% and an upper bound of 9.5%. Decreasing the lower bound further splits lineage W and *E. argillaquus* into several lineages each.

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Table 3. Population indices for all main cytochrome oxidase subunit I lineages

	No. Ind.	No. Hapl.	No. Pools	h ± SD	π ± SD
Eocyzicus parooensis	17	6	3	0.691 ± 0.103	0.002 ± 0.002
E. argillaquus	84	24	16	0.889 ± 0.022	0.013 ± 0.007
Z	31	15	7	0.948 ± 0.024	0.012 ± 0.006
Y	21	12	7	0.900 ± 0.046	0.009 ± 0.005
X	32	17	11	0.960 ± 0.016	0.010 ± 0.006
W	44	10	11	0.696 ± 0.075	0.011 ± 0.006
U	68	21	21	0.856 ± 0.034	0.005 ± 0.003
T	7	2	2	0.667 ± 0.160	0.003 ± 0.002
S	5	1	1	0	0
R	1	1	1	–	–
Q	1	1	1	–	–

For each lineage, the number of individuals (# ind.), the number of COI haplotypes (# hapl.), the number of pools featuring the respective lineage (# pools) and its haplotype (h) and nucleotide (π) diversity are given plus standard deviations (SD).

The results of the phylogenetic and network analyses of ITS2 and EF1α are not as straightforward as those of the COI analyses, although except in a few cases there is no direct conflict with the main lineages derived using COI. Individuals of the same main lineage generally cluster together and are not randomly distributed across the trees/networks or among lineages (Fig. 4, Figs S1 and S2). However, some main lineages are not supported as monophyletic (Fig. 4, Figs S1 and S2), and genetic distances within and between lineages partially overlap in some cases (Table 4). All genetic distances obtained for ITS2 and EF1α are generally lower than those obtained for COI. However, compared with the interlineage genetic distances, the intralineage genetic distances are relatively large (Table 4). This is particularly true in the case of EF1α. Several lineages are characterized by distinct indels in ITS2. As these were accounted for only in the network analyses, the separation of some lineages is more pronounced here than the genetic distances or Bayesian analyses. Lineages X and S are nested within each other in analyses of EF1α and ITS2 and even share an identical sequence in analyses of the latter (Fig. 4, Figs S1 and S2). Lineages R and Z share an identical EF1α sequence, but are clearly separated in ITS2 (where lineage R is most closely related to *E. parooensis*). Lineage T is nested within lineage Y in EF1α, but the two lineages are clearly distinct in ITS2 (Fig. 4, Fig. S1 and S2). The Western Australian specimen of lineage U appears not to be part of the cluster of the remaining ITS2 sequences of lineage U (Fig. 4), although in the Bayesian analysis, the whole lineage including this specimen is monophyletic (Fig. S2). In all other instances, individuals of different main lineages never share identical sequences even if they occur sympatrically or even syntopically and always cluster with members of their own main lineage

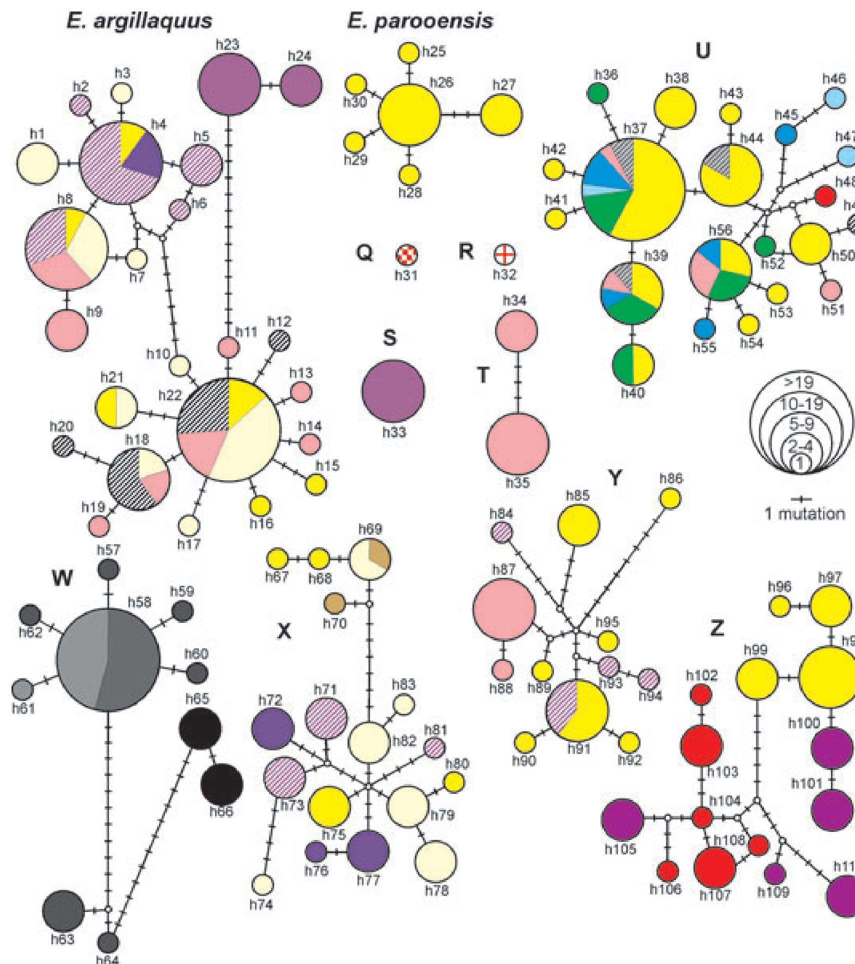

Fig. 2. Median joining haplotype networks of all 312 *Eocyzicus* cytochrome oxidase subunit I sequences grouped into eleven main lineages. Main lineages are indicated. Colours code for the locations in which specimens were collected as shown in Fig. 1

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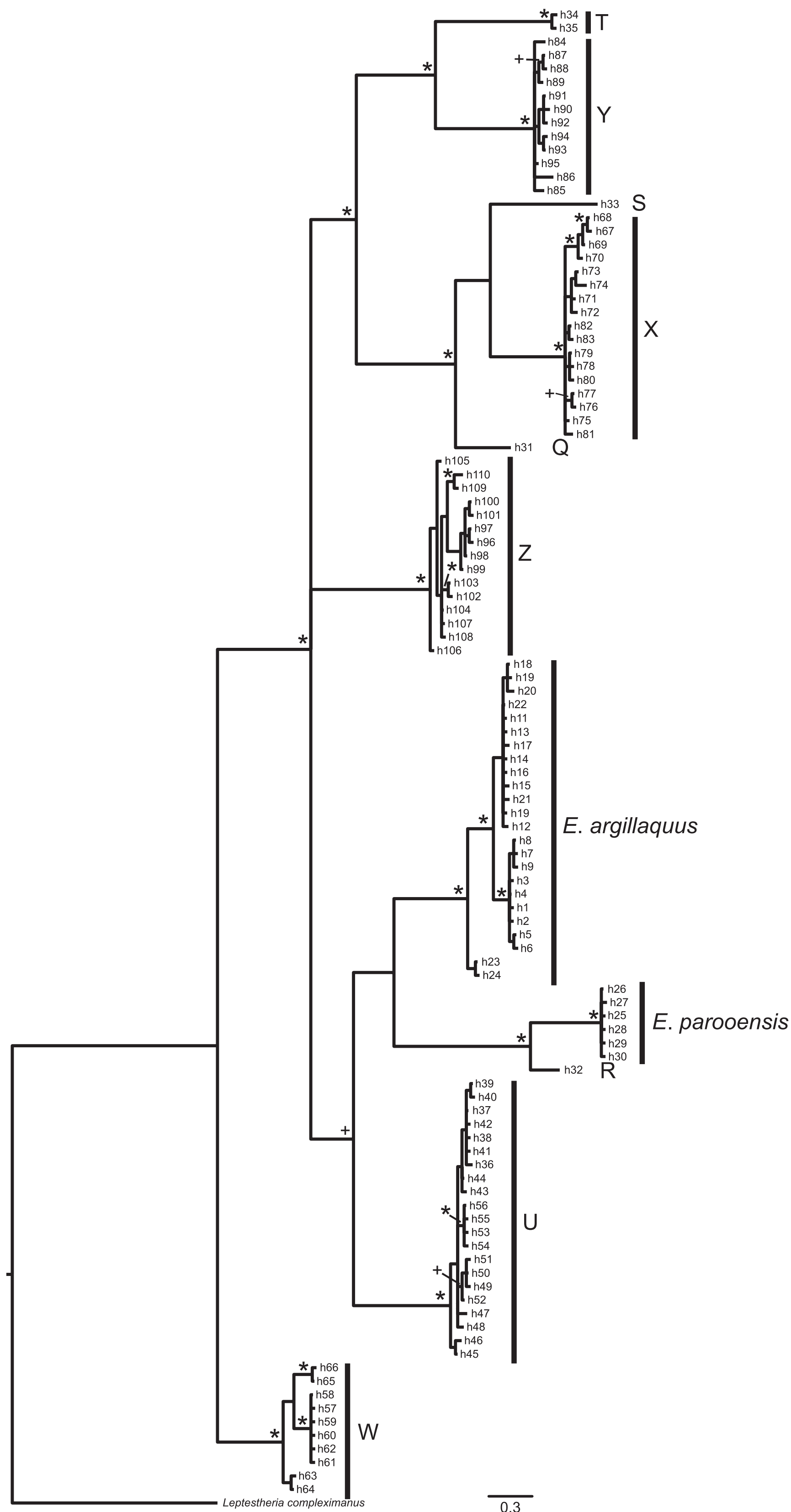

Fig. 3. Bayesian inference majority rule tree based on cytochrome oxidase subunit I. Each haplotype was included only once. Main genetic lineages are indicated. *, posterior probability ≥ 0.95; +, posterior probability ≥ 0.90

(Fig. 4). Interestingly, the large range of intralineage genetic distances is observable not only among individuals but also among clones of the same individual (e.g. individuals '1', '5', '8', '9', '10', '14', '18', '19', '21' and '25' in Fig. 4). Several instances where the monophyly of main lineages is not supported are due to intraindividual genetic diversity.

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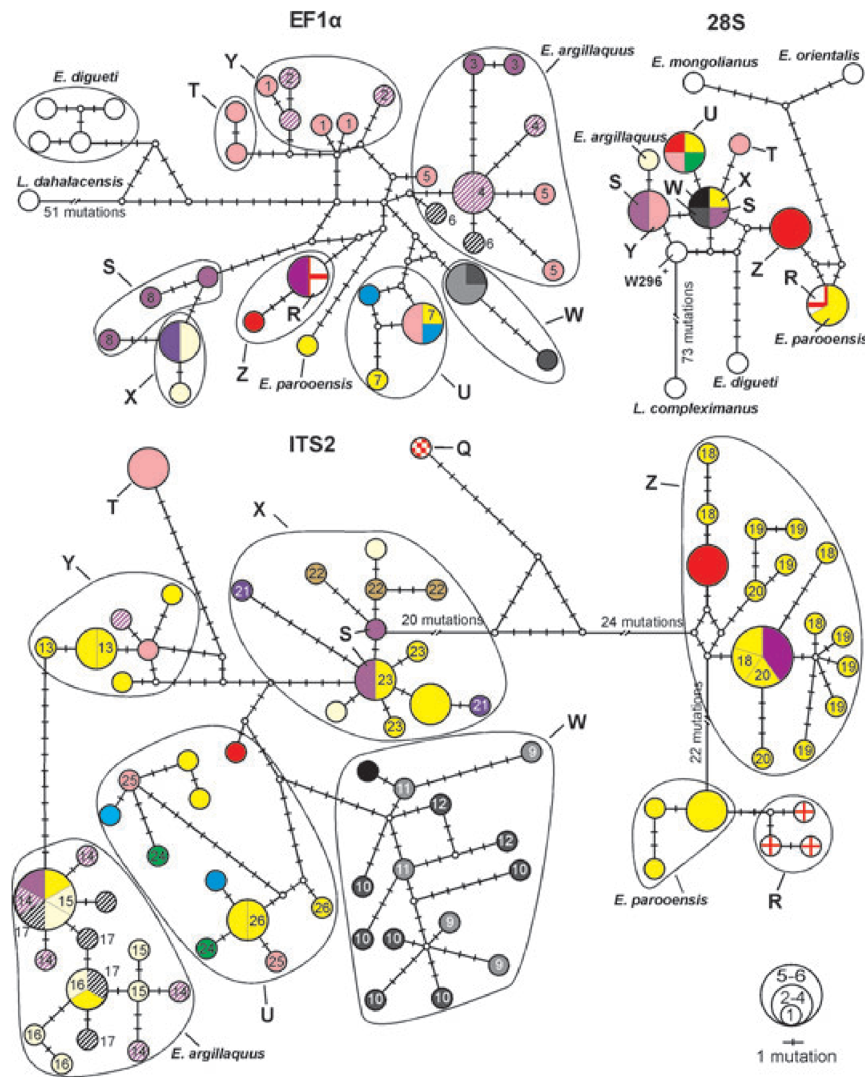

Fig. 4. Median joining networks of EF1α, 28S and ITS2. Affiliation of specimens to the main genetic lineages derived from the cytochrome oxidase subunit I data set is indicated by circles. Colours code for the locations in which specimens were found and correspond to Figs 1 and 2. Outgroups and non-Australian specimens are shown in white. The EF1α and ITS2 networks feature several sequences obtained by cloning. In these cases, more than one sequence per specimen is included. These specimens have designated numerals to indicate cloned sequences: 1 = P.89581; 2 = P.89574; 3 = P.89407; 4 = P.89414; 5 = P.89384; 6 = P.89400; 7 = P.89501; 8 = P.89450; 9 = P.89558; 10 = P.89544; 11 = P.89556; 12 = P.89537; 13 = P.89565; 14 = P.89413; 15 = P.89360; 16 = P.89376; 17 = P.89399; 18 = P.89586; 19 = P.89585; 20 = P.89599 21 = P.89642; 22 = P.89618; 23 = P.89615; 24 = P.89484; 25 = P.89523; 26 = P.89503. +, from Weeks et al. 2009 (specimen stems from South Australia)

In 28S, genetic diversity is very low and lineages are only marginally differentiated, if at all. Only lineage S is represented by two different sequences, one shared by lineage Y and the other by X and W. Lineages U, T and Z, and *E. argillaquus* are differentiated from all other lineages by one or two mutations each (Table 4, Fig. 4). *Eocyzicus parooensis* and lineage R sequences are identical and differentiated from other lineages. Notably, the Australian lineages appear phylogenetically to be more closely related to *E. digueti* from North America than to the two Asian species *E. mongolianus* and *E. orientalis*.

The phylogenetic relationships among the eleven lineages are not fully resolved in any of the analyses, and the proposed sister group relationships varied dramatically from analysis to analysis. Proposed phylogenetic sister group relationships among lineages recovered in analyses of at least two different markers are T and Y (COI, ITS2 and EF1α), S and X (COI, ITS2 and EF1α), U and W (ITS2 and EF1α), and *E. parooensis* and R (COI, ITS2 and 28S). A close relationship between the latter group and lineage Z is indicated by some markers (ITS2, EF1α and 28S).

Co-occurrences of two main lineages within a single pool (syntopic) are rare (Table 5): *E. argillaquus* co-occurs with lineages Y (pool 9) and X (pools 59 and 71), and lineage Z co-occurs with lineage U (pools 1 and 46; Table 1). Nevertheless, most of the lineages do occur sympatrically in certain areas (Figs 1,25,and, Table 5): six lineages occur in the central Paroo River catchment (bright yellow), four in the Bulloo River catchment (pink) and five in central Australia (shades of purple). Only lineages W, R and Q were not sympatrically recorded with any other main lineage. Although no detailed habitat parameters were available, certain habitat preferences are evident: most lineages occur in either clear or turbid freshwater habitats (Table 6). Lineages W and U are commonly recorded from both turbid and clear habitats, although this is most striking in the latter. Four lineages occurred in hyposaline habitats: R, U, Z and *E. parooensis* (Table 6). None of these except U were recorded in freshwater habitats too. While lineage Z occurred in habitats with low salinities and relatively fresh periods at the beginning of the filling cycle (Timms 1997), the habitats in which *E. parooensis*

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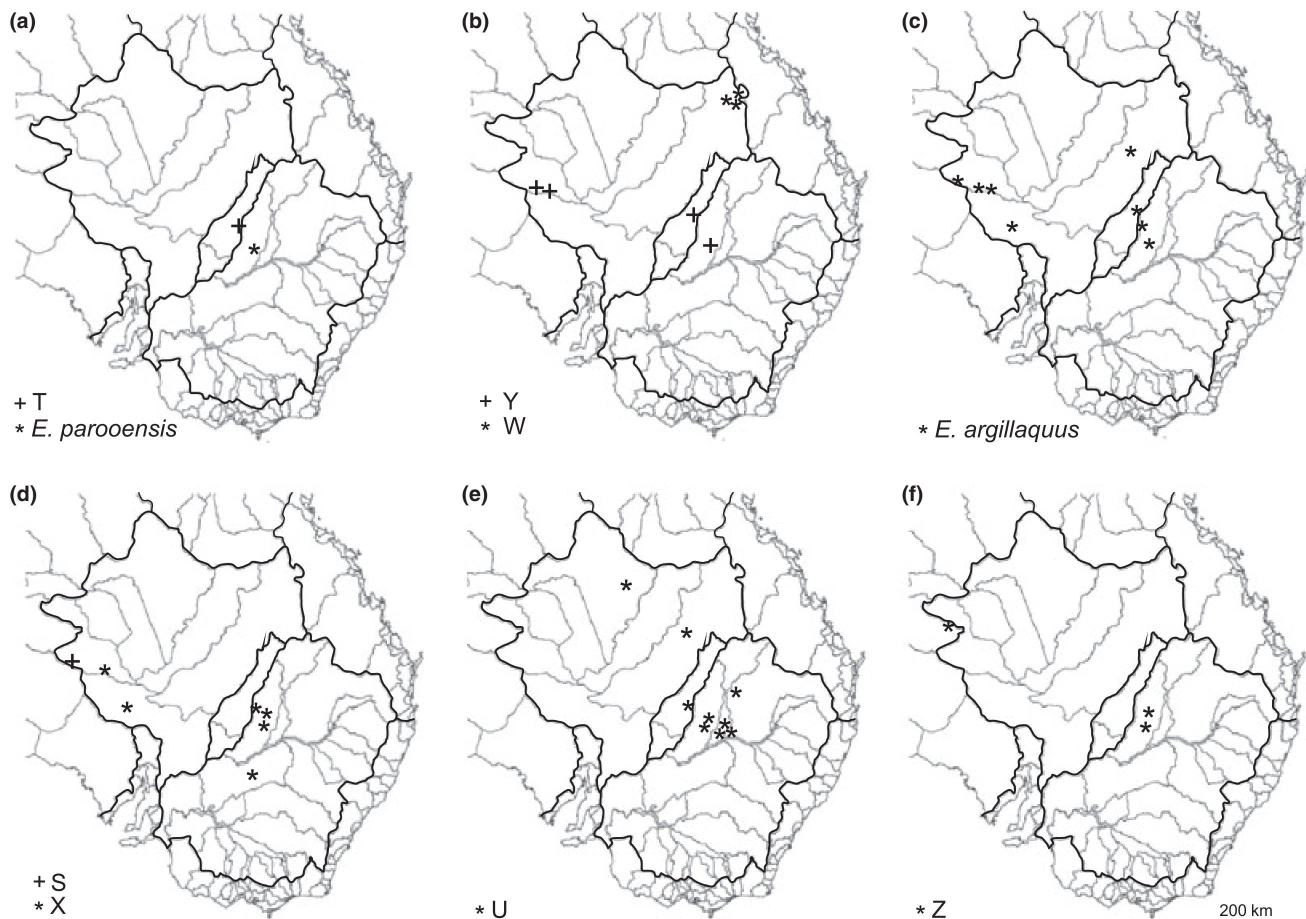

Fig. 5. Distribution of main *Eocyzicus* lineages in central and eastern Australia. Main drainage systems are indicated in bold. Note that several lineages occur in Western Australia as well, some lineages even exclusively, which is not depicted here (see. Figs 1 and 2)

(Timms and Richter 2009) and lineage R were found consistently featured higher salinities.

Population differentiation

Several of the main COI lineages are distributed over large parts of eastern and central Australia (e.g. *E. argillaquus*, lineages U, X, Y and Z; Fig. 5) with some even extending into the far west of Australia (lineages U and Z). COI haplotypes with wide geographical distributions occur in lineages Y and U and *E. argillaquus* (Fig. 2). Lineages X and Z feature closely related haplotypes (e.g. only a few mutational steps apart; Fig. 2 in geographically widely separated localities. The other lineages (*E. parooensis* and lineages Q, R, S, T and W) were only found in one single or a few closely associated pools (Figs 2 and 4). Genetic differentiation measured by Φ_{ST} revealed some fairly consistent patterns among several of the lineages. Among populations occurring within the MDB and/or eastern or northern areas of the LEB (albeit not in the north-east), little to no genetic differentiation is observable, with Φ_{ST} values low and mostly non-significant (*E. argillaquus* and lineage U; Table S3). However, genetic differentiation between the former and populations occurring in central (e.g. western areas of the LEB and closely associated populations in the far east of the Western Plateau Division) or Western Australia is strong (*E. argillaquus*, lineages X and Z; Table S3). In most cases, Φ_{ST} values here are high and significant. Furthermore, genetic differentiation in central Australia is usually high and often significant, even among comparatively closely associated populations (*E. argillaquus* and lineage Z; Table S3). Even after the conservative Bonferroni correction, about half of those pairwise comparisons significant at the 0.05 level were still significant. If the Bonferroni correction had been carried out for each species separately, almost all pairwise comparisons would have remained significant. A case that stands out is lineage W, the only lineage to inhabit the north-eastern area of the LEB ('Buchanan'). Despite the close proximity of its populations, genetic differentiation is high and, in several instances, significant at the 0.05 level, especially between those populations furthest to the north.

Discussion

Translating main lineages into species

An important prerequisite for most biological disciplines, for example biogeography, ecology and conservation, is a solid understanding of the diversity and taxonomy of the species inhabiting the habitats or regions studied. In 2005, Richter and Timms (2005) listed 23 spinicaudatan species known to occur in Australia, of ~150 species globally (Brendonck et al. 2008). In the few years since, ten additional species have been described or recorded from Australia (Timms 2009a,b; Timms and Richter 2009; Schwentner et al. 2012a), suggesting that overall spinicaudatan diversity is greater than previously thought. So far, the species listed include two species of *Eocyzicus*. Although *E. parooensis* is reported to prefer hyposaline habitats and *E. argillaquus* turbid water bodies (Timms and Richter 2009), both

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Table 4. Uncorrected *p*-distances within and among main lineages

	Eocyzicus parooensis	*E. argillaquus*	Z	Y	X	W	U	T	S	R	Q	*E. digueti*
E. parooensis	0.0–0.6 – 0.0–0.3	14.2–15.5 1.5–2.2 3.4–4.1	15.3–16.9 1.5–1.8 1.8–2.7	16.0–17.1 1.6–1.8 3.4–3.9	16.6–17.5 2.5–2.6 3.3–4.2	15.5–16.9 1.6–2.1 3.6–4.4	13.8–15.1 1.6–1.9 3.7–4.8	17.7–18.2 1.8–1.9 4.1–4.4	16.8–17.1 2.2–2.8 3.4–3.8	7.1–7.3 0.6 0.2–0.6	16.0–16.4 – 3.6–3.7	– 3.7–4.0 –
E. argillaquus		0.0–3.8 0.0–1.6 0.0–1.0	11.6–14.3 0.9–2.1 3.1–4.4	14.8–16.8 0.9–1.9 1.5–2.4	13.6–15.5 1.9–2.8 1.5–2.9	14.7–16.0 0.9–2.3 1.8–3.1	11.0–12.8 0.7–2.1 1.9–3.4	16.2–17.3 1.0–1.8 2.1–2.9	14.4–15.3 1.6–2.9 1.6–2.4	13.0–15.1 0.6–1.5 3.6–4.2	13.0–14.0 – 2.5–3.1	– 2.8–4.0 –
Z			0.0–2.4 0.3 0.0–1.6	14.1–16.0 1.3–1.8 3.0–4.1	13.8–16.0 1.6–2.1 2.8–4.5	14.7–16.4 1.2–2.1 3.0–4.4	12.7–14.5 1.3–2.1 3.3–4.9	15.8–17.5 1.5–1.6 3.6–4.8	14.4–15.8 1.3–2.2 3.0–4.0	15.3–16.8 0.0–0.3 1.9–2.7	13.8–14.8 – 3.3–4.1	– 2.8–3.2 –
Y				0.0–2.4 0.1–1.0 0.0–0.5	13.6–15.3 1.8–2.2 0.6–1.8	16.6–18.4 1.0–2.1 1.0–1.9	15.6–17.3 0.9–1.5 0.8–2.1	12.8–13.8 0.4–1.0 0.8–1.3	16.2–17.3 1.6–2.3 0.5–1.0	17.2–18.1 1.0–1.2 3.7–4.9	15.4–16.0 – 2.0–2.3	– 2.6–3.2 –
X					0.0–2.4 0.0–0.1 0.0–1.3	14.7–16.8 2.2–2.9 0.3–2.1	13.9–15.5 2.1–2.6 0.3–2.1	14.7–15.8 2.2–2.4 1.1–2.1	10.0–11.4 0.3–0.7 0.0–1.0	16.6–17.3 1.3–1.5 3.3–4.4	9.7–10.5 – 2.1–2.8	– 3.2–3.7 –
W						0.0–3.0 0.0–0.6 0.0–1.3	14.2–16.0 0.4–1.3 0.8–2.1	15.3–16.4 1.5–2.2 1.1–1.9	15.9–16.8 1.9–3.1 0.5–1.2	16.2–17.5 0.9–1.3 3.6–4.8	14.3–15.0 – 2.0–2.8	– 2.8–3.4 –
U							0.0–1.7 0.0–0.4 0.0–1.1	17.7–19.0 1.3–1.6 1.1–2.1	13.9–14.7 1.8–2.7 0.3–1.2	14.0–14.9 1.0–1.3 3.8–4.5	13.0–13.8 – 2.3–3.1	– 2.8–3.7 –
T								0.0–0.6 0.1 0.0	17.5–18.1 1.8–2.6 0.8–1.0	18.2–18.4 1.2–1.3 4.1–4.5	16.8–17.0 – 2.6–2.8	– 3.1–3.5 –
S									0.0 0.3–0.9 0.0	17.1–17.3 1.0–1.3 3.4–4.0	10.1 – 2.0–2.1	– 2.9–3.8 –
R										– – 0.0–0.5	17.4 – 3.7–4.0	– 2.4–2.7 –
Q											– – –	– – –
E. digueti	COI EF1α ITS2											– 0.1–0.4 –

–, No sequence or only a single sequence available.
From top to bottom, each pairwise comparison features cytochrome oxidase subunit I (COI), elongation factor 1 α (EF1α) and ITS2 genetic distances, respectively (see bottom left corner). The lineages correspond to the main lineages derived from the COI data set. Note: indels are not accounted for so ITS2 distances are slightly underestimated.

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Table 5. Overview of the sympatric and syntopic occurrences of all main lineages

	E. argillaquus	Z	Y	X	W	U	T	S	R	Q
E. parooensis										
E. argillaquus			S	S						
Z						S				
Y										
X										
W										
U										
T										
S										
R										

Grey cells mark lineage pairs that occur sympatrically, those that occur syntopically as well are indicated by an 'S'.

species were assumed to inhabit a wide range of habitats throughout Australia.

DNA barcoding as a means of species delimitation involves using a barcoding gap to separate intraspecific variability from interspecific variation. Originally, a universal threshold value of ~3% was proposed for the lower bound of the barcoding gap for COI (Hebert et al. 2003), but this idea has been refuted by several authors (Meyer and Paulay 2005; Meier et al. 2006), and taxon-specific thresholds for determining barcoding gaps have been suggested instead (Puillandre et al. 2012). The lower bound of the barcoding gap is usually determined by the largest intraspecific distance observed. In this study, this corresponds to the 3.8% observed within *E. argillaquus*. The lower bound of ~2.5% suggested by the ABGD method, on the other hand, reflects the distance by which each sequence is linked to at least one other sequence within the respective lineage/species (Puillandre et al. 2012). Although this may be the more accurate approximation of the lower bound of the barcoding gap, it makes comparisons with most literature data rather difficult. Similar barcoding gap thresholds have been applied to other branchiopod species, where intraspecific distances were usually below 5% or 6% and interspecific distances >10% (most studies use corrected distances, which are generally larger than the uncorrected *p*-distances used here; Adamowicz et al. 2004; Penton et al. 2004; Puillandre et al. 2012; Schwentner et al. 2011). Exceptionally low interspecific distances were observed in Anostraca Ketmaier et al. (2003) and Muñoz et al. (2008). Applying these barcoding gap estimates to our material made it possible to delineate all eleven main lineages as distinct species under the barcoding approach.

In the following, we discuss the extent to which the inclusion of nuclear markers permits inferences to be made about species monophyly (the defining criterion for the PSC and, -implicitly, for the ESC) and reproductive isolation (the defining criterion for the BSC and the HSC), to establish the number of species that can be distinguished under the various species concepts. With few exceptions, individuals of the same main lineage derived on the basis of COI cluster together in the analyses of the nuclear markers ITS2 and EF1α and are not randomly associated with members of other lineages. This implies that most of the main lineages constitute monophyletic species (relevant here is the monophyly of the species, not of single gene trees). The lack of genetic differentiation in 28S restricts its value as a basis for inferences about monophyly and reproductive isolation. Six lineages co-occurred sympatrically or even syntopically in the central Paroo River catchment (MDB; bright yellow Figs 1 and 2): *E. argillaquus, E. parooensis* and lineages U, X, Y and Z. All six are clearly differentiated from each other in EF1α and ITS2. Consequently, these six lineages are most likely reproductively isolated. All *E. argillaquus* individuals from the most western habitat (pool 8) are differentiated in COI (2.6–3.8%, haplotypes h24 and h25; Fig. 2) and EF1α (Fig. 4; ITS2 and 28S data not available). The nearest populations are only about 100 km further east, but gene flow is not apparent. This either indicates reproductive isolation or restricted dispersal in this area (see section Phylogeographical patterns). Lineage T is nested within Y (as a result of intraindividual genetic variation in Y) in analyses of EF1α, but clearly differentiated in ITS2 and 28S. As T and Y are sympatric in the Bulloo River catchment, the non-monophyly of Y in EF1α may be explained by incomplete lineage sorting of an ancestral polymorphism rather than reproduction. Lineage sorting is a random process driven by the loss of ancestral polymorphisms through genetic drift, meaning that monophyly is achieved for each marker independently (Jennings and Edwards 2005) and achieved more rapidly by mitochondrial markers than by nuclear markers (Moore 1995). Consequently, gene trees of nuclear markers more often result in non-monophyletic (i.e.

Table 6. Habitat preferences of the eleven main cytochrome oxidase subunit I lineages

	Claypans and cgs	Turbid lakes	Swamps (except cgs)	Clear lakes	Artificial water bodies*	Turbid	Clear	Fresh	Hyposaline	Summary/notes
E. ns	x	x			x	x		x		Usually very turbid, fresh
X	x	x				x		x		Turbid, fresh
W	x			x		x	x	x		Mainly turbid, rarely clear, fresh
S	x					x		x		Turbid, fresh
Q	x					x				Turbid, fresh
U	x	x	x	x	x	x	x	x	(x)	Clear to turbid, usually fresh, rarely hyposaline
E. parooensis				x			x		x	Clear, always hyposaline
R				x			x		x	Clear, always hyposaline
Z			x	x			x	(x)	x	Clear, limited vegetation, pools hyposaline at times
Y			x		x	(x)	x	x		Clear, rarely turbid, well vegetated, fresh
T			x		x		x	x		Clear to slightly turbid, well vegetated, fresh

cgs, cane grass swamps; * e.g. dams or dugouts.
In clear habitats, the bottom of the respective water body is usually visible. Claypans and cane grass swamps were considered a single habitat type as many water bodies were transitional (e.g. featuring only a small amount of cane grass).

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non-resolved or paraphyletic) lineages than those of mitochondrial markers even when the lineages concerned represent distinct species (Moore 1995; Funk and Omland 2003). Given the clear differentiation in ITS2 and 28S, reproductive isolation can be assumed to exist between lineages Y and T. Lineages R and Z share an identical EF1α sequence, but are clearly differentiated in ITS2 and even 28S. In the latter two markers and in COI, lineage R is more closely related to *E. parooensis* than to lineage Z. Lineage R can therefore be assumed to be distinct from Z (the identical EF1α sequence could represent an ancestral polymorphism/incomplete lineage sorting or historic introgression event). R and its putative sister species *E. parooensis*, however, are allopatrically distributed: one occurs in the east and the other in the west of Australia. Similarly, lineages Q and W are allopatrically distributed from all other lineages. Lineage Q is genetically well differentiated from all other lineages, and its putative sister species was not apparent from the phylogenetic analyses. The putative sister species to lineage W is U, which occurs a few hundred kilometres further south. The clear differentiation between them in all markers (including 28S) strongly suggests reproductive isolation, although this does not appear to have been tested in nature through sympatry. Lineages X and S are not differentiated from each other by any other marker apart from COI, but are well differentiated from other lineages. This can be interpreted either as an effect of incomplete lineage sorting in rather young species or as ongoing gene flow within a single species. For the time being, we go with the latter as no genetic differentiation is detectable in any nuclear marker, whereas all other lineage/species pairs studied are differentiated in at least one nuclear marker. The two lineages also have very similar habitat preferences.

In summary, there is good evidence that the eleven main lineages can be delineated into ten species under the PCS (Mishler and Theriot 2000a): *E. argillaquus, E. parooensis*, Q, R, T, U, W, X+S, Y and Z. All the main lineages – except X + S – are most likely monophyletic, although monophyly may not be established yet in all markers. The divergent lineage within *E. argillaquus* may be an additional species but whether or not it is 'worthy of recognition' stands to debate due to its relatively small genetic and ecological differentiation. Translating the main lineages into species following the ESC (Wiley and Mayden 2000a) would probably result in the same ten species as under the PCS. Monophyly, genetic differentiation, ecological specialization (see section Habitat preferences and niche differentiation) and, potentially, reproductive isolation suffice to infer 'independent evolutionary fate and historical tendencies'. The more emphasis is placed on differentiation in COI, the stronger the argument for delineating lineages X and S, too, under the ESC. We believe, however, that the lack of differentiation in any nuclear marker prohibits the assumption of independent evolutionary fates for this pair of lineages. We deemed reproductive isolation between lineages – as required by the BSC (Mayr 1942) and the HSC (Meier and Willmann 2000) – to exist if the lineages in question were differentiated in their nuclear genome and additionally occurred in sympatry or syntopy. Consequently, only seven species (*E. argillaquus, E. parooensis*, T, U, X, Y and Z) can be delineated under these species concepts. The status of lineages R, Q and W remains ambiguous as reproductive isolation has not yet been tested by sympatry/syntopy in nature. The obvious differentiation in the nuclear genes of these lineages does not suffice under the BSC or HSC. Resolution is only likely to be achieved by the discovery of sympatric/syntopic co-occurrences in the future.

If the main lineages X and S do represent a single species (X + S), the maximum intraspecific genetic distance this species would exhibit would be 11.4%, a value in excess of the genetic distance observed between most other pairs of putative species. A similarly high distance, it should be noted, was observed between two otherwise indistinguishable populations of *Limnadopsis parvispinus* (Schwentner et al. 2011), exemplifying the fact that genetic distances of COI alone – as proposed by DNA barcoding – are not a suitable means of unambiguously delineating species compatible with common species concepts.

Noteworthy is the high degree of intraindividual genetic variation observed for ITS2 and EF1α, which spanned the entire range of observed intralineage variation observed within several main lineages. With the few exceptions noted above, intraindividual variation did not interfere with the differentiation of main lineages, even where lineages occurred in sympatry or syntopy. This adds further weight to the delineation of the main lineages as distinct species under all species concepts. The reason for this level of intraindividual diversity cannot be pinned down: either populations within lineages are well admixed, frequently exchanging newly differentiated genetic variation (this seems to be the case in several instances, see Phylogeographical patterns), or the level of intraindividual diversity reflects ancestral polymorphisms maintained within each population.

Habitat preferences and niche differentiation

The idea that each Australian *Eocyzicus* species might inhabit a wide range of habitats throughout Australia (Timms and Richter 2009) was clearly based on an erroneous assumption of low species richness. It appears now that most species have narrowly defined habitat requirements and are often restricted in their distribution. The distribution and assemblages of Branchiopoda and other aquatic invertebrates have been shown to be largely shaped by abiotic or biotic habitat properties (Vanschoenwinkel et al. 2007; Nhiwatiwa et al. 2011). Important factors can be the duration and frequency of inundation, conductivity, vegetation cover, habitat size and turbidity (Timms and Sanders 2002; Vanschoenwinkel et al. 2009; Nhiwatiwa et al. 2011). The combination of preclusive habitat properties and priority effects determines species assemblage within single habitats by inhibiting the effective establishment of other species (Vanschoenwinkel et al. 2007). This may explain why the syntopic occurrence of *Eocyzicus* species is so rare (observed in just five of 78 studied water bodies (Table 1), despite sympatry and good dispersal ability (see Phylogeographical patterns). Species are able to co-exist within a habitat if their niches are clearly differentiated (Leibold 1995), otherwise the species that is better adapted to the habitat in question or that colonized the habitat first (priority effect) will outcompete the other species, and later immigration will be inhibited by competitive exclusion (Waters 2011). Accordingly, the Australian *Eocyzicus* species either have niches, which are not fully differentiated from each other, resulting in permanent competition and mutual exclusion, or have habitat requirements so specialized that each species can only inhabit a narrow range of habitats (focusing on the habitat aspects of niche formation only). A large number of water bodies featuring other spinicaudatans were not found to contain any *Eocyzicus* species (Timms and Richter 2002; unpublished data), suggesting that interspecific competition between *Eocyzicus* species may not be the only factor or even the dominating factor the species' distribution. We propose that their habitat requirements may be so specialized that many habitats (especially certain types of swamp) cannot be successfully colonized. On the basis of the limited ecological data available, four types of habitat specialization can be distinguished, all similar to those identified for Anostraca in the central Paroo River catchment (Timms and Sanders 2002).

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Eocyzicus can thus be divided into halophilic species (*E. parooensis*, lineages R and Z), turbid freshwater species (*E. argillaquus*, lineages X/S and Q), clear freshwater species (lineages Y and T) and generalists. The generalists occur in turbid and clear freshwater habitats and sometimes even in hyposaline habitats (lineages W and U). Salinity and turbidity are thus possibly the most important factors involved in shaping the species' distributions (Timms and Sanders 2002), although other factors such as surrounding terrestrial vegetation, degree of submerged vegetation and amount and type of organic matter may be involved as well, especially in determining the distribution of species with similar requirements for salinity and turbidity (possibly by affecting the availability of particular food sources). This may explain the high density and diversity of *Eocyzicus* species observed in the central Paroo River catchment (MDB), an area with a high density and diversity of temporary water bodies ranging from hyposaline lakes to claypans, various types of swamp and creek pools (Kingsford and Porter 1999; Timms and Boulton 2001). Not surprisingly, the anostracan (Timms and Sanders 2002) and spinicaudatan (Timms and Richter 2002) fauna in this area is rich, and of the six *Eocyzicus* species recorded there, only two or three species are recorded in the neighbouring Currawinya National Park (*E. argillaquus* and species X) and Bulloo River catchment (*E. argillaquus* and species U and Y) as well. Conversely, the Bulloo River catchment only featured a single species not recorded from the central Paroo River catchment (species T).

It is worth noting that the rare syntopic occurrences of *Eocyzicus* species brought to light by this study usually involved one species not typically found in the habitat in question (e.g. lineage U in a hyposaline pool or lineage Y in a turbid habitat). It is possible that the species in question were introduced from neighbouring pools by local flooding, a phenomenon that has also been observed for some species of Anostraca (Timms and Sanders 2002). If this is the case, 'true' syntopic occurrences of *Eocyzicus* species, with both species exhibiting stable populations over several inundation periods, may hardly exist.

Phylogeographical patterns

Species inhabiting permanent freshwater habitats across inland Australia are genetically differentiated according to drainage system borders (e.g. Carini and Hughes 2004; Hughes et al. 2004, 2009; Hughes and Hillyer 2006; Faulks et al. 2010). This does not seem to apply to the 'large branchiopods' that inhabit temporary water bodies, however, which exhibit a low level of genetic differentiation among populations across large areas of eastern and central Australia, despite the high level of genetic differentiation (Schwentner et al. 2012b, 2013). This finding is well corroborated by the results of the present study. The *Eocyzicus* species we investigated displayed little to no genetic differentiation over large parts of the study area, namely the MDB, the Bulloo River catchment and the northern and eastern LEB (with the exception of the north-eastern LEB), as far as the species occurred at all in these regions. Drainage system borders do not constitute barriers to dispersal, it seems. On the contrary, the low and usually non-significant Φ_{ST} values obtained indicate that gene flow across drainage borders has occurred relatively recently on a large scale and may still be occurring now. As assumed in the case of the *Limnadopsis* species (Schwentner et al. 2012b), the low level of genetic differentiation in this area may be linked to the presence of vast numbers of highly nomadic water birds that visit the Paroo River catchment (MDB), the Bulloo River catchment and the southern and central areas of the Cooper (eastern LEB), in particular to breed during wet seasons (Kingsford and Porter 1999; Kingsford et al. 1999). These birds migrate up to 300 km a day (Roshier et al. 2006, 2008), making it easy for them to disperse resting eggs without being restricted by drainage system borders. Furthermore, telemetric data (Roshier et al. 2008) obtained for Grey Teal (*Anas gracilis*) and the modelling of potential water bird movement during wet seasons (Roshier et al. 2001) indicate regular movement within the area of little to no genetic differentiation in *Eocyzicus*. Studies into branchiopods and other invertebrates that produce resting eggs (or other dormant stages) in other parts of the world have often revealed strong genetic differentiation even on a local scale (reviewed in De Meester et al. 2002). This has been attributed to the priority effects of the first colonizers, local adaptation and the buffering effects of the resting egg bank (Boileau et al. 1992), all of which inhibit the establishment of newly arriving migrants (Monopolization Hypothesis, De Meester et al. 2002). In these studies, then, effective gene flow between populations was limited despite theoretically high dispersal rates. Because the same patterns of genetic differentiation are not observed in the regions of eastern Australia under discussion here, the dispersal rate mediated by water birds must be high enough to overcome such limitations to gene flow, at least from time to time (Schwentner et al. 2012b).

Contrasting our findings for *Eocyzicus*, in which most populations from central Australia (namely western LEB and the eastern Western Plateau Division) are significantly differentiated from those further to the east, *Limnadopsis tatei* and *L. birchii* failed to display genetic differentiation in central Australia either (Schwentner et al. 2012b). These contrasting patterns of genetic differentiation are surprising, as the overall dispersal potential via migratory water birds should be similar for both taxa. A possible explanation may be differences in habitat requirements. As argued before, because *Eocyzicus* species hardly ever occur in syntopy, they can be assumed to be either harshly competitive due to incomplete niche differentiation or very specialized with narrowly defined habitat requirements. In contrast, the *Limnadopsis* species of eastern Australia regularly occur syntopically (Timms and Richter 2002), implying less competition and/or less restrictive habitat requirements. This may explain the higher effective dispersal rate of *Limnadopsis* species towards central Australia, as the diversity and density of suitable water bodies is much lower here than further east (especially compared with the central Bulloo River catchment and neighbouring MDB; Roshier et al. 2001; Timms 2012). This fact, coupled with the lower number of nomadic water birds in central Australia (Reid et al. 2009), may mean that for species that are highly specialized or in strong competition for regular effective dispersal, the chances of reaching a suitable habitat may simply be too low. Moreover, differentiation among the *Eocyzicus* populations in central Australia is not only apparent but quite pronounced, despite the fact that the geographical distances among them are similar to or smaller than those among eastern Australian populations. It seems that intraspecific lineages that differentiated in eastern and Western Australia come into secondary contact in central Australia, but not within single pools. For example, potential Western Australian haplotypes (h23 and h24 from population 8; Fig. 2 and Table 1) of *E. argillaquus* are <100 km apart from a population featuring eastern Australian haplotypes (h2, h4, h5, h6 and h8 from populations 9, 10 and 15). In the case of lineage Z, <25 km separate such populations (h100 and h101 from population 6 versus h105, 109 and h110 from population 5). Lineages S and X may also constitute intraspecific lineages that differentiated allopatrically in eastern and Western Australia and came into secondary contact in central Australia. The dispersal rate in this region may be too low – due to the lower number of nomadic water birds (Reid et al. 2009) – to overcome the effective dispersal-limiting effects described above. This may be the cause

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that prevents regular gene flow between western and eastern Australian populations in many species. A notable exception is lineage U, the species that inhabits the widest range of habitat types. Although this species was not recorded in central Australia, genetic differentiation between western and eastern haplotypes appears minimal, suggesting recent effective long-distance dispersal.

Interestingly, the north-eastern region of the LEB (referred to as 'Buchanan' in Schwentner et al. 2012a,b in connection with *Limnadopsis*) appears to be isolated and genetically well differentiated from all other regions studied. The only *Eocyzicus* species to occur here – lineage W – was not recorded anywhere else. Populations of *Limnadopsis birchii* and *L. parvispinus* also exhibited strong genetic differentiation from populations occurring further to the south in the MDB and even from others within the LEB (Schwentner et al. 2012b). This may be explained by a lack of sufficient temporary habitats in the area in between, reducing the connectivity of temporary water bodies for nomadic water birds (Roshier et al. 2001, 2008). Nevertheless, the potential for dispersal between the north-eastern LEB and the MDB has been demonstrated for *Cyclestheria hislopi*, whose establishment of new populations in the central Paroo River catchment (MDB; Schwentner et al. 2013; Timms 2012) is evidence of very recent dispersal and effective gene flow. *Cyclestheria hislopi* is known from the (sub)tropical regions of northern Australia (Timms 1986) and had never been recorded in the MDB before. *Cyclestheria hislopi* may have benefitted from two important factors: (1) no population of *C. hislopi* or other very closely related species existed in the central Paroo River catchment at the time, meaning that priority effects did not negatively affect the establishment of immigrating individuals, and (2) *C. hislopi* reproduces parthenogenetically, allowing new populations to be established even from a single specimen. For spinicaudatans and possibly other aquatic invertebrates with similar life history traits (e.g. obligate sexual with resting eggs), an important biogeographical break appears to separate north-eastern Australia. Dispersal rates to and from this region may not be high enough to overcome gene flow-limiting factors for these species.

Areas accommodating high genetic diversity may have been important long-term refugia during the climatic changes in the Pleistocene. Australia was not glaciated, although the cold/warm cycles corresponded to dry/wet phases, respectively (Martin 2006). In eastern Australia, the central Paroo River catchment and neighbouring parts of the Bulloo River catchment most likely constituted the most important refugial area for spinicaudatans and harboured the greatest genetic diversity and differentiation of *Eocyzicus* and *Limnadopsis* species (Schwentner et al. 2012b). Another important refugial area was the north-eastern LEB, evidenced by its distinct set of species and intraspecific lineages (Schwentner et al. 2012b). Whether central Australia was a refugial area itself or whether it was colonized from other refugial areas remains unclear, as data stemming from Western Australia are still scarce. However, the available data do suggest a close affinity between central Australian haplotypes and those found in Eastern or Western Australia, which may indicate colonization from other refugial areas. The distinctiveness of the Western Australian 'large branchiopod' fauna in general (Timms 2012) also supports the long-term persistence of spinicaudatan species there.

The fact that the Australian *Eocyzicus* species have a closer phylogenetic relationship with *E. digueti* from North America than with the two Asian species is surprising and suggests a more recent exchange between Australia and North America than between Australia and Asia. A similar biogeographical link indicating long-distance intercontinental dispersal between Australia and North America was recently detected for *Triops* (Vanschoenwinkel et al. 2012).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Bayesian inference majority rule tree based on elongation factor 1 α.

Figure S2. Bayesian inference majority rule tree based on ITS2.

Table S1. List of each specimen’s registration number at the Australian Museum (Sydney) and all GenBank accession numbers.

Table S2. Occurrence and frequency of haplotypes for each studied population.

Table S3. Pairwise Φ_{ST} values among populations of several main *Eocyzicus* lineages.

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