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Species, ESUs or populations? Delimiting and describing morphologically cryptic diversity in Australian desert spring amphipods

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Abstract. Cryptic species are frequently being discovered in refugial habitats, such as desert springs and groundwater systems. Unfortunately, many of these taxa remain as unnamed entities years after their initial discovery. Recent advances in the use of molecular data and coalescent analyses allow DNA-based delimitation of species to move from single locus, tree-based methods to multilocus coalescent analyses. This study compares two DNA-based approaches to delimit species of putatively cryptic freshwater amphipods (Chiltoniidae) from desert springs in central Australia. In addition, a morphometric analysis of 11 characters was undertaken to determine whether the DNA-delimited species were morphologically distinguishable. The single locus method results in identification of lineages that are not supported as species under the multilocus coalescent analyses. We conclude that *Wangiannachiltonia guzikae* King, 2009, as currently circumscribed, represents six genetically distinct amphipod species, and we describe and name these species despite no clear diagnosable morphological differences. Critically, all of these newly recognised species have extremely limited distributions, which increases the biodiversity significance of their desert spring habitat.

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Introduction

Whether by ensuring that unrecognised species get protection, or that over-split taxa do not waste precious resources, correctly describing species plays a major role in conservation outcomes (Frankham *et al*. 2012). Cryptic species, those that are morphologically indistinguishable, therefore provide challenges for conservation. These species are readily identifiable using molecular studies (Brower 2010) and, while criticisms of DNA methods of taxonomy exist (e.g. Ebach 2011), molecular analyses have contributed significantly to the understanding of global biodiversity (Cook *et al*. 2010). However, there remains a large disconnection between the recognition of cryptic species using DNA, and delimiting and describing taxa, as many authors have not ascribed names to their newly found species (including studies by us, e.g. Murphy *et al*. 2013). Consequently, many taxa remain undescribed (Goldstein and DeSalle 2011), potentially impeding critical conservation management.

Recent analytical advances utilising the coalescent (Fujita *et al*. 2012) have enabled DNA-based species delimitation to move away from threshold measures, such as degree of genetic divergence and the phylogenetic species concept. The use of the coalescent for species delimitation provides solid, objective-based criteria that are philosophically rooted in the process of lineage diversification. Coalescent theory provides a framework to determine the shape and pattern of trees to identify species as independently evolving lineages, thus fitting the assumptions of many species concepts.

Recent studies in Australian groundwater systems have identified a suite of previously unknown evolutionary diversity, including many cryptic species, dramatically increasing the importance of these water sources for preserving unique biodiversity (e.g. Leys *et al*. 2003; Cooper *et al*. 2007; Juan *et al*. 2010; Harvey *et al*. 2011; Murphy *et al*. 2013). Most of these cryptic taxa have incredibly small distributions, and therefore are inherently at risk of extinction. So the discovery of this fauna has had a dramatic impact on the conservation of groundwater fauna in Australia, and on the use of groundwater resources (Nevill *et al*. 2010). Common to most of these studies is the use of molecular information to describe the diversity, and also that many of the cryptic taxa remain undescribed and therefore not formally recognised as species.

One of the major reasons for the lack of formal description in these studies of groundwater biota is the greater emphasis on DNA-based methods than on traditional morphological

approaches, and the general reluctance to use only DNA data for species descriptions (Cook *et al*. 2010). For the few Australian groundwater species that have been studied morphologically, differences have been found indicating that the species are not as cryptic as originally believed (e.g. King *et al*. 2012). For many of these taxa, morphological differences are limited to species that have been evolving independently for tens of millions of years (Abrams *et al*. 2013; Murphy *et al*. 2013). However, for many of the species-groups that appear to have evolved allopatrically, under the same selective pressures (i.e. non-adaptive radiation), no clear morphological differentiation is evident (e.g. King 2009) despite millions of years of independent evolution. For these taxa, formal description is unlikely without information provided from DNA data.

A compelling need for a coalescent-based taxonomy is in the diverse chiltoniid amphipod crustaceans inhabiting artesian desert springs in central Australia. These amphipods are endemic to the springs of the Great Artesian Basin and represent lineages that evolved before the formation of their desert spring habitat (Murphy *et al*. 2009). Initially considered to be a single species, molecular studies using nuclear and mitochondrial genes demonstrated several reciprocally monophyletic amphipod lineages that have been identified as evolutionary significant units (ESUs), including some restricted to single, small, degraded springs (Murphy *et al*. 2013). Morphological systematic studies have presently distinguished significant variation only between the lineages that diverged ~20 million years ago (King 2009), and the majority of ESUs are allopatric, making it difficult to apply a strict species concept of reproductive isolation. The phylogenetic species concept (PSC) could be used to delimit these desert spring amphipods but, with a significant level of genetic divergence also evident within each ESU, assigning species under the PSC is subjective.

This paper takes advantage of recent developments in multi-species coalescent methods to delimit and then describe species of potentially morphologically cryptic, yet genetically divergent, amphipods from the Lake Eyre desert springs. In addition, detailed analyses of multidimensional morphological data were undertaken to confirm the cryptic nature of these newly described species.

Materials and methods

Population sampling

Murphy *et al*. (2013) identified 13 ESUs among amphipods from the Lake Eyre group of desert springs fed by the Great Artesian Basin (GAB springs) in central Australia (Fig. 1*A*). We focus here on the four ESUs (and populations within them) identified within *Wangiannachiltonia guzikae* King, 2009 (WG1–4). The Lake Eyre desert springs system is divided into geographic clusters or spring complexes, which can be further divided into spring groups (Ponder *et al*. 1995), comprising all spring vents around a single area of geological weakness. The four ESUs within *W. guzikae* are distributed allopatrically around the southernmost spring groups (Fig. 1*B*) and, within them, 13 distinct allopatric populations have been identified using mitochondrial DNA (Fig. 1*C*). This nested series of ESUs and populations is ideal for applying coalescent species delimitation, which is best suited to the comparison of population versus species evolutionary processes (Fujita *et al.* 2012).

Molecular methods

Sequence data were collected for nine loci: one mitochondrial (cytochrome oxidase 1, COI) (from Murphy *et al*. 2013) and eight anonymous nuclear loci (ANL) (GenBank accession numbers KT221634–KT221775) (Table S1, available as 'Supplementary material' on the journal website). Anonymous markers were developed following the method of Karl and Avise (1993). The choice of loci was based on polymerase chain reaction (PCR) amplification reliability, not levels of variability, so as to not introduce an ascertainment bias (e.g. Wakeley *et al*. 2001). Polymerase chain reaction amplifications were carried out in 25 µL containing PCR buffer, 0.2 mM of each dNTP, 0.4 µM of each primer (see 'Supplementary material' for primer information), 2 mM of $MgCl_2$, 0.5 units of AmpliTaq Gold DNA Polymerase (Thermo Fisher (www.thermofisher.com), formally Applied Biosystems) and 25–100 ng of genomic DNA. Thermocycling conditions were: an initial denaturation step of 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, an annealing temperature of 50°C for 30 s, and an extension temperature of 72°C for 30 s. Sequencing reactions were performed using ABI Big Dye Terminator Chemistry and fragments were resolved on an ABI 3700 sequencer (www.appliedbiosystems.com). From the nuclear genotypes, haplotype phases were reconstructed using the DnaSP v. 5 (Librado and Rozas 2009) implementation of PHASE 2.1 (Stephens and Donnelly 2003), keeping the most probable haplotypes for subsequent analyses.

For each locus, the number of segregating sites (s), genetic diversity (π), haplotype diversity (h) and assumptions of neutrality (using Tajima's *D*) were computed with DnaSP v. 5.0 for the entire dataset. Models of molecular evolution were chosen for each locus using jModelltest (Posada 2008) and using the Akaike information criteria to discriminate among models (Table 1). Gene trees for each locus were estimated using maximum likelihood analyses implemented in PHYML (Guindon *et al*. 2010) and robustness assessed using 500 bootstrap pseudoreplicates.

Bayesian species delimitation

Bayesian species delimitation was conducted using Bayesian phylogenetics and phylogeography (BPP 2.1) (Yang and Rannala 2010) with the full phased dataset for the nine loci. Bayesian phylogenetics and phylogeography performs a multilocus, coalescent-based approach that includes prior information about population size and divergence times using reversible jump Markov chain Monte Carlo (rjMCMC) to estimate the posterior distribution for different species delimitation events (Yang and Rannala 2010) associated with each bifurcation in a guide tree. The BPP analyses result in an estimation of the speciation probability for a branching event in the guide tree.

Bayesian phylogenetics and phylogeography requires a guide tree to test models of speciation, which first requires the selection of independent populations. To limit the influence of the choice of populations on the guide tree and subsequent species

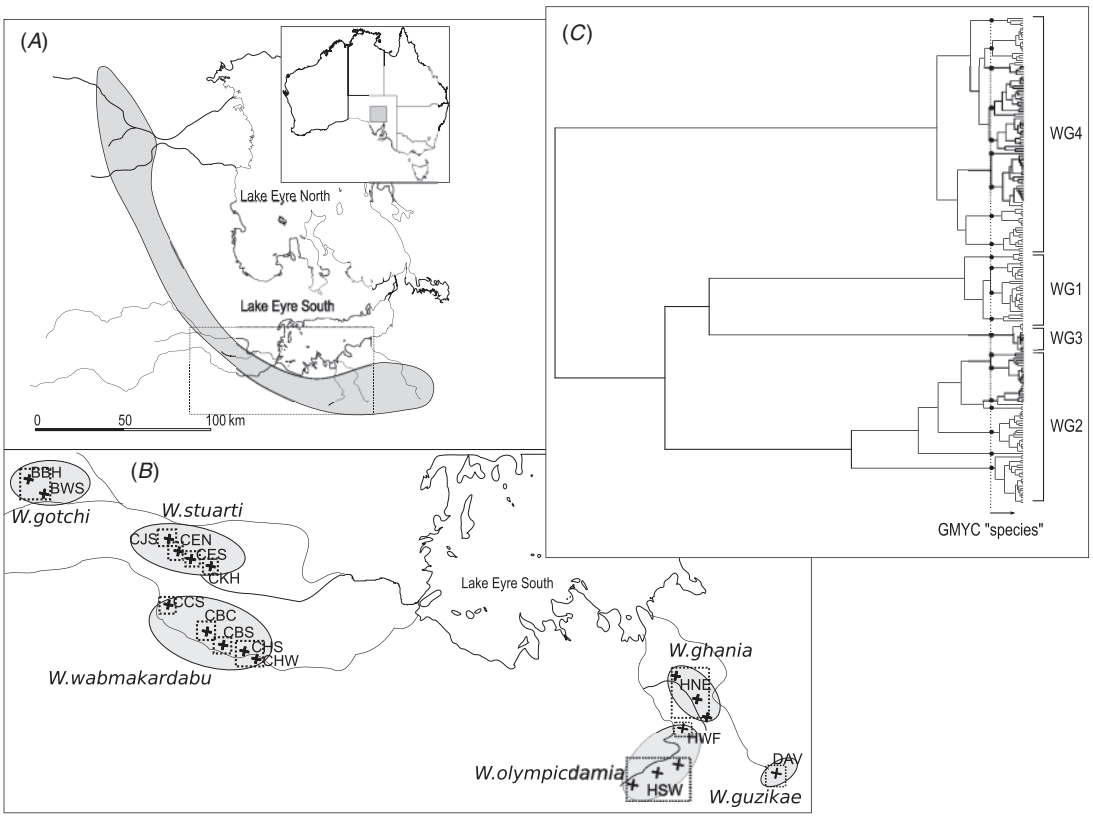

Fig. 1. Map of study sites, distribution and identification of previously identified evolutionary significant units (ESUs) of desert spring amphipods. (*A*) Study area showing locations of Lake Eyre desert springs (shaded area) and location within Australia (inset). (*B*) Distribution of previously identified amphipod ESUs (shaded ovals), and reciprocally monophyletic spring populations (dotted boxes). BBH, Beresford springs; BWS, Warburton Springs; CJS, Jersey Springs; CEN, Elizabeth north; CES, Elizabeth south; CKH, Kewson Hill; CCS, Coward Springs; CBC, Blanche Cup; CBS, Butttercup Springs; CHS, Horse Springs; CHW, Horse West; HNE, north-eastern Hermit Hill springs (i.e. Hermit, Old Woman and Old Finniss springs); HWF, west Finniss Springs; HSW, south-western Hermit Hill springs (i.e. Dead Boy, Sulphuric and Bopeechee springs); DAV, Davenport Springs. (*C*) Mitochondrial cytochrome oxidase 1 phylogenetic tree (modified from Murphy *et al*. 2013) showing the identified ESUs and the demarcation (dotted line) of the 21 'species' (circles) identified using a generalised mixed Yule-coalescent (GMYC) analysis. Scale in millions of years.

Table 1. Overall genetic diversity summary statistics and model of substitution used for each locus

COI, Cytochrome Oxidase Subunit 1; Bp, base pairs; s, number of segregating sites; *h*, haplotype diversity; π, nucleotide diversity; *D*, significance of Tajima's *D*; model, substitution model; GTR, General Time Reversible; HKY, Hasegawa, Kishino and Yano; +G, Gamma correction

Locus	bp	s	*h*	π	*D*	Model
COI	573	168	0.992	0.079	ns	GTR+G
1307	438	90	0.94	0.027	ns	HKY+G
1309	172	53	0.987	0.06	ns	GTR+G
1313	561	154	0.981	0.029	ns	GTR+G
1315	276	53	0.904	0.019	ns	HKY+G
1317	501	60	0.922	0.017	ns	HKY
1319	519	112	0.908	0.026	ns	HKY+G
1321	692	87	0.941	0.042	ns	HKY
1323	205	49	0.915	0.028	ns	HKY+G

delimitation, two sets of guide trees were examined. First, a guide tree containing only the four previously identified ESUs (WG1–4) was examined, and second, a more complex model treating each of the 13 allopatric populations (hereafter referred to as 13pop) as independent evolutionary units. BEAST v. 1.6 (Heled and Drummond 2010) was used to estimate species trees using COI and the phased nuclear alleles, generating posterior samples of the nine individual gene trees using an unlinked substitution model (Table 1), and the overall species trees. Each analysis was repeated twice for a total of 100 million generations, excluding the first 10% as burn-in, and convergence was determined by examining trace files.

Bayesian phylogenetics and phylogeography analysis of both the ESU and 13pop guide trees was undertaken by running multiple rjMCMC analyses for 500 000 generations with a burn-in of 10 000 from different starting seeds to check for consistency. Adequate rjMCMC mixing was achieved using algorithm 0 and the fine-tuning parameter = 15.0. As the prior distribution of ancestral population size (θ) and root age (τ_0) can affect the posterior probabilities, and because it is likely that the potential speciation events within these amphipods range from old to relatively recent, we used three different combinations of priors: large ancestral population size and deep divergence (θ~G (1, 10), τ_0~G(1, 10)); small ancestral population size and shallow

divergence (θ~G(2, 2000), τ_0~G(2, 2000)); and a combination of large ancestral population size and shallow divergence (θ~G(1, 10), τ_0~G(2, 2000)).

Missing data and guide tree implications

Not all loci were amplifiable in all lineages. The impacts of these missing data were tested by running BPP analyses of the most complete population dataset (i.e. no missing populations) for both the 4pop and the 13pop guide trees, and comparing the results with the complete locus set (i.e. with some loci/population combinations missing). Incorrect guide trees may lead to incorrect species delimitation (Leaché and Fujita 2010), so BPP analysis was also undertaken on all alternative guide trees for the 4pop model. For the 13pop model, relationships among the inter-lineage populations were analysed separately (e.g. within the four populations of the Coward, Elizabeth and Southern groups respectively) to minimise the computational time required for examining alternative tree topologies.

Alternative examination of Bayesian delimitation

Species defined by the single locus general mixed Yule-coalescent (GMYC) model (Pons *et al*. 2006) were compared with the BPP analyses. Analyses using GMYC are locus-specific and most reported studies to date have used COI datasets alone, but it has been shown that species inferences using this method can be misleading (Carstens *et al*. 2013). The GMYC model tests whether both the mixed Yule (i.e. species branching) and coalescent (i.e. within population branching) models can be fitted to an ultrametric phylogenetic tree and infers species differences from the estimated switch from inter- to intra-species branching. This analysis was undertaken as per Murphy *et al*. (2013) based on ultrametric trees estimated under gene-specific model parameters, with a relaxed uncorrelated log-normal clock and branch lengths estimated using a coalescent (constant size) prior.

Morphological methods

We used 11 morphological characters, including those commonly used to distinguish between chiltoniid species and those outlined by King (2009) in the original description of *Wangiannachiltonia guzikae* (i.e. lengths of antennae, male gnathopod 2 and uropod; Table S2), to examine whether the species identified using the DNA-based methods outlined above were morphologically cryptic. Chiltoniid amphipods exhibit slight sexual dimorphism, more apparent in the second gnathopod, which, in males, is robust for grasping females during sex, and in uropods 1 and 2, in which the rami can be shorter and more robust in large males. Unless eggs are visible within the marsupium, females are difficult to distinguish from juveniles and so analyses were restricted to adult males only (determined by presence of penes and robust gnathopod 2 development). Analyses were based on 359 individuals from three discrete springs (herein defined as an individual sample) per each putative cryptic species (determined by the molecular results), covering the range of the *W. guzikae* species complex. For each population, 14–24 individuals were measured, with sample sizes restricted by the number of males available in existing collections at the South Australian Museum. Measurements were taken using a camera lucida at 20× magnification. Total body length (TL) was measured through the midline of the animal, from the head to the telson.

Morphological analyses

Morphological analyses were undertaken in the R statistical environment (R Development Core Team 2011). We used principal component analysis (PCA) to assess the structure of variation in the ratios of body measurements among amphipods from the 18 populations examined above. Data were log-transformed and mean-centred (on the geometric mean) before analysis. The data were scaled by a measure of isometric size giving equal weight to all body measurements in the analysis. We used the PCA ratio spectrum (Baur and Leuenberger 2011) to interpret the results of the analysis in terms of body ratios that represent shape differences. This method displays the body measurements as points along a spectrum where the distance between them indicates their ratio; measures distant from one another have large ratios and contribute substantially to the principal component. The statistical stability of the ratio comparisons was assessed using 95% bootstrap confidence intervals (1000 replicates); measures separated by a distance greater than the empirical confidence intervals of each measure are statistically distinct. Additionally, we examined the influence of allometric relationships with body ratios using the allometry ratio spectrum (Baur and Leuenberger 2011).

Results

Gene trees

The amphipod ESUs WG1 and WG4 were clearly defined and well supported in the majority of the nine gene trees (Fig. S1), although the relationships among them varied. In contrast, WG2 was found to be monophyletic in only two of the nine gene trees (COI, ANL-1313) and, in most trees, exhibited longer branch lengths and greater haplotype diversity (Fig. S1) than the other three populations. WG3 showed a lack of genetic diversity, with identical genotypes across individuals for the majority of loci except at COI and ANL-1309.

Among the 13 allopatric populations defined by the COI tree (13pop dataset) (Fig. 1*C*), only the population from Davenport (DAV) springs was monophyletic in analyses of nuclear genes (ANL-1313 and ANL-1323 only). None of the other allopatric populations found in the COI tree were recovered as monophyletic in analyses of the nuclear genes.

Species tree inference

The BEAST analysis of the ESU model (4pop) (Fig. S2*A*) resulted in strong support for a clade comprising WG1, WG2 and WG3 (0.98 posterior probability (PP)), separate from WG4. Within this clade, WG2 and WG3 were estimated as sisters but without support (PP = 0.65). The 13pop model (Fig. S2*B*) supported the same relationships among these ESUs, and provided strong support (PP ≥ 0.98) for the monophyly of the spring groups within each of them. Relationships among the WG4 spring groups shows that the placement of the north-eastern Hermit Hills (HNE) populations had low support (PP ≥ 0.78), while there was strong support for the relationship between West Finniss (HWF) and south-western Hermit Hills (HSW) populations

(PP = 1.0), whereas relationships among the spring groups in WG2 (PP~0.60) and WG1 (PP = 0.41) were not supported.

Bayesian species delimitation

All iterations of the ESU (4pop) species tree resulted in speciation probabilities (i.e. the probability that a branch in a tree equals a speciation event) of 1.0 for each ESU (Fig. 2*A*). Missing data, alternative prior distributions, and different guide trees had no impact on the final result; therefore, this analysis clearly supports the delineation of these ESUs as species. For the 13pop model (Fig. 2*B*) (i.e. including populations within each of the ESUs), again all four ESUs have speciation probabilities of 1.0; however, within these ESUs, species delineation is dependent on the prior distributions. Using a speciation probability of 0.95 as a minimum requirement for support, there is no support for speciation events among the allopatric populations within WG1 (independent of alternative guide trees and prior distributions). Within WG2, the prior distributions have a large impact on delineation of populations, changing from zero delineation (i.e. WG2 as just one species) to total species delineation (all four populations as separate species). Within WG4, delineation of both the DAV and HNE populations was supported by all prior distributions, while species delineation between the HWF and HSW populations in Hermit Hills was not supported. Six species from the *W. guzikae* species complex are supported by all prior distribution iterations: WG1 (comprising CCS, CBC, CBS and CHW) (*W. wabmakadarbu*, sp. nov.); WG2 (comprising CEN, CES, CJS and CKH) (*W. stuarti*, sp. nov.); WG3 (comprising BBH) (*W. gotchi*, sp. nov.); and three within WG4 – DAV (*W. guzikae sensu stricto*), HNE (*W. ghania*, sp. nov.) and HSW and HWF combined (*W. olympicdamia*, sp. nov.).

General mixed Yule-coalescent

In contrast to the Bayesian species delimitation, the GMYC analysis of the COI dataset for *W. guzikae* (Fig. 1*C*) divides this group into 21 putative species: WG3, four species within WG1, seven within WG2, and nine within WG4.

Morphological analyses

There is no clear morphological distinction between the six species recognised by the Bayesian species tree analyses (Figs 3, S3; Tables S2, S3). Statistically stable ratios (showing that the largest eigenvalues are sufficiently distinct) explain the first principal component (Fig. 3*A*), but not the second component (Fig. 3*B*), while allometric comparisons are consistent with ratios identified for PC1 (Fig. 3*C*). More specifically, there is some discrimination along the first principal component (antenna 1 length and uropodal rami length ratios had the greatest influences, see Table S2), between the three species identified from the southern springs (WG4: *W. ghania*, sp. nov., *W. guzikae*

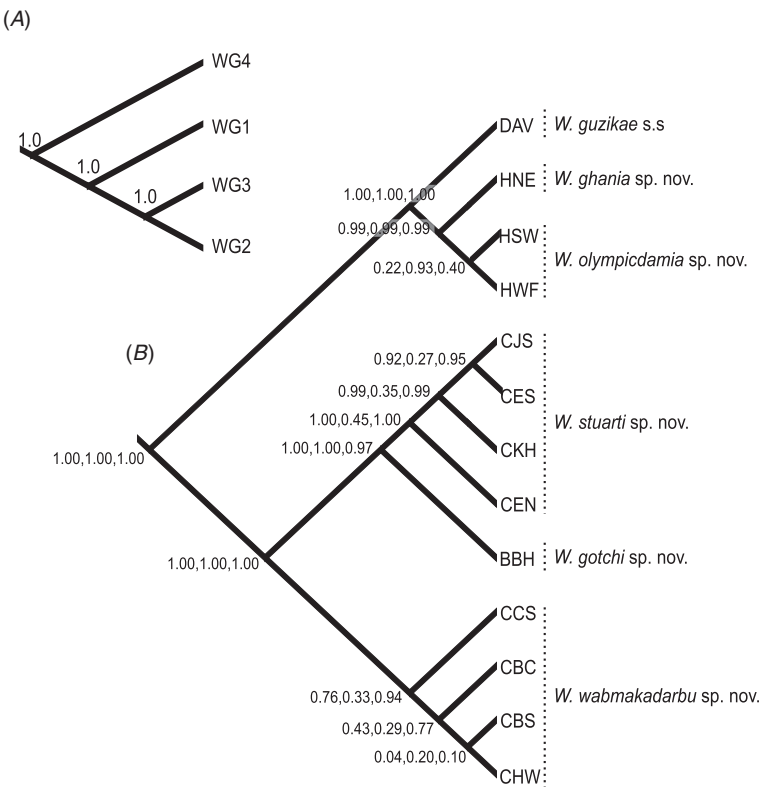

Fig. 2. Results of Bayesian species delimitation of (*A*) the '4pop' (i.e. among the ESUs) and (*B*) '13pop' (i.e. among the spring groups) models. The speciation probabilities are provided for each branching event under the combination of priors for θ and τ_0: left, prior means = 0.1; middle, prior means = 0.001; right, prior mean θ = 0.1, prior mean τ_0 = 0.001. For the '4pop' tree, all priors resulted in posterior probabilities of 1.0.

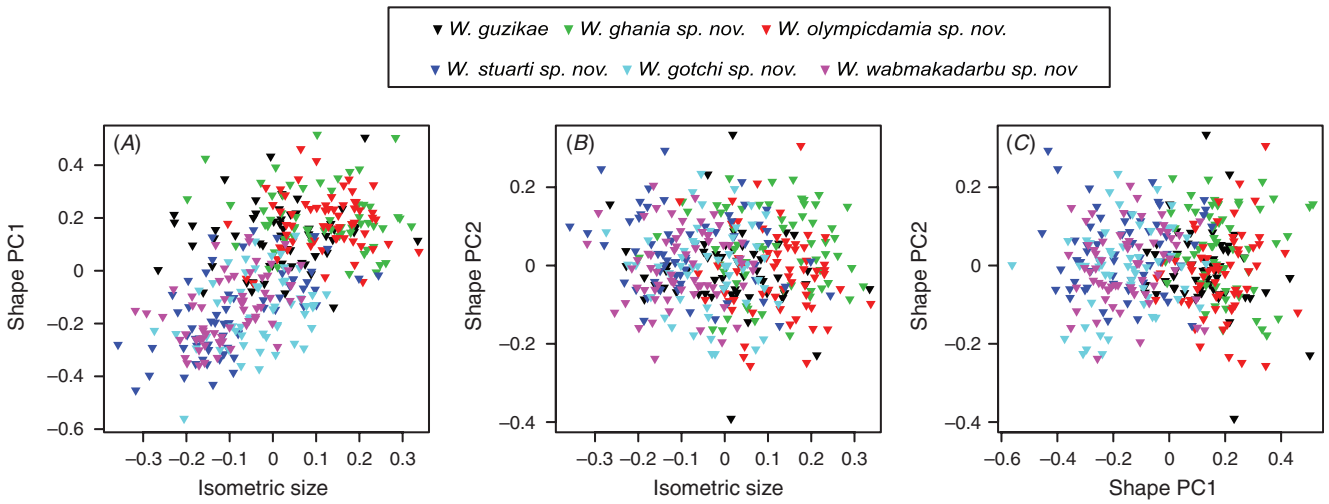

Fig. 3. Scatterplots of isometric size versus (*A*) the first and (*B*) the second principal component of amphipod shape, and (*C*) the scatterplot of the first and second principal components. The average size of *Wangiannachiltonia ghania*, sp. nov. and *W. olympicdamia*, sp. nov. is greater than *W. stuarti*, sp. nov., *W. gotchi*, sp. nov. and *W. wabmakadarbu*, sp. nov., whereas *W. guzikae* is not distinguishable from either group.

and *W. olympicdamia*, sp. nov.) and those from the more northerly springs. (WG1–3: *W. gotchi*, sp. nov., *W. stuarti*, sp. nov., *W. wabmakadarbu*, sp. nov.). Also, it is possible to distinguish between these two broad species groupings based on the average size of mature males, with *W. ghania*, sp. nov. and *W. olympicdamia*, sp. nov. generally much larger than *W. gotchi*, sp. nov., *W. sturti*, sp. nov. and *W. wabmakadarbu*, sp. nov. (~2.9–3.1 mm versus ~2.3–2.4 mm). However, there is still some overlap between the groups, and *W. guzikae* remains indistinguishable from the other species with its broad range of mature male sizes (Fig. S3). Within the populations analysed, the variability of morphological ratios is minimal (~1–5%), indicating that morphology (measurements of individual specimens) is not a discriminating method for diagnosing these species, even with measurements carried out on a large scale, as was done here. Thus, identification to species level could not be carried out using these characters (antennae lengths, uropodal lengths, gnathopod shape), which are usually species-level variables within the Chiltoniidae (e.g. King *et al.* 2012), and the putative species within the *W. guzikae* complex remain morphologically cryptic.

Discussion

Using a multispecies coalescent approach with multiple genetic markers, we recognise six morphologically cryptic species of endemic amphipods from the desert springs south-west of Lake Eyre in central Australia (Fig. 1*B*). All of these newly identified species are distributed allopatrically and have very small distributions. Due to the small ranges, it is possible that all six species will qualify for protection under the *Australian Environmental Protection and Biodiversity Conservation Act* (EPBC) as a range of <10 km^2 can lead to a designation of critically endangered. The most extreme of these small distributions is that of *W. gotchi*, sp. nov. from the Beresford spring group, which consists of only three flowing springs, all of which show signs of habitat degradation (N. Murphy, pers. obs.).

Molecular species delimitation is not new and is regularly used in understanding microbiological communities (Hughes *et al.* 2001) and, as demonstrated in this study and others (e.g. Cook *et al.* 2010; Leaché and Fujita 2010; Jörger and Schrödl 2013), should also command a more prominent place in the delineation of complex organisms, particularly those that are difficult to discriminate morphologically (Jörger and Schrödl 2013). However, it is critical that molecular delineation be undertaken in a rigorous manner. In particular, the use of any single-locus dataset to delimit species is fraught with potential error because of the risk of relying on the phylogenetic reconstruction of gene history, as opposed to lineages (Carstens *et al.* 2013). This is demonstrated by our analyses using a single-locus GMYC test, which supported the recognition of 21 species. This is in line with Carstens *et al.*'s (2013) finding that the GMYC method is prone to over-splitting, particularly if species are structured into populations with no recent gene flow (e.g. Lohse 2009), which is clearly the case in these desert springs (Robertson *et al.* 2014). The coalescent approach captures the evolutionary assumptions of the biological species concept (reproductive isolation and independent evolutionary lineages), and provides an objective means for DNA-based species discovery (Fujita *et al.* 2012). In this new age of sequencing, where multiple locus data will eventually be as easy to obtain as a COI barcode or a morphological description, coalescent-based species delimitation methods should be used more frequently to provide an independent test for any species hypothesis.

In principle, we agree that morphologically based species diagnoses should take place alongside comprehensive DNA analyses, as this allows for multiple sources of evidence to determine species boundaries, and provides tools for field-based identification (Dayrat 2005). However, morphological diagnoses are not possible in these desert spring amphipods. There is some evidence of morphological differentiation at the deeper evolutionary levels, but miniscule change has occurred despite millions of years of independent evolution (Murphy *et al.* 2013). This apparent morphological stasis has been hypothesised

for several taxa inhabiting this type of environment (Guzik *et al*. 2012). Thus, these results provide support for a non-adaptive radiation (Rundell and Price 2009) in these desert springs, as speciation has occurred despite strong stabilising selection pressures that have constrained phenotypic evolution.

It is apparent that the delimited populations identified in this study represent non-interbreeding entities (species), on independent evolutionary trajectories, albeit morphologically cryptic. Critically, the species identified and described in this study live in a degraded habitat, where there is increasing pressure to use the water that enables their persistence for human activities (e.g. Fensham *et al*. 2011). This is of particular importance given that only *W. stuarti*, sp. nov. and *W. wabmakadarbu*, sp. nov. are found within protected areas (Wabma Kadarbu Mound Springs Conservation Park). The newly recognised species described below provide further evidence for the increasingly strong case that the biodiversity of the desert springs of the Lake Eyre Basin has been greatly underestimated.

Systematics

Given that the newly recognised species are morphologically cryptic, the previous morphological description of *Wangiannachiltonia guzikae* now stands as the morphological description for the six species within the *W. guzikae* species complex, and the definition of *W. guzikae sensu stricto* is now based on a much narrower distribution. Our newly described species can be diagnosed using both DNA synapomorphies and geographic data, in combination with the morphological diagnoses of the entire species complex.

Infraorder **TALITRIDA** Rafinesque, 1815

Superfamily **TALITROIDEA** s.s. Rafinesque, 1815

Family **CHILTONIIDAE** Barnard, 1972

Genus ***Wangiannachiltonia*** King, 2009

Wangiannachiltonia guzikae King, 2009 cryptic species complex

Composition. *W. ghania*, sp. nov., *W. gotchi*, sp. nov., *W. guzikae* King, 2009 (type species), *W. olympicdamia*, sp. nov., *W. stuarti*, sp. nov., *W. wabmakadarbu*, sp. nov.

Morphological diagnosis (based on mature males)

As for generic diagnosis (King 2009), this diagnosis is also relevant for all species within the complex.

Coxae 1–4 longer than pereon segments are deep. Pereopod 4 coxa with distinct proximal excavated corner. Uropod 1 inner ramus with robust seta at mid-length, outer ramus without seta at mid-length. Uropod 2 peduncle dorsal margin with few scattered setae along entire length; inner ramus with robust seta at mid-length, outer ramus without seta at mid-length. Gnathopod 2 propodus without proximal lobe covering distal margin of carpus; ventral-distal corner marked with a distinct robust seta on each of the inner and outer faces (larger than those along ventral distal margin (adjacent to dactylus).

Morphological description

Male

Head about as long as deep. Antenna 1 peduncular article 1 twice as long as broad; peduncular article 2 ~0.75× length of article 1, twice as long as broad; peduncular article 3 similar length to article 2, twice as long as broad; flagellum similar length to peduncle, of five articles, with one ventral aesthetasc on the proximal margin of the last two articles. Antenna 2 ~0.7× length of antenna 1; peduncular article 1 broader than long; peduncular article 2 longer than article 1, slightly longer than broad; peduncular article 3 longer than article 2, twice as long as broad; flagellum similar length to peduncle, of five articles.

Upper lip slightly broader than long, apically bluntly rounded, with numerous short setae along apical margin. Lower lip with rounded lateral lobes, apical margins rounded, apical and inner margins with numerous short setae. Left mandible with incisor of five teeth, *lacinia mobilis* of four teeth, spine row of three plumose setae and triturative molar. Right mandible with incisor of six teeth, *lacinia mobilis* of three teeth, spine row of two plumose setae and triturative molar with a long plumose seta. Maxilla 1 outer plate with seven setulate robust setae; inner plate with two long apical plumose setae. Maxilla 2 outer plate with an apical row of 15 simple setae; inner plate with an apical row of 16 simple setae, with a plumose seta on the inner lateral margin below the apical row. Maxilliped inner plate apical margin with three short spine-like robust setae, with plumose seta along apical and inner lateral margins; outer plate with numerous simple setae along apical and inner lateral margins; palp articles 1 and 2 similar width, palp article 2 with numerous simple setae on inner lateral margin; palp article 3 not as broad as articles 1 and 2, with numerous simple setae on inner lateral and distal margins; palp article 4 short, about half as broad as article 3, with unguis and seta on distal margin.

Gnathopod 1 coxa distal margin with 14 short simple setae, ventral margin with two plumose setae at mid-length; basis, ischium, and merus ventral margins with setae; carpus with ventral-lateral lobe and row of nine setulate setae becoming longer distally, dorsal-distal margin with long setulate setae, inner face with a single plumose seta; propodus triangular in shape, around 1.5× as long as broad, ventral-distal corner with two robust setae (near where tip of dactylus touches), ventral-distal margin (adjacent to dactylus length) with long simple and short plumose setae, dorsal-distal margin with long simple setae, inner face with three robust plumose setae; dactylus curved, fitting just behind ventral-distal corner of propodus, with dorsal plumose seta. Gnathopod 2 coxa distal margin with nine short simple setae, ventral margin with three plumose setae at mid-length; basis dorsal and ventral margins with setae; ischium and merus with scattered setae on ventral margins; carpus dorsal-distal margins with long setulate setae; propodus about as long as broad, without proximal lobe covering distal margin of carpus (see *Arabunnachiltonia murphyi* King, 2009), ventral-distal corner marked with a robust seta on each of the inner and outer faces, ventral-distal groove present on inner face to accommodate the tip of the dactylus, ventral distal margin with numerous apically bifid robust setae (not as robust as those marking the ventral-distal corner). Pereopod 3 coxa distal margin with nine short simple setae; basis and ischium

dorsal and ventral margins with setae; merus with strong dorsal-distal lobe, dorsal margin with plumose seta, dorsal-distal margin with long plumose setae, ventral-distal margin with long simple setae; carpus ventral margin with two apically bifid robust setae and scattered simple setae; propodus ventral margin with five apically bifid robust setae in four groups (distal seta smallest); dactylus dorsal margin with plumose seta, ventral margin with simple seta, unguis present. Pereopod 4 coxa with distinct proximal excavated corner, distal margin with 18 short simple setae; basis and ischium dorsal margins with setae; merus with strong dorsal-distal lobe, dorsal margin with long simple setae; carpus ventral margin with two apically bifid robust setae and scattered simple setae; propodus ventral margin with six apically bifid robust setae in four groups (distal seta smallest); dactylus dorsal margin with plumose seta, ventral margin with simple seta, unguis present. Pereopod 5 coxa ventral margin with six short simple setae; basis about as long as broad, dorsal margin with four apically divided robust setae along length, distal end of dorsal margin with two robust setae, ventral margin subtly crenulated and with eight short simple setae along length; ischium dorsal margin with distal robust setae; merus with strong postero-distal lobe, dorsal margin with robust setae in three clusters, ventral margin with robust setae in three clusters; carpus shorter than merus, dorsal margin with robust setae in two clusters, ventral margin with robust setae in one distal cluster; propodus similar length to merus, dorsal margin with five apically divided robust setae in four clusters (distal seta smallest), distal margin with long simple setae; dactylus with plumose seta on ventral margin, unguis present. Pereopod 6 coxa ventral margin with five short simple setae; basis slightly longer than broad, dorsal margin with five apically divided robust setae along length, distal end of dorsal margin with two robust setae, ventral margin subtly crenulated and with nine short simple setae along length; ischium dorsal margin with distal robust setae; merus with strong postero-distal lobe, dorsal margin with robust setae in three clusters, ventral margin with robust setae in three clusters; carpus shorter than merus, dorsal margin with robust setae in two clusters, ventral margin with robust setae in one distal cluster; propodus similar length to merus, dorsal margin with five apically divided robust setae in four clusters (distal seta smallest), distal margin with long simple setae; dactylus with plumose seta on ventral margin, unguis present. Pereopod 7 coxa margin with five short simple setae; basis about as long as broad, dorsal margin with four apically divided robust setae along length, distal end of dorsal margin with two robust setae, ventral margin subtly crenulated and with 14 short simple setae along length; ischium dorsal margin with distal robust setae; merus with strong postero-distal lobe, dorsal margin with robust setae in three clusters, ventral margin with robust setae in two clusters; carpus shorter than merus, dorsal margin with robust setae in three clusters, ventral margin with robust setae in one distal cluster; propodus similar length to merus, dorsal margin with seven apically divided robust setae in four clusters (distal seta smallest), distal margin with long simple setae; dactylus with plumose seta on ventral margin, unguis present.

Pleopods 1–3 similar, unmodified (as in *Chiltonia*), peduncle inner margins with two distal retinacula (coupling hooks).

Uropod 1 peduncle distinctly longer than rami, dorsal margin with robust setae in three clusters along length; outer ramus distal margin with four robust setae of varying lengths; inner ramus with single seta at mid-length, distal margin with five robust setae of varying lengths. Uropod 2 peduncle similar length to rami, dorsal margin with four long robust setae; outer ramus slightly smaller than inner ramus, distal margin with four robust setae of varying lengths; inner ramus with two robust setae at mid-length, distal margin with five robust setae of varying lengths. Uropod 3 with one article, distal margin with one short robust seta and two long robust setae apically and one long seta subapically.

Telson slightly broader than long, apically blunt with robust setae at each distal corner.

Female

Similar morphology to male except for the following.

Antenna 1 flagellum of four articles. Antenna 2 flagellum of four articles.

Gnathopod 1 coxa distal margin with seven short simple setae, ventral margin with one plumose seta at mid-length; carpus with ventral-lateral lobe and row of six setulate setae becoming longer distally; propodus almost rectangular in shape, nearly twice as long as broad, ventral-distal corner with one robust seta (near where tip of dactylus touches), ventral-distal margin (adjacent to dactylus length) with long and short simple setae, dorsal-distal margin with long simple setae, inner face with one robust plumose seta; dactylus curved, fitting against ventral-distal corner of propodus, with dorsal plumose seta. Gnathopod 2 coxa distal margin with eight short simple setae; basis dorsal and ventral margins with setae; ischium and merus with setae on ventral margins; carpus with ventral-lateral lobe and row of six setulate setae becoming longer distally; propodus almost rectangular in shape, nearly twice as long as broad, ventral-distal corner with one robust seta (near where tip of dactylus touches), ventral-distal margin (adjacent to dactylus length) with long and short simple setae, dorsal-distal margin with long simple setae, inner face with one robust plumose seta; dactylus curved, fitting against ventral-distal corner of propodus, with dorsal plumose seta.

Uropod 3 distal margin with three long robust setae apically.

Oostegites present on coxae 2 to 5 to form the marsupium, margins with scattered curved hooks.

Remarks

Using primarily molecular evidence, six morphologically cryptic species across the Lake Eyre spring supergroup are now recognised from material previously identified as *W. guzikae*, which is now restricted to populations from Davenport and Welcome Springs (Fig. 1*B*).

Species of the *Wangiannachiltonia guzikae* cryptic species complex can be easily distinguished from the other mound spring chiltoniid, *Arabunnachiltonia murphyi*, by the presence of a distinct excavated proximal corner on coxal plate 4, gnathopod 2 propodus of males having a short propodus, uropod 1 peduncle with two to four dorsally scattered setae (not row of five to seven robust setae), and antenna 1 peduncular article 1 being smooth (lacking robust ventral seta).

There is a large degree of overlap in the size of adult males (Fig. S3); however, the average sizes (adult males) of both *W. ghania*, sp. nov. (2.9 mm) and *W. olympicdamia*, sp. nov. (3.1 mm) are greater than for *W. gotchi*, sp. nov., *W. stuarti*, sp.

nov. and *W. wabmakadarbu*, sp. nov. (2.3–2.4 mm each); the average size (males) of *W. guzikae* was ~3.0 mm, but it had the largest distribution of sizes and was indistinguishable from the other species in terms of body size (Fig. S3).

Wangiannachiltonia ghania Murphy & King, sp. nov.

W. guzikae (in part) King (2009): 11.
http://zoobank.org/zoobank.org:act:8833A487-9A4E-48D2-B7A1-AA8E221F56FE

Material examined

Holotype. ♂, SAM C8287, 2.88 mm, Old Finniss Springs, Hermit Hills springs complex, South Australia, 29°34.97′S 137°26.79′E, Coll. W. Zeidler, 10.vi.1983.

Allotype. ♀, SAM C8288, 2.50 mm, collected with holotype.

Additional material examined. SAM C6833, Old Finniss Springs, Hermit Hills springs complex, 29° 35.08′S 137° 27.0′E, Coll. W. Zeidler and K. Gowlett-Holmes, 21.ix.1989. SAM C8289, Old Finniss Springs, Hermit Hills springs complex, South Australia, 29° 35.08′S 137° 27.0′E, Coll. W.F. Ponder, 12.vi.1983. SAM C8290, Old Finniss Springs, Hermit Hills springs complex, South Australia, 29° 34.97′S 137° 26.79′E, Coll. W.F. Ponder, 10.vi.1983.

Diagnosis

This species includes all populations in the Hermit, Old Woman and Old Finniss spring groups from the Hermit Hills spring complex (Fig. 1*B*) in the Lake Eyre spring supergroup. Molecular diagnosis (COI gene, with reference to GenBank accession number HM068085): the combination of ‘C’ at base 393 and ‘C’ at base 569.

Etymology

Named for the historic Old Ghan railway, which followed the overland telegraph line through the Lake Eyre region where the mound springs were the only source of fresh water.

Wangiannachiltonia gotchi Murphy & King, sp. nov.

W. guzikae (in part) King (2009): 11.
http://zoobank.org/zoobank.org:act:ACB91F0D-7F18-4273-81BF-86A6DC184DF0

Material examined

Holotype. ♂, SAM C8303, 2.39 mm, Beresford Spring group, South Australia, 29° 16.0′S 136° 39.7′E, Coll. W. Zeidler, 10.ix.1981.

Allotype. ♀, SAM C8304, 1.73 mm, collected with holotype.

Additional material examined. SAM C6838, Beresford Spring, South Australia, 29° 16.0′S 136° 39.7′E, Coll. W. Zeidler and K. Gowlett-Holmes, 24.ix.1989. SAM C8305, Warburton Spring, South Australia, 29° 16.68′S 136° 40.31′E, Coll. W.F. Ponder, 7.vi.1983. SAM C8306, Warburton Spring, South Australia, 29° 16.68′S 136° 40.31′E, Coll. W. Zeidler, 7.vi.1983.

Diagnosis

This species includes all populations in the Beresford and Warburton spring groups from the Beresford spring complex (Fig. 1*B*) in the Lake Eyre spring supergroup. Molecular diagnosis (COI gene, with reference to GenBank accession number HM068085): ‘C’ at base 29, ‘T’ at base 74, ‘C’ at base 158, ‘C’ at base 167, ‘T’ at base 240, ‘A’ at base 242, ‘T’ at base 269, ‘T’ at base 275, ‘C’ at base 335, ‘A’ at base 413 and ‘A’ at base 545.

Etymology

Named for Travis Gotch, South Australian Arid Lands Natural Resource Management Board, who has provided invaluable assistance in our exploration of the mound springs.

Wangiannachiltonia guzikae King, 2009 *sensu stricto*

http://zoobank.org/zoobank.org:act:F76CF11A-DAD5-4188-83A7-CB2A21173C52

Material examined

Holotype. ♂, SAM C6829, 2.9 mm, Davenport Springs, South Australia, 29°39′44.388 S 137°35′ 6.7914 E, Coll. M. Guzik and N. Murphy, 31.x.1997.

Allotype. ♀, SAM C6830, 2.6 mm, collected with holotype. Paratypes, 3 ♂, 1 ♀, 1 juvenile, collected with holotype.

Other material examined. SAM C6831, Davenport Springs, South Australia, 29°40.09′S 137°36.31′E, Coll. W. Zeidler and K. Gowlett-Holmes, 21.ix.1989. SAM C6832, Welcome Springs, South Australia, 29°40.75′S 137°49.8′E, Coll. W. Zeidler and K. Gowlett-Holmes, 28.ix.1989.

Diagnosis

This species is now restricted to populations in the Davenport and Welcome springs groups from the Wangianna spring complex (Fig. 1*B*) in the Lake Eyre spring supergroup. Molecular diagnosis (COI gene, with reference to GenBank accession number HM068085): the combination of ‘A’ at base 182, ‘G’ at base 269, ‘T’ at base 293, ‘C’ at base 393 and ‘G’ at base 416.

Wangiannachiltonia olympicdamia Murphy & King, sp. nov.

W. guzikae (in part) King (2009): 11.
http://zoobank.org/zoobank.org:act:E605731A-2145-47C0-ADB6-636B369141DA

Material examined

Holotype. ♂, SAM C8291, 2.83 mm, west Finniss Springs, Hermit Hills spring complex, South Australia, 29°35.68′S 137°24.66′E, Coll. W. Zeidler, 9.vi.1983.

Allotype. ♀, SAM C8292, 1.98 mm, collected with holotype.

Additional material examined. SAM C6834, Bopeechee Spring, Hermit Hills spring complex, South Australia, 29°36.49′S 137°23.15′E, Coll. W. Zeidler and K. Gowlett-Holmes, 1989. SAM C6835, Dead Boy Spring, Hermit Hills spring complex, South Australia, 29°36.08′S 137°24.44′E, Coll. W. Zeidler and K. Gowlett-Holmes, 22.ix.1989. SAM C6836, west Finniss Springs, Hermit Hills spring complex, South Australia, 29°35.68′S 137°24.66′E, Coll. W. Zeidler and K. Gowlett-Holmes, 22.ix.1989. SAM C8293, west Finniss Springs, Hermit Hills spring complex, South Australia, 29°35.68′S 137°24.66′E, Coll. W.F. Ponder, 9.vi.1983. SAM C8294, Bopeechee Springs, Hermit Hills spring complex, South Australia, 29°36.49′S 137°23.15′E, Coll. W. Zeidler, W.F. Ponder, 9.vi.1983.

Diagnosis

This species includes all populations in the west Finniss, Dead Boy, Sulphuric and Bopeechee spring groups from the Hermit

Hills spring complex (Fig. 1*B*) in the Lake Eyre spring supergroup. Molecular diagnosis (COI gene, with reference to GenBank accession number HM068085): the combination of 'T' at base 407 and 'G' at base 416, or 'T' at base 194, 'C' at base 520 and 'G' at base 585, or 'C' at base 105 and 'C' at base 461.

Etymology

Named for the Olympic Dam mine, as this is the species located closest to the Olympic Dam bore field.

Wangiannachiltonia stuarti Murphy & King, sp. nov.

W. guzikae (in part) King (2009): 11.
http://zoobank.org/zoobank.org:act:77158139-8F03-4727-BCAC-698EDCA9678E

Material examined

Holotype. ♂, SAM C8299, 2.19 mm, Jersey Springs, Coward springs complex, South Australia, Coll. W. Zeidler, 15.v.1981.

Allotype. ♀, SAM C8300, 1.66 mm, collected with holotype.

Additional material examined. SAM C6837, Elizabeth Springs, South Australia, 29°21.36′S 136°46.30′E, Coll. W. Zeidler and K. Gowlett-Holmes, 24.ix.1989. SAM C8301, Jersey Springs, Coward springs complex, South Australia, 29°20.81′S 136°45.37′E, Coll. W.F. Ponder, R. Herschler, P. Winn, 5.ix.1983 (Mound Springs Survey Station Number 770B). SAM C8302, Kewson Hill, South Australia, 29°22.28′S 136°47.16′E, Coll. W.F. Ponder, R. Heschler, D. Bushell, 21.viii.1983 (Mound Springs Survey Station Number 741).

Diagnosis

This species includes all populations in the Elizabeth north, Elizabeth south, Jersey and Kewson Hill spring groups from the Coward spring complex (Fig. 1*B*) in the Lake Eyre spring supergroup. Molecular diagnosis (COI gene, with reference to GenBank accession number HM068085); 'A' or 'G' at base 86, 'G' or 'A' at base 467 and a combination of; 'G' or 'A' at base 107 and 'C' at base 128; or 'G' at base 119 but not 'T' at base 74; or 'T' at base 461 and/or 'C' at base 464, but not 'A' at base 455 or 'T' at base 74; or 'G' at base 632 or 'A' at base 626 but not 'T' at base 74.

Etymology

Named for John McDouall Stuart, who visited these springs several times during his expeditions.

Wangiannachiltonia wabmakadarbu Murphy & King, sp. nov.

W. guzikae (in part) King (2009): 11.
http://zoobank.org/zoobank.org:act:0D69E06B-438D-4208-A935-DFF1842C6CC8

Material examined

Holotype. ♂, SAM C8295, 2.07 mm, Little Bubbler Spring, Blanche Cup springs group, Coward springs complex, South Australia, 29°27.35′S 137°51.91′E, Coll. W. Zeidler, 27.xi.1983.

Allotype. ♀, SAM C8296, 1.75 mm, collected with holotype.

Additional material examined. SAM C6227, Coward Springs, Coward springs complex, South Australia, 29°24.78′S 136°47.28′E, Coll. W. Zeidler and K. Gowlett-Holmes, 23.iv.1989. SAM C8297, unnamed spring, Blanch Cup springs group, Coward springs complex, South Australia, Coll. W. Zeidler, 27.xi.1983. SAM C8298, Little Bubbler Spring, Blanche Cup springs group, Coward springs complex, South Australia, 29°27.35′S 137°51.91′E, Coll. W.F. Ponder, B. Jenkins, C. Wollard, 1.ix.1983 (Mound Spring Survey Station Number 744B).

Diagnosis

This species includes all populations in the Coward, Blanche Cup, Buttercup, Horse West and Horse spring groups from the Coward spring complex (Fig. 1*B*) in the Lake Eyre spring supergroup. Molecular diagnosis (COI gene, with reference to GenBank accession number HM068085): 'G' at base 20, 'G' at base 62, 'T' at base 86, 'G' at base 122, 'A' at base 215, 'T' at base 246, 'A' at base 326, 'G' at base 431, 'G' at base 518, and 'T' at base 608.

Etymology

Named for the Wabma Kadarbu Mound Springs Conservation Park, in which the springs are located.

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