A peer-reviewed version of this preprint was published in PeerJ on 17 January 2017.

View the peer-reviewed version (peerj.com/articles/2759), which is the preferred citable publication unless you specifically need to cite this preprint.

Luna-Ramirez K, Miller AD, Rašić G. (2017) Genetic and morphological analyses indicate that the Australian endemic scorpion *Urodacus yaschenkoi* (Scorpiones: Urodacidae) is a species complex. PeerJ 5:e2759
https://doi.org/10.7717/peerj.2759
Genetic and morphological analyses indicate that the Australian endemic scorpion *Urodacus yaschenkoi* (Scorpiones: Urodacidae) is a species complex

Karen Luna-Ramirez 1, Adam D Miller 2,3, Gordana Rašić Corresp. 3

1 Projektgruppe "Bioressourcen", Fraunhofer-Institut für Molekularbiologie und Angewandte Ökologie IME, Gießen, Hessen, Germany
2 Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Burwood, Victoria, Australia
3 School of BioSciences, Bio21 Institute, The University of Melbourne, Parkville, Victoria, Australia

Corresponding Author: Gordana Rašić
Email address: gordana.rasic@unimelb.edu.au

**Background.** Australian scorpions have received far less attention from researchers than their overseas counterparts. Here we provide the first insight into the molecular variation and evolutionary history of the endemic Australian scorpion *Urodacus yaschenkoi*. Also known as the inland robust scorpion, it is widely distributed throughout arid zones of the continent and is emerging as a model organism in biomedical research due to the chemical nature of its venom. **Methods.** We employed Bayesian Inference (BI) methods for the phylogenetic reconstructions and divergence dating among lineages, using unique haplotype sequences from two mitochondrial loci (*COXI*, *16S*) and one nuclear locus (*28S*). We also implemented two DNA taxonomy approaches (GMYC and PTP/dPTP) to evaluate the presence of cryptic species. Linear Discriminant Analysis was used to test whether the linear combination of 21 variables (ratios of morphological measurements) can predict individual’s membership to a putative species. **Results.** Genetic and morphological data suggest that *U. yaschenkoi* is a species complex. High statistical support for the monophyly of several divergent lineages was found both at the mitochondrial loci and at a nuclear locus. The extent of mitochondrial divergence between these lineages exceeds estimates of interspecific divergence reported for other scorpion groups. The GMYC model and the PTP/bPTP approach identified major lineages and several sub-lineages as putative species. Ratios of several traits that approximate body shape had a strong predictive power (83–100%) in discriminating two major molecular lineages. A time-calibrated phylogeny dates the early divergence at the onset of continental-wide aridification in late Miocene and Pliocene, with finer-scale phylogeographic patterns emerging during the Pleistocene. This structuring dynamics is congruent with the diversification history of other fauna of the Australian arid zones. **Discussion.** Our results indicate that the taxonomic status of *U. yaschenkoi* requires revision, and we provide recommendations for such future
efforts. A complex evolutionary history and extensive diversity highlights the importance of conserving *U. yaschenkoi* populations from different Australian arid zones in order to preserve patterns of endemism and evolutionary potential.
Title: Genetic and morphological analyses indicate that the Australian endemic scorpion *Urodacus yaschenkoi* (Scorpiones: Urodacidae) is a species complex

Authors: Luna-Ramírez K\(^a\), Miller AD\(^{b,c}\), Rašić G\(^b\)

\(^a\) Museum Victoria, 11 Nicholson St., Carlton Gardens, Melbourne, VIC 3053, Australia. (Current affiliation: Fraunhofer-Institut für Molekularbiologie und Angewandte Oekologie IME, Projektgruppe "Bioressourcen" Heinrich-Buff-Ring 58/62, Gießen 35392; Germany).

\(^b\) Pest and Environmental Adaptation Research Group, School of BioSciences, The University of Melbourne, Victoria 3010, Australia.

\(^c\) Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Victoria 3280, Australia.

*Corresponding author: Gordana Rašić, Pest and Disease Vector Group, School of BioSciences, The University of Melbourne, Victoria 3010, Australia; phone number: +61 3 9035 5237, fax number: +61 3 8344 2279, Email: rasic.gordana@gmail.com*
Running title: Scorpion *Urodacus yaschenkoi* is a species complex
Abstract

Background. Australian scorpions have received far less attention from researchers than their overseas counterparts. Here we provide the first insight into the molecular variation and evolutionary history of the endemic Australian scorpion *Urodacus yaschenkoi*. Also known as the inland robust scorpion, it is widely distributed throughout arid zones of the continent and is emerging as a model organism in biomedical research due to the chemical nature of its venom.

Methods. We employed Bayesian Inference (BI) methods for the phylogenetic reconstructions and divergence dating among lineages, using unique haplotype sequences from two mitochondrial loci (*COXI*, *16S*) and one nuclear locus (*28S*). We also implemented two DNA taxonomy approaches (GMYC and PTP/dPTP) to evaluate the presence of cryptic species. Linear Discriminant Analysis was used to test whether the linear combination of 21 variables (ratios of morphological measurements) can predict individual’s membership to a putative species.

Results. Genetic and morphological data suggest that *U. yaschenkoi* is a species complex. High statistical support for the monophyly of several divergent lineages was found both at the mitochondrial loci and at a nuclear locus. The extent of mitochondrial divergence between these lineages exceeds estimates of interspecific divergence reported for other scorpion groups. The GMYC model and the PTP/bPTP approach identified major lineages and several sub-lineages as putative species. Ratios of several traits that approximate body shape had a strong predictive power (83–100%) in discriminating two major molecular lineages. A time-calibrated phylogeny dates the early divergence at the onset of continental-wide aridification in late Miocene and Pliocene, with finer-scale phylogeographic patterns emerging during the Pleistocene.
structuring dynamics is congruent with the diversification history of other fauna of the Australian arid zones.

Discussion. Our results indicate that the taxonomic status of *U. yaschenkoi* requires revision, and we provide recommendations for such future efforts. A complex evolutionary history and extensive diversity highlights the importance of conserving *U. yaschenkoi* populations from different Australian arid zones in order to preserve patterns of endemism and evolutionary potential.
Introduction

Scorpions represent an ancient arthropod lineage that first appeared in the Silurian, and fossil records indicate their bodyplan remained largely unchanged since the Paleozoic period (Dunlop 2010; Jeram 1997; Kjellesvig-Waering 1986). Given this relative morphological stasis over long periods of time, the placement of scorpions within Arachnida and internal evolutionary relationships inferred solely from morphological characters have long been contentious (Prendini & Wheeler 2005; Sharma et al. 2014; Shultz 2007; Soleglad & Fet 2003). A recent phylogenomic study based on the transcriptome-wide variation suggested non-monophyly of all scorpion superfamilies and several families, largely contradicting the traditional morphology-based hypotheses (Sharma et al. 2015).

The well-supported phylogenetic reconstructions and taxonomy of scorpions are critical for their effective conservation. Scorpion populations can be sensitive to environmental changes due to a low reproductive rate (long generation time, long gestation time, small litter size) and high mortality of immature females (Fet et al. 1998; Lourenço & Cuellar 1995). Several species have gained threatened status due to over-harvesting for the souvenir and exotic pet trades (CITES, Appendix II, http://www.cites.org/eng/app/appendices.php). Scorpions might also become more harvested for their venom that is increasingly regarded as a source of new therapeutic and insecticidal agents (Gurevitz et al. 2007; Possani et al. 2000; Rodríguez de la Vega et al. 2010).

An extensive venom characterization can be found for individual taxa (e.g. (Luna-Ramírez et al. 2013; Xu et al. 2014), but a deeper understanding of the evolution of scorpion venoms and their molecular characteristics has been limited by the lack of underlying species tree (Sharma et al. 2015).
Extant scorpions inhabit a diversity of terrestrial habitats across all continents except Antarctica, with the greatest species diversity found in tropical and subtropical regions of the world (Lourenço 2001; Prendini 2010). Australian scorpions have received far less attention from researchers than their overseas counterparts. Over 40 scorpion species described in Australia are traditionally organized into four families: Buthidae, Bothriuridae, Urodacidae and Hormuridae (Koch 1977; Monod & Prendini 2015; Volschenk et al. 2008). The Urodacidae is an Australian endemic family found across the continent, except on the south-eastern seaboard. The family was first described by Koch (1977) that under the current classification includes two genera: *Urodacus* and the recently described troglobitic *Aops* (Volschenk & Prendini 2008). The genus *Urodacus* contains 20 species described based on morphological characters (Volschenk et al. 2012), with many likely undescribed species.

*Urodacus yaschenkoi* (Birula 1903), commonly known as the inland robust scorpion, occupies Australian desert habitats stretching from north-western Victoria through South Australia and across to Western Australia (Walker et al. 2003. ) Fig1). It is emerging as a model organism in toxinology because it produces large volumes of venom compared with other *Urodacus* species (Luna-Ramírez et al., 2013; Luna-Ramírez et al., 2014). This scorpion has had several synonyms throughout its taxonomic history, starting from the original description as *Hemihoplopus yaschenkoi* (Birula 1903), followed by *Urodacus granifrons* (Kraepelin 1916), *U. fossor* (Kraepelin 1916), and *U. kraepelini* (Glauert 1963), and finally by *U. yaschenkoi* (Birula) (Koch 1977). Since then, studies of variation in *U. yashenkoi* populations have not been conducted.

Here we provide the first molecular analysis of phylogenetic patterns and history of *U. yaschenkoi* sampled across its native range. DNA sequence data from mitochondrial and nuclear loci, complemented with the analysis of several body-proportion characters, showed that *U.
yaschenkoi shares a complex diversification history with other Australian arid-adapted fauna. Moreover, the existence of several deeply divergent lineages that also differ in body-shape indicate that further revision of this taxon is warranted.

Materials and Methods

Biological material

Samples of Urodacus yaschenkoi were obtained from field and museum collections (Table 1). Live specimens were collected from eight locations (approximately 500 m²) in the semi-arid and arid regions of Central Australia in December 2010 and October 2011 (Table 1 and Fig1). Individuals were collected at night from pitfall traps set in front of their burrows, and those outside their burrows were detected using ultraviolet (UV) lamps that reveal soluble fluorescent components (β-carboniles) in the scorpion exoskeleton (Stachel et al. 1999). Captured scorpions were kept alive and transported to the laboratory for morphological identification according to Koch (1977). Key diagnostic feature that distinguishes U. yaschenkoi from other Urodacus species is a very small terminal prolateral tarsus unguis. All specimens were handled according to good animal practices defined by the Government of Australia, and all institutions and museums involved approved the animal handling work. Scorpions were anaesthetized by cooling in a refrigerator (4°C) for 5 min before removing ~1 mm² of leg muscle tissue, which was stored in 90% ethanol at 4°C or –20°C for subsequent DNA extraction. Additional samples were obtained from collections at the South Australian Museum (SAM) and Western Australian Museum (WAM) containing specimens collected between 2000 and 2010 (Table 1).
**DNA extraction, amplification and sequencing**

Total DNA was extracted from the stored muscle tissue using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands) following the manufacturer’s instructions. Two mitochondrial loci (cytochrome oxidase subunit I, COXI; large ribosomal subunit, 16S) and a single nuclear locus (28S) were amplified by PCR with a reaction volume of 20 μl containing 0.5 ng of template DNA, 10 μl of Go Taq Master Mix (Promega, Madison, Wisconsin, USA), 0.5 μl of 10 nM primers and 7 μl of RNase-free water (Qiagen). The primer sequences and PCR amplicon sizes are summarized in Table 2.

Primers previously designed for the insect COXI gene (Simon et al. 1994; Tanaka et al. 2001) were used to amplify a 630-base pair (bp) fragment from the 3' end of the locus. The amplification conditions comprised an initial denaturing step at 95°C for 5 min followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 52°C for 40 s, and extension at 72°C for 45 s, and a final extension phase at 72°C for 5 min. For the mitochondrial 16S gene, the scorpion-specific primer pairs modified by (Gantenbein et al. 2005b) were used to amplify a 425-bp region at the 3' end of the locus. The amplification conditions comprised an initial denaturing step at 94°C for 4 min followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 47.5°C for 30 s, and extension at 72°C for 30 s, and a final extension phase at 72°C for 7 min. The COXI and 16S gene fragments were also amplified from three specimens keyed out as *Urodacus manicatus* (Um2714, Um1814) and *U. novaehollandiae* (Un2112, Table 1). Sequences from these taxa were used as outgroups in downstream phylogenetic reconstruction. Primer pairs R1S and R1AS, and R2S and R2AS, designed by (Arabi et al. 2012), were used to amplify 1158-bp and 1246-bp fragments of the 28S locus, respectively. Each set of primers amplifies a different region of the gene, which overlaps by 327 bp, and their sequences were concatenated to form a larger product.
of 2076 bp. The amplification conditions for both sets of primers comprised an initial denaturing step at 94°C for 4 min, followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and a final extension phase at 72°C for 7 min.

Museum specimens that were not stored under ideal conditions for preservation failed to yield COXI amplicons suitable for direct sequencing. To address this issue, additional PCR primers were designed to amplify smaller fragments for COXI locus (Table 2), resulting in amplicons of 150 bp that were used for subsequent analysis. For the SAM specimens, the amplification of the 28S nuclear gene failed entirely and these samples were excluded from further analysis of the nuclear gene variation. All amplicons were sequenced in both directions using the PCR amplification primers, and carried out on an Applied Biosystems 3130 genetic analyzer by Macrogen Inc. (Seoul, South Korea).

Sequences were aligned and edited in Geneious Pro v6.1 (Biomatters Ltd) using the MUSCLE alignment option with default parameters. All chromatograms were checked for the presence of multiple peaks (which indicate heterozygosity), and authenticity of the COXI coding gene was validated by checking for indels and premature stop codons. After this editing process, the alignment of the mitochondrial gene fragments yielded 616-bp and 396-bp products for the COXI and 16S genes respectively, and the final 28S alignment was 2076 bp in length. The final dataset contained 68 sequences for each of the mitochondrial genes and 27 sequences for the 28S locus (Table 1, [GenBank accession # KP176717-KP176786]). Shared haplotypes were identified and the uncorrected pairwise genetic distances (%) were calculated using Geneious Pro v6.1 (Biomatters Ltd). This simple distance measure was implemented to achieve reliable estimates of both intraspecific and interspecific genetic variation.
Phylogenetic analysis

Phylogenetic reconstructions and divergence dates among lineages were calculated using unique haplotypes and Bayesian Inference (BI) methods implemented in BEAST v2.1.3 (Bouckaert et al. 2014). We used jModeltest v0.1.1 (Posada 2008) to select the best-fit model of evolution, based on Akaike Information Criteria (AIC) (Akaike & Company 1981) for each of the mitochondrial and nuclear genes (GTR + G in each case). Mitochondrial loci were combined for analysis due to their similar modes of evolution (GTR+R), as indicated by the incongruence-length difference (ILD) tests (Farris et al. 1995) implemented in PAUP_4.0b10 (Swofford 2002). The nuclear gene (28S) was analyzed independently due to inconsistencies in taxon sampling (Table 1).

Operators were auto-optimized, and five independent Markov Chain Monte Carlo (MCMC) runs were performed using a Yule (speciation) tree-prior, each running for 5 x 10^6 generations, sampling every 10,000 states. Log files were examined with Tracer v1.5 (Drummond & Rambaut 2007) to ensure that runs were sampling from the same posterior distribution, to determine appropriate burn-in, and to ensure that effective sample sizes (ESSs) of parameters of interest were greater than 1000. Tree files of independent runs were then combined using LogCombiner v2.1.3 (Drummond et al. 2012), discarding the first 20% and re-sampling at a lower frequency of 15,000. The maximum clade credibility (MCC) tree was recovered from a sample of 10,000 posterior trees, and branch support was annotated using TreeAnnotator v2.1.3 (Drummond et al. 2012). Each analysis started with a random starting tree and seed with no root specified.

Sequence data from species of the same genus (*U. manicatus* and *U. novaehollandiae*) were used to estimate the root of the mitochondrial gene tree.
Additional phylogenetic constructions were also performed using a truncated COXI alignment to test the influence of missing data on the final tree topology. Because numerous museum collections yielded short COXI gene products, we trimmed the alignment to 150-bp to exclude regions of the alignment with high levels of missing data. This exercise demonstrated that the inclusion/exclusion of missing data had little influence on the phylogenetic reconstructions. Consequently, all results presented from this point reflect those from the non-truncated COXI alignment.

Species delineation based on molecular data
We implemented two DNA taxonomy approaches to evaluate the presence of cryptic species. First, the general mixed Yule coalescent (GMYC) approach (Fujisawa & Barraclough 2013; Pons et al. 2006) was applied to an ultrametric tree (produced using BEAST) in R v2.15.3 (R Development Core Team 2008) with the Splits package (http://splits.r-forge.r-project.org). The GMYC model is a process-based approach that detects the threshold in a gene tree at which within-species processes (i.e. coalescence) shift to between-species processes (i.e. speciation and extinction). Second, we combined the Poisson Tree Processes model for species delimitation (PTP) and a Bayesian implementation of PTP (bPTP) to infer putative species boundaries on a given phylogenetic input tree (Zhang et al. 2013). The PTP/bPTP model, unlike the GMYC model, requires a bifurcated phylogenetic tree rather than an ultrametric tree. PTP/dPTP models speciation or branching events in terms of the number of substitutions. The following parameters were used: MCMC, 500,000 generations; thinning, 100; burn-in, 0.1; seed, 123, and assessed convergence in each case to ensure the reliability of the results.
Delineation based on the analyses of morphological measurements

Proportions of several characters that approximate body shape were assessed in 39 female adult specimens that were keyed out as *U. yaschenkoi* (according to Koch, 1977) and were collected at 26 locations (Table 1, Fig1). Gender was determined by examining the genital opercula of adult scorpions, with males having a small finger-like projection known as the genital papilla. Because our collection contained only three males, the analyses were done only with females.

The following traits were measured under a microscope using an ocular ruler with 1-mm precision: carapace length (CL), metasoma segment V length (MVL), telson length (SL), pedipalp length (PL), chela length (ChL), pecten length (PecL) and pecten width (PecW). Ratios of traits (e.g. CL/MVL, SL/PL etc.) gave in total 21 variables scored in each individual (Supplemental file 4). These variables were treated as predictors in the Linear Discriminant Analysis (LDA) implemented in the R package “MASS” (Venables & Ripley 2002). LDA was used to test whether the linear combination of 21 variables (ratios of morphological measurements) can predict individual’s membership to a mitochondrial lineage (putative species). Strong predictive power of morphological variation on the observed molecular divergence would provide additional support for a species complex in *U. yaschenkoi*.

Divergence time estimation

The mitochondrial gene tree was time calibrated with divergence times of nodes inferred from 95% highest posterior density (HPD) intervals. Scorpion-specific mutation rates of 0.007 substitutions/site/million years for *COXI* and 0.005 substitutions/site/million years for *16S* (Gantenbein et al. 2005a; Gantenbein & Largiadèr 2003) were used to calibrate the tree. These estimates are derived from buthid scorpions and have been used to estimate divergence times among various scorpion lineages including non-buthid taxa (Bryson et al. 2013a; Bryson et al.
Substitution rates were set in BEAUti v1.7.3 (Drummond et al. 2012) using relaxed clock log normal priors. Tracer was then used to obtain parameter estimates for time to most recent common ancestor (tMRCAs) for nodes within the gene tree.

Results
We identified 31 unique mitochondrial haplotypes with uncorrected distances between haplotypes ranging from 0.3–7.6% (mean ± standard deviation = 3.0% ± 0.4%) and distances from the outgroup taxa of 8.4–10.2% (mean ± standard deviation = 9.4% ± 1.4%) (Supplemental File 1). A total of 13 nuclear 28S haplotypes were identified with uncorrected $p$-distances of 0.1–0.5% (mean ± standard deviation = 0.2% ± 0.1%) (Supplemental File 2). A list of haplotypes for sample locations is provided in Supplemental File 3.

Phylogenetic analysis
Mitochondrial markers
Bayesian inference analysis of the mitochondrial dataset identified several genetically divergent lineages (three major lineages represented as black, red and green clades in Fig2), with strong statistical support for their respective monophyly (posterior probability >0.95). Sublineages within the black clade are broadly distributed across Victoria, South Australia and Western Australia, whereas the red and green clades are restricted to Western Australia (Fig1). From this point forward we will refer to the black, red and green clades as the south-central (SC), western (W) and central-western (CW) lineages, respectively.
Mean uncorrected pairwise genetic distances between the three major lineages (SC, CW and W) ranged from 6.4 to 6.9% (overall mean ± standard deviation = 6.6% ± 0.9%). The mean sub-lineage distances ranged from 2.2% ± 0.4% and 0.8% ± 0.2%, respectively (not calculated for the W lineage due to only a single recorded haplotype). Mean uncorrected distances between the three major lineages and the outgroups ranged from 9.3 to 10.3% (mean ± standard deviation = 9.4% ± 1.4%).

**Nuclear marker**

Despite low level of variation in the 28S dataset, Bayesian analysis produced a nuclear gene topology that was largely concordant with the mitochondrial gene tree. Three genetically divergent clades were identified, corresponding to those from the mitochondrial dataset (SC, CW and W, Fig3). In each case, strong statistical support for the monophyly of each clade was found (posterior probability >0.95). The unresolved interrelationships among lineages within each clade in the nuclear gene tree prevented any reliable inferences of phylogeographic patterns.

**Molecular-based species delineation**

Among the 31 unique mitochondrial haplotypes described above, the GMYC model identified nine entities and the PTP/bPTP approach identified seven, each representing putative species (Table 3). The assignment of haplotypes to putative species groups is shown in Fig2, where conspecifics share a common number. Species assignments were highly consistent when comparing each of the methods, but we presented the PTP/bPTP results as they are more accurate when the evolutionary distances between lineages are small (Zhang et al. 2013). In summary, SC, W and CW clades were recognized as putative species groups, as were the sub-lineages within the SC ancestral grouping (SC-1 to 5, Fig2).
Discriminant power of morphological variation

None of the *U. yaschenkoi* specimens that were characterized at 21 morphological ratio variables were assigned to the W mitochondrial clade, hence the LDA was done on 39 females assigned to the SC and the CW clades. Individuals were categorized into four groups (putative species) based on the results of the PTP/bPTP molecular species delineation analysis: 18 females from SC-1, 12 from SC-3, three from the SC-4, and six from the CW clade (Fig2). Because our dataset contained four groups, we could find a maximum of three discriminant functions that separate these groups.

The first discriminant function (LD1) achieved 93.7% of the separation, reflecting the morphological distinction of the CW clade from the SC clade (Fig4). Further separation of the three putative groups within the SC clade was weak (LD2-3, Fig4). We then grouped samples into two putative species (CW and SC clade) and tested the accuracy of prediction using 100 jackknife resampling steps. The grouping into two molecular clades based on morphological variation was 100% accurate (33/33) for the SC clade and 83.3% accurate (5/6) for the CW clade. Therefore, our results indicate strong predictive power of body proportion variation on the observed molecular divergence, and suggest the existence of at least two distinct taxa within *U. yaschenkoi*.

The most discriminating uncorrelated proportions were of the telson and chela length (SL/ChL) and pedipalp and pecten length (PL/PecL). Overall, members of the CW clade tend to have disproportionately shortened chela and enlarged pecten when compared to the members of the SC clade.
Divergence dating

Our time calibrated mitochondrial phylogeny suggested that the split between the major *U. yaschenkoi* clades (SC, CW and W lineages) occurred during the late Miocene/early Pliocene (4–7 MYA) (Fig2). Lineage diversifications within SC appear to have occurred during the Pliocene and early Pleistocene (1.8–4 MYA), while finer-scale phylogeographic patterning within the sub-lineages arose during the late Pleistocene (<1 Mya). Divergence time estimates should be interpreted with some caution, as the nucleotide substitution rate was derived from a different scorpion family (Buthidae) and there are large errors margins around 95% HPD estimates.

Biogeographic patterns

The SC lineage showed substantial geographic structure. The most divergent sub-lineage (SC-5) was found in Western Australia in sympatry with the CW lineage (Fig1). SC-1 was found west of the Central Ranges, through to the Eyre Peninsula in South Australia, while SC-3 had a distribution extending from the Central to Mt Lofty Ranges in South Australia, and across to north-western Victoria. SC-4 had a narrow north-south distribution in the central inland and coastal regions of South Australia (Fig1).

Discussion

Our analyses reveal strong genetic and morphological diversification in *U. yaschenkoi* across its range, pointing to the existence of a species complex with at least three putative species. High statistical support for the monophyly and the extent of genetic divergence between the main three lineages (6.4–6.9%) exceeds estimates of interspecific divergence previously reported for other scorpion and arthropod groups (Bryson et al. 2014; Tourinho et al. 2012; Wysocka et al. 2011).
DNA-based species delineation approaches (GMYC and bPTP) provided significant statistical support for the recognition of the three lineages (SC, CW, W) as distinct species, and potential further cryptic speciation within the south-central clade (SC1-5, Fig2).

We also demonstrated a strong association between this molecular divergence and morphological variation. Namely, ratios of several traits that approximate body shape had a strong predictive power (83-100%) in discriminating two major molecular clades (CW and SC). The two clades differ most notably in proportions involving chela and pecten. Because of their great variation in shape, scorpion chalae have been used as one of the key characters to delineate different ecomorphotypes (van der Meijden et al. 2012). Until now *U. yaschenkoi* has been distinguished from other congeneric species by its much smaller terminal prolateral tarsal ungues and by the production of large amounts of venom (Koch 1977). Based on our results from a limited sample size, detailed analyses of morphological variation in *U. yaschenkoi* are warranted.

Our time-calibrated phylogeny suggests that the split between the CW, W and SC clades occurred during the mid-Miocene to early Pliocene (approximately 5-9 Mya). This geological time was marked by a shift to a much drier climate, the significant contraction of rainforests and the expansion of arid habitats (Martin 2006). Further diversification within the major ancestral *U. yaschenkoi* lineages appears to have occurred throughout the Pliocene (3-5 Mya), which was a consistently dry period. This is followed by further lineage divergence during the mid and late Pleistocene when the climate was highly dynamic (< 1 Mya), with wetter and drier episodes corresponding to interglacial and glacial cycles (McLaren & Wallace 2010).

The spatio-temporal dynamics of diversification observed in *U. yaschenkoi* parallels those reported in other Australian arid biota. Reviewing tens of dated phylogenies of the south-western
Australian terrestrial fauna, including arthropods like crayfish and spiders, (Rix et al. 2015) found a compelling commonality in the basal east-west lineage diversification during the first half Miocene (until 10 Mya). The more xeric taxa currently occupying semi-arid and arid zones seemed to have experienced this divergence in late Miocene (6-10 Mya) (Rix et al. 2015), which we also inferred in the desert scorpion *U. yaschenkoi* (Fig2). A strong genetic and morphological divergence between the *U. yaschenkoi* lineages from the western (CW, W) and south-central (SC) Australia could be partly explained by the Miocene east-west vicariance hypothesis (Rix et al. 2015) (Fig1). After a longer period of range contraction, arid-adapted taxa such as *U. yaschenkoi* likely underwent significant range expansions during the Pliocene. Separation of SC-5 from other SC sub-lineages was estimated to have occurred during this time (Fig2), with SC-5 moving easterly. This sub-lineage is now sympatric with the CW clade (Fig1), suggesting their secondary contact. Further diversification within the SC clade (SC1-4) coincides with transition to the Pleistocene severe glacial cycles and expansion of the Australian deserts during the last 1 My (beginning of the “dusty world”, (Rix et al. 2015)). Like the Bynoe's gecko (Fujita et al. 2010) and lizards (Dubey & Shine 2010; Pepper et al. 2011), *U. yaschenkoi* is another arid-adapted Australian taxon whose diversification and distribution were profoundly affected by the opening of desert biomes during this hyper-arid, unstable climatic history. Teasing out the relative importance of vicariance, putative refugia (e.g. Pilbara, Kimberley, central Ranges, (Pepper et al. 2013)), or dispersal (Melville et al. 2016) (Fig1) in shaping this diversity would require extensive sampling, particularly at the western and northern parts of *U. yaschenkoi* distribution.
Revising the *U. yaschenkoi* taxonomy – future directions

Our results provide solid baseline data on the historical and spatial extent of diversification in *U. yaschenkoi* and offer some guidelines for future integrative taxonomic approaches in delimiting species within this taxon. We found an agreement among disciplines (morphology, nuclear and mitochondrial genetic information) during a primary exploration, which strengthens the argument for a taxonomic revision (Pante et al. 2014; Schlick-Steiner et al. 2009). Congruent morphological and molecular phylogenetic signals are particularly compelling for a scorpion taxon, given that this is not the case in many scorpion lineages (Sharma et al. 2015).

The level of mitochondrial sequence divergence observed between *U. yaschenkoi* lineages satisfy the requirements for species delineation based on the principles of the phylogenetic species concept (De Queiroz 2007; Wheeler 1999). The three major lineages (SC, CW, W) can be considered the putative species. Because genetic ‘yardstick’ approaches provide crude taxonomic measures and nucleotide substitution rates often vary considerably between taxonomic groups, some caution is needed when considering findings of these analyses alone. Additional DNA-based species delineation approaches (GMYC and bPTP) indicated extensive cryptic speciation in *U. yaschenkoi* (Fig. 2). The GMYC method has been criticized for over-splitting species with a pronounced genetic structure (Satler et al. 2013), yet several recent studies have shown that it is highly robust (Fujisawa & Barraclough 2013; Talavera et al. 2013). The obvious next step is to characterize the nuclear genome-wide variation in *U. yaschenkoi* sampled extensively within the “type” locality (28°35’S, 138°33’E), as well as western and northern parts of the distribution. We certainly advise against a pool-sequencing phylogenomic approach (e.g. samples from the same location are pooled to achieve cost-efficiency), given that the putative species have been found in sympathy.
The proportions of various morphological characters are routinely used in species descriptions or
identification keys, particularly for arthropods where morphologically similar species often differ
significantly in body proportions but not in qualitative characters. (Baur & Leuenberger 2011).
Arguably, the results of multivariate analyses summarizing the overall body shape differences
between groups are not easily interpreted. Yet, our initial results suggest that further analyses of
e.g. chela shape might reveal more easily quantifiable diagnostic characters for _U. yaschenkoi_.
Several parameters of chela shape were found to be correlated with the amount of strain stress
they can withstand. Specifically, slender chela morphologies may be less suitable for high-force
functions such as burrowing and defence (van der Meijden et al. 2012). Given that _U. yaschenkoi_
putative species (SC and CW) show marked shape differences involving chela, further
exploration of burrowing behavior or prey preference might provide additional characters to
describe the _U. yaschenkoi_ species complex.

Finally, it is important to note that we cannot exclude the possibility that some of the cryptic
lineages have already been described as species, and we are not able to compare our genetic data
against other _Urodacus_ sequences as none published at the time of our study. Also, our sampling
did not cover the exact “type” locality (28°35’S, 138°33’E). The samples closest to this area
belong to the SC clade and likely represent the “type” lineage. These data gaps would need to be
addressed in further studies aiming to revise the taxonomy of the Australian desert scorpion _U._
yaschenkoi.

**Conclusions**

Our study provides the first insight into the molecular phylogeny of the endemic Australian
scorpion _Urodacus yaschenkoi_. We show that this scorpion shares a complex diversification
history with other Australian arid-adapted fauna. Concordance between the mitochondrial and nuclear data, along with the morphological variation, all suggest that *U. yaschenkoi* is a species complex that requires further taxonomic revision. Our findings highlight the importance of conserving populations from different Australian arid zones in order to preserve patterns of endemism and evolutionary potential.
References

Akaike H, and Company N-hP. 1981. Likelihood of a model and information criteria. *Journal of Econometrics* 16:3-14. 10.1016/0304-4076(81)90071-3

Arabi J, Judson MLI, Deharveng L, Lourenço WR, Cruaud C, and Hassanin A. 2012. Nucleotide composition of CO1 sequences in Chelicerata (arthropoda): Detecting new mitogenomic rearrangements. *Journal of Molecular Evolution* 74:81-95. 10.1007/s00239-012-9490-7

Baur H, and Leuenberger C. 2011. Analysis of Ratios in Multivariate Morphometry. *Systematic Biology*. 10.1093/sysbio/syr061

Birula A. 1903. Sur un nouveau genre et une nouvelle espèce de scorpions, provenant d'Australie.] Exploration du Parc National de l'Upemba. *Mission G F de Witte* 8:xxxiii-xxxiv.

Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu CH, Xie D, Suchard MA, Rambaut A, and Drummond AJ. 2014. BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. *PLoS Computational Biology* 10:e1003537. 10.1371/journal.pcbi.1003537

Bryson RW, Prendini L, Savary WE, and Pearman PB. 2014. Caves as microrefugia: Pleistocene phylogeography of the troglobilphic North American scorpion Pseudouroctonus reddelli. *BMC Evolutionary Biology* 14:9. 10.1186/1471-2148-14-9

Bryson RW, Riddle BR, Graham MR, Smith BT, and Prendini L. 2013a. As Old as the Hills: Montane Scorpions in Southwestern North America Reveal Ancient Associations between Biotic Diversification and Landscape History. *PLoS ONE* 8:e52822. 10.1371/journal.pone.0052822

Bryson RW, Savary WE, Prendini L, and Parmakelis A. 2013b. Biogeography of scorpions in the Pseudouroctonus minimus complex (Vaejovidae) from south-western North America: Implications of ecological specialization for pre-Quaternary diversification. *Journal of Biogeography* 40:1850-1860. 10.1111/jbi.12134

De Queiroz K. 2007. Species concepts and species delimitation. *Systematic Biology* 56:879-886. 10.1080/10635150701701083

Drummond A, and Rambaut A. 2007. Tracer: MCMC trace analysis tool. 1.5.0, Program distributed by the authors.

Drummond AJ, Suchard MA, Xie D, and Rambaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* 29:1969-1973. 10.1093/molbev/mss075

Dubey S, and Shine R. 2010. Evolutionary Diversification of the Lizard Genus <italic>Bassiana</italic> (Scincidae) across Southern Australia. *PLoS ONE* 5:e12982. 10.1371/journal.pone.0012982

Dunlop JA. 2010. Geological history and phylogeny of Chelicerata. *Arthropod structure & development* 39:124-142.

Farris JS, Källersjö M, Kluge AG, and Bult C. 1995. Constructing a Significance Test for Incongruence. *Systematic Biology* 44:570-572. 10.1093/sysbio/44.4.570

Fet V, Polis GA, and Sissom WD. 1998. Life in sandy deserts: the scorpion model. p 609-622.

Fujisawa T, and Barraclough TG. 2013. Delimiting species using single-locus data and the generalized mixed yule coalescent approach: A revised method and evaluation on simulated data sets. *Systematic Biology* 62:707-724. 10.1093/sysbio/syt033
Fujita MK, McGuire JA, Donnellan SC, and Moritz C. 2010. Diversification and persistence at the arid-monsoonal interface: australia-wide biogeography of the Bynoe's gecko (Heteronotia binoei; Gekkonidae). Evolution 64:2293-2314. 10.1111/j.1558-5646.2010.00993.x

Gantenbein B, Fet V, Gantenbein-Ritter IA, and Balloux F. 2005a. Evidence for recombination in scorpion mitochondrial DNA (Scorpiones: Buthidae). Proceedings of the Royal Society B: Biological Sciences 272:697-704. 10.1098/rspb.2004.3017

Gantenbein B, Fet V, Gantenbein-Ritter IA, and Balloux F. 2005b. Evidence for recombination in scorpion mitochondrial DNA (Scorpiones: Buthidae). Proceedings Biological Sciences / The Royal Society 272:697-704. 10.1098/rspb.2004.3017

Gantenbein B, and Largiadèr CR. 2003. The phylogeographic importance of the Strait of Gibraltar as a gene flow barrier in terrestrial arthropods: A case study with the scorpion Buthus occitanus as model organism. Molecular Phylogenetics and Evolution 28:119-130. 10.1016/S1055-7903(03)00031-9

Glaubert L. 1963. Notes on Urodacus scorpions. Western Australian Naturalist 8:132-135.

Graham MRMR, Oláh-Hemmings V, and Fet V. 2012. Phylogeography of co-distributed dune scorpions identifies the Amu Darya River as a long-standing component of Central Asian biogeography: (Scorpiones: Buthidae. Zoology in the Middle East 55:95-110. 10.1080/09397140.2012.10648924

Gurevitz M, Karbat I, Cohen L, Ilan N, Kahn R, Turkov M, Stankiewicz M, Stühmer W, Dong K, and Gordon D. 2007. The insecticidal potential of scorpion β-toxins. p 473-489.

Jeram AJ. 1997. Phylogeny, classification and evolution of Silurian and Devonian scorpions. Proceedings of the 17th European colloquium of arachnology, Edinburgh. p 17-31.

Kjellesvig-Waering EN. 1986. A restudy of the fossil Scorpionida of the world: Paleontological Research Institution.

Koch LE. 1977. The taxonomy, geographic distribution and evolutionary radiation of Australo-Papuan scorpions. Records of the Western Australian Museum 5:79-79.

Kraepelin K. 1916. Results of Dr. E. Mjöbergs Swedish Scientific Expeditions to Australia 1910-1913. 4. Scolopendriden und Scorpiione. Arkiv för Zoologi 10:1-43.

Lourenço WR. 2001. The scorpion families and their geographical distribution.

Lourenço WR, and Cuellar O. 1995. Scorpions, scorpionism, life history strategies and parthenogenesis.

Luna-Ramírez K, Quintero-Hernández V, Vargas-Jaimés L, Batista CVF, Winkel KD, and Possani LD. 2013. Characterization of the venom from the Australian scorpion Urodacus yaschenkoi: Molecular mass analysis of components, cDNA sequences and peptides with antimicrobial activity. Toxicon 63:44-54. 10.1016/j.toxicon.2012.11.017

Martin HA. 2006. Cenozoic climatic change and the development of the arid vegetation in Australia. Journal of Arid Environments 66:533-563. 10.1016/j.jaridenv.2006.01.009

McLaren S, and Wallace MW. 2010. Plio-Pleistocene climate change and the onset of aridity in southeastern Australia. Global and Planetary Change 71:55-72. http://dx.doi.org/10.1016/j.gloplacha.2009.12.007

Melville J, Haines ML, Hale J, Chapple S, and Ritchie EG. 2016. Concordance in phylogeography and ecological niche modelling identify dispersal corridors for reptiles in arid Australia. Journal of Biogeography 43:1844-1855. 10.1111/jbi.12739
Monod L, and Prendini L. 2015. Evidence for Eurogondwana: the roles of dispersal, extinction and vicariance in the evolution and biogeography of Indo-Pacific Hormuridae (Scorpiones: Scorpionoidea). *Cladistics* 31:71-111. 10.1111/cla.12067

Pante E, Schoelinck C, and Puillandre N. 2014. From Integrative Taxonomy to Species Description: One Step Beyond. *Systematic Biology*. 10.1093/sysbio/syu083

Pepper M, Doughty P, and Keogh JS. 2013. Geodiversity and endemism in the iconic Australian Pilbara region: a review of landscape evolution and biotic response in an ancient refugium. *Journal of Biogeography* 40:1225-1239. 10.1111/jbi.12080

Pepper M, Ho SYW, Fujita MK, and Scott Keogh J. 2011. The genetic legacy of aridification: Climate cycling fostered lizard diversification in Australian montane refugia and left low-lying deserts genetically depauperate. *Molecular Phylogenetics and Evolution* 61:750-759. [http://dx.doi.org/10.1016/j.ympev.2011.08.009](http://dx.doi.org/10.1016/j.ympev.2011.08.009)

Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S, Kamoun S, Sumlin WD, and Vogler AP. 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology* 55:595-609. 10.1080/10635150600852011

Posada D. 2008. jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution* 25:1253-1256. 10.1093/molbev/msn083

Prendini L. 2010. Order Scorpiones C.L. Koch, 1837 scorpions. In: Gerlach J, and Marusik Y, eds. *Arachnida and Myriapoda of the Seychelles islands*. Manchester, UK: Siri Scientific Press, 321–330.

Prendini L, and Wheeler WC. 2005. Scorpion higher phylogeny and classification, taxonomic anarchy, and standards for peer review in online publishing. *Cladistics* 21:446-494. 10.1111/j.1096-0031.2005.00073.x

Rix MG, Edwards DL, Byrne M, Harvey MS, Joseph L, and Roberts JD. 2015. Biogeography and speciation of terrestrial fauna in the south-western Australian biodiversity hotspot. *Biological Reviews* 90:762-793. 10.1111/brv.12132

Rodríguez de la Vega RC, Schwartz EF, and Possani LD. 2010. Mining on scorpion venom biodiversity. p 1155-1161.

Satler JD, Carstens BC, and Hedin M. 2013. Multilocus species delimitation in a complex of morphologically conserved trapdoor spiders (mygalomorphae, antrodiaetidae, Aliatypus). *Systematic Biology* 62:805-823. 10.1093/sysbio/syt041

Schlick-Steiner BC, Steiner FM, Seifert B, Stauffer C, Christian E, and Crozier RH. 2009. Integrative Taxonomy: A Multisource Approach to Exploring Biodiversity. *Annual Review of Entomology* 55:421-438. 10.1146/annurev-ento-112408-085432

Sharma PP, Fernández R, Esposito LA, González-Santillán E, and Monod L. 2015. Phylogenomic resolution of scorpions reveals multilevel discordance with morphological phylogenetic signal. *Proceedings of the Royal Society B: Biological Sciences* 282.

Sharma PP, Kaluziak ST, Pérez-Porro AR, González VL, Hormiga G, Wheeler WC, and Giribet G. 2014. Phylogenomic interrogation of Arachnida reveals systemic conflicts in phylogenetic signal. *Molecular Biology and Evolution*. 10.1111/j.1096-3642.2007.00284.x
Simon C, Frati F, Beckenbach A, Crespi B, Liu H, and Flook P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved PCR primers. *Annals of the Entomological Society of America* 87:651-701.

Soleglad ME, and Fet V. 2003. High-level systematics and phylogeny of the extant scorpions (Scorpiones: Orthosterni). *Euscorpius* 11:1–175.

Stachel SJ, Stockwell SA, and Van Vranken DL. 1999. The fluorescence of scorpions and cataractogenesis. *Chemistry and Biology* 6:531-539. 10.1016/S1074-5521(99)80085-4

Swofford DL. 2002. PAUP* phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. *Sinauer Associates*. 10.1159/000170955

Talavera G, Dincă V, Vila R, and Paradis E. 2013. Factors affecting species delimitations with the GMYC model: insights from a butterfly survey. *Methods in Ecology and Evolution* 4:1101-1110. 10.1111/2041-210x.12107

Tanaka H, Roubik DW, Kato M, Liew F, and Gunsalam G. 2001. Phylogenetic position of Apis nuluensis of northern Borneo and phylogeography of A. cerana as inferred from mitochondrial DNA sequences. p 44-51.

Tourinho JL, Sole-Cava AM, and Lazoski C. 2012. Cryptic species within the commercially most important lobster in the tropical Atlantic, the spiny lobster Panulirus argus. *Marine Biology* 159:1897-1906. [http://dx.doi.org/10.1007/s00227-012-1977-7](http://dx.doi.org/10.1007/s00227-012-1977-7)

van der Meijden A, Kleinteich T, and Coelho P. 2012. Packing a pinch: functional implications of chela shapes in scorpions using finite element analysis. *Journal of Anatomy* 220:423-434. 10.1111/j.1469-7580.2012.01485.x

Venables W, and Ripley B. 2002. Modern Applied Statistics with S. Fourth Edition ed. New York: Springer.

Volschenk ES, Harvey MS, and Prendini L. 2012. A new species of Urodacus (Scorpiones: Urodacidae) from Western Australia. *American Museum Novitiates* 3748:1-18. 10.1206/3748.2

Volschenk ES, Mattoni CI, and Prendini L. 2008. Comparative anatomy of the mesosomal organs of scorpions (Chelicerata, Scorpiones), with implications for the phylogeny of the order. p 651-675.

Volschenk ES, and Prendini L. 2008. Aops oncodactylus, gen. et sp. nov., the first troglobitic urodacid (Urodacidae:Scorpiones), with a re-assessment of cavernicolous, troglobitic and troglomorphic scorpions. *Invertebrate Systematics* 22:235-257. 10.1071/IS06054

Walker, K.L, Yen AL, and Milledge, G.A.. 2003. *Spiders and Scorpions commonly found in Victoria*. Melbourne, Australia: The Royal Society of Victoria.

Wheeler QD. 1999. Why the phylogenetic species concept?-Elementary. *Journal of nematology* 31:134-141.

Wysocka A, Krzysztofiak L, Krzysztofiak A, Zohnierkiewicz O, Ojdowska E, and Sell J. 2011. Low genetic diversity in Polish populations of sibling ant species: Lasius niger (L.) and Lasius platythorax Seifert (Hymenoptera, Formicidae). *Insectes Sociaux* 58:191-195. 10.1007/s00040-010-0135-9

Xu X, Duan Z, Di Z, He Y, Li J, Li Z, Xie C, Zeng X, Cao Z, Wu Y, Liang S, and Li W. 2014. Proteomic analysis of the venom from the scorpion Mesobuthus martensii. *Journal of Proteomics* 106:162-180. [http://dx.doi.org/10.1016/j.jprot.2014.04.032](http://dx.doi.org/10.1016/j.jprot.2014.04.032)

Zhang J, Kapli P, Pavlidis P, and Stamatakis A. 2013. A general species delimitation method with applications to phylogenetic placements. *Bioinformatics* 29:2869-2876.

10.1093/bioinformatics/btt499
Web references:

Department of the Environment, Water, Heritage and the Arts (12 February 2010). "Species Urodacus yaschenkoi (Birula, 1903)". *Australian Biological Resources Study: Australian Faunal Directory*. Commonwealth of Australia. Retrieved 20 July 2015.

CITES Appendix II, http://www.cites.org/eng/app/appendices.php; accessed on Sep 10, 2014.

Medscape, http://emedicine.medscape.com/article/168230-overview; accessed on Aug 20, 2014.
Table 1. *Urodacus yaschenkoi* specimen location and analyses made. List of *Urodacus yaschenkoi* collected from the field as live specimens (Field) or obtained from the Australian museum collections (South Australian Museum - SA, Western Australian Museum - WA). Geographic position (lat/log) and the geographic region details are reported for each sample. List of haplotypes (mito, 28S) and GenBank Accession # scored in each individual. Morphological variation scored ($\bar{e}$), Museum ID.

| Sample | Source | Latitude | Longitude | Geographic Region | Haplotype: mito | Haplotype: 28S | GenBank: (mito / 28S) | Morpho | Museum ID/Reg.No. |
|--------|--------|----------|-----------|-------------------|----------------|----------------|----------------------|--------|------------------|
| BKA11  | Field  | -33.2283 | 141.3011  | NSW               | 20             | 1              | KP176775 / KP176743 | NA     |                  |
| BKA12  | Field  | -33.2283 | 141.3011  | NSW               | 20             | 2              | KP176775 / KP176744 | NA     |                  |
| BKB08  | Field  | -33.2199 | 141.3089  | NSW               | 20             | 1              | KP176775 / KP176743 | NA     |                  |
| BKB12  | Field  | -33.2242 | 141.3061  | NSW               | 20             | 1              | KP176775 / KP176743 | NA     |                  |
| BK13   | Field  | -33.2283 | 141.3011  | NSW               | 20             | 1              | KP176775 / KP176743 | NA     |                  |
| MARR1  | Field  | -26.3400 | 133.2000  | SA                | 28             | 3              | KP176783 / KP176745 | NA     |                  |
| MARR2  | Field  | -26.3400 | 133.2000  | SA                | 28             | 3              | KP176783 / KP176745 | NA     |                  |
| PIM1   | Field  | -31.2509 | 136.5089  | SA                | 1              | 4              | KP176756 / KP176746 | NA     |                  |
| PIM2   | Field  | -31.2509 | 136.5089  | SA                | 1              | 5              | KP176756 / KP176747 | NA     |                  |
| PIM5   | Field  | -31.2509 | 136.5089  | SA                | 1              | 4              | KP176756 / KP176746 | NA     |                  |
| PIM6   | Field  | -31.2509 | 136.5089  | SA                | 1              | 1              | KP176756 / KP176746 | NA     |                  |
| PIM8   | Field  | -31.2509 | 136.5089  | SA                | 1              | 1              | KP176756 / KP176743 | NA     |                  |
| POP1   | Field  | -33.0710 | 141.6372  | NSW               | 20             | 1              | KP176775 / KP176743 | NA     |                  |
| POP4   | Field  | -33.0710 | 141.6372  | NSW               | 20             | -              | KP176775            | NA     |                  |
| POP5   | Field  | -33.0710 | 141.6372  | NSW               | 20             | -              | KP176775            | NA     |                  |
| SAMS    | SAM   | X    | Y    | Q | ID    | P1   | P2   |
|---------|-------|------|------|---|-------|------|------|
| SAM1397 | SAM   | -30.7667 | 138.1767 | SA | 2 | KPI176757 | » | NS1397 |
| SAM1399 | SAM   | -27.1192 | 132.8300 | SA | 6 | KPI176761 | » | NS1399 |
| SAM1400 | SAM   | -27.1191 | 132.8300 | SA | 6 | KPI176761 | » | NS1400 |
| SAM1403 | SAM   | -26.6453 | 132.8858 | SA | 4 | KPI176759 | » | NS1403 |
| SAM1406 | SAM   | -31.2878 | 136.5831 | SA | 1 | KPI176756 | » | NS1406 |
| SAM1412 | SAM   | -26.2747 | 137.3269 | SA | 20 | KPI176775 | » | NS1412 |
| SAM1415 | SAM   | -33.8555 | 140.5361 | SA | 20 | KPI176775 | » | NS1415 |
| SAM1416 | SAM   | -34.0583 | 140.1500 | SA | 20 | KPI176775 | » | NS1416 |
| SAM1606 | SAM   | -26.6922 | 134.1722 | SA | 23 | KPI176778 | » | NS1606 |
| SAM1607 | SAM   | -26.5767 | 137.1933 | SA | 22 | KPI176777 | » | NS1607 |
| SAM1812 | SAM   | -33.3267 | 137.0931 | SA | 15 | KPI176770 | » | NS1812 |
| SAM1823 | SAM   | -33.7511 | 140.2747 | SA | 20 | KPI176775 | » | NS1823 |
| SAM1825 | SAM   | -33.7230 | 140.1238 | SA | 20 | KPI176775 | » | NS1825 |
| SAM1831 | SAM   | -33.7183 | 139.9300 | SA | 20 | KPI176775 | » | NS1831 |
| SAM1834 | SAM   | -33.7236 | 139.0438 | SA | 20 | KPI176775 | » | NS1834 |
| SAM1835 | SAM   | -33.7236 | 139.0438 | SA | 21 | KPI176776 | » | NS1835 |
| SAM1837 | SAM   | -33.7400 | 139.0816 | SA | 20 | KPI176775 | » | NS1837 |
| SAM1917 | SAM   | -32.6244 | 135.0322 | SA | 24 | KPI176779 | » | NS1917 |
| SAM1939 | SAM   | -33.1233 | 136.0214 | SA | 3  | KPI176758 | » | NS1939 |
| SAM2038 | SAM   | -33.1167 | 136.0000 | SA | 3  | KPI176758 | » | NS2038 |
| SAM2053 | SAM   | -24.4036 | 132.8886 | NT | 14 | KPI176769 | » | NS2053 |
| SAM2054 | SAM   | -28.4627 | 129.0102 | SA | 5  | KPI176760 | » | NS2054 |
| SAM2055 | SAM   | -28.4627 | 129.0102 | SA | 5  | KPI176770 | » | NS2055 |
| SAM2056 | SAM   | -28.4627 | 129.0102 | SA | 10 | KPI176765 | » | NS2056 |
| SAM2060 | SAM   | -28.4977 | 129.3205 | SA | 11 | KPI176766 | » | NS2060 |
| SAM2061 | SAM   | -28.4977 | 129.3205 | SA | 11 | KPI176766 | » | NS2061 |
| SAM2062 | SAM   | -24.5060 | 129.2619 | NT | 9  | KPI176764 | » | NS2062 |
| SAM2067 | SAM   | -32.0033 | 135.6558 | SA | 3  | KPI176758 | » | NS2067 |
| SAM2070 | SAM   | -28.8969 | 132.7575 | SA | 12 | KPI176767 | » | NS2070 |
| SAM2071 | SAM   | -28.8969 | 132.7575 | SA | 13 | KPI176768 | » | NS2071 |
| SAM2073 | SAM   | -28.5319 | 131.6903 | SA | 19 | KPI176774 | » | NS2073 |
| Code   | Type | Name  | Lat   | Long   | State | Code  | Lat   | Long   | State |
|--------|------|-------|-------|--------|-------|-------|-------|--------|-------|
| SAM2076 | SAM  | -29.7706 | 131.1081 | SA     | 18    | -     | KP176773 | NS2076 |
| SAM2120 | SAM  | -31.9972 | 140.0644 | SA     | 20    | -     | KP176775 | NS2120 |
| SAM2125 | SAM  | -29.1286 | 135.6997 | SA     | 25    | -     | KP176780 | NS2125 |
| SAM2126 | SAM  | -29.1286 | 135.6997 | SA     | 20    | -     | KP176775 | NS2126 |
| SAM2133 | SAM  | -32.4947 | 135.3644 | SA     | 7     | -     | KP176762 | NS2133 |
| SAM2140 | SAM  | -29.4053 | 132.8556 | SA     | 26    | -     | KP176781 | NS2140 |
| WAM20   | WAM  | -27.4867 | 122.3119 | WA     | 31    | 7     | KP176786/| 85020  |
| WAM31   | WAM  | -27.4867 | 122.3119 | WA     | 31    | 8     | KP176786/| 85031  |
| WAM32   | WAM  | -27.4867 | 122.3119 | WA     | 30    | 8     | KP176785/| 85032  |
| WAM36   | WAM  | -27.3893 | 115.1847 | WA     | 29    | 9     | KP176784/| 78236  |
| WAM37   | WAM  | -27.6145 | 121.9947 | WA     | 17    | 10    | KP176772/| 112637 |
| WAM38   | WAM  | -26.4408 | 115.3661 | WA     | 29    | 9     | KP176784/| 78238  |
| WAM46   | WAM  | -28.7333 | 123.8667 | WA     | 16    | 11    | KP176771/| 80246  |
| WAM55   | WAM  | -27.4867 | 122.3119 | WA     | 31    | 7     | KP176786/| 83855  |
| WAM56   | WAM  | -27.4867 | 122.3119 | WA     | 30    | 7     | KP176785/| 83856  |
| WAM75   | WAM  | -27.4867 | 122.3119 | WA     | 31    | 12    | KP176786/| 83875  |
| WAM88   | WAM  | -25.9307 | 128.4526 | WA     | 8     | 13    | KP176763/| 95988  |
| Um1814  | SAM  | -33.1997 | 138.2189 | SA     | NA    | NA    | NS0001814 |        |
| Um2714  | SAM  | -33.1997 | 138.2189 | SA     | NA    | NA    | NS0002714 |        |
| Un2112  | SAM  | -31.6597 | 129.1083 | SA     | NA    | NA    | NS0002112 |        |

599 NSW: New South Wales; SA: South Australia; WA: Western Australia; NT: Northern Territory
600 NA: Not applicable.
Table 2. List of primer sequences and corresponding amplicons sizes for the three *Urodacus yaschenkoi* loci (COXI, 16S rRNA, 28S rRNA).

| Marker | Primer   | Primer sequence                        | Size (bp) | Reference                  |
|--------|----------|----------------------------------------|-----------|----------------------------|
| COXI   | F C1-J-2183 | 5’-CAACATTTATTTTTGGATTTTTTGG - 3' | 550-630   | (Simon et al., 1994)       |
|        | R COXIKG-R2 | 5’- GATATTAATCCTAAAAAATGTTGAGG-3' |           | (Tanaka et al., 2001)      |
| COXI   | Nested F   | 5’-AGGAACCTTTTGGGGCTTT-3'            | 150       |                           |
| COXI   | Nested R   | 5’-AGGAACCTTTTGGGGCTTT-3'            |           |                           |
| 16S    | F 16SF    | 5’- AACAAAACCCACAGCTCACA- 3'         | 422       | (Gantenbein et al., 2005)  |
|        | R 16SR    | 5’- GTGCAAAGGTAGCATAATCA- 3'         |           |                           |
| 28S    | R1        | F R1S (5’-ACCCGCTGAATTTAAGCAT-3’),  | 1158      | (Arabi et al., 2012)       |
|        |           | R R1AS (5’-GCTATCCTGAGGGAAACTTC-3’)  |           |                           |
|        | R2        | F R2S (5’-CGACCCGTCTTGAAACACGGA-3’), | 1246      |                           |
|        |           | R R2AS (5’-CACCTTGAGACCTGCTGGAT-3’)  |           |                           |
Table 3. Species delineation analyses in *Urodacus yaschenkoi* based on 31 unique mitochondrial haplotypes.

| Analysis type         | # Entities | Statistics                                                                 |
|-----------------------|------------|-----------------------------------------------------------------------------|
| GMYC                  | 9          | Likelihood null model: 32.7519; likelihood best model: 33.36569; likelihood ratio: 1.2255; P-value, 0.0001, confidence interval: 1-10 |
| PTP/bPTP (ML and BL)  | 7          | Acceptance rate: 0.50975; merge: 49942; split: 50058                        |
Supplemental Information

The data sets supporting the results of this article are included within the article and its additional files in Supplemental_Files_1-4.xlsx.

Supplemental File 1. Pairwise uncorrected $p$-distance between 31 unique *U. yaschenkoi* haplotypes and three outgroup haplotypes (*U. novaehollandiae* and two *U. manicatus*). Haplotypes were generated from the concatenated partial sequences of COXI and 16S loci.

Supplemental File 2. Pairwise uncorrected $p$-distance between 13 unique *U. yaschenkoi* haplotypes generated from the partial 28S sequence.

Supplemental File 3. List of haplotype numbers assigned to the *U. yaschenkoi* samples.

Supplemental File 4. Measures (in mm) of seven morphological traits in *U. yaschenkoi* adult females.
Figure captions

**Fig1. Urodacus yaschenkoi sampling locations** across its distribution range (in dark yellow, adapted from (Koch 1977)). Numbers 1 to 5 designate individuals belonging to the sub-lineages (SC1-5) of the south-central major clade (SC); members of the central-western (CW) clade and western (W) clades are marked in green and red color, respectively. Different hypotheses about diversification in various Australian taxa (vicariance, refugia, dispersal corridors) are adapted from (Melville et al. 2016; Pepper et al. 2011; Rix et al. 2015).

**Fig2. Dated phylogeny** (Bayesian tree) for *Urodacus yaschenkoi* based on the concatenated COXI and 16S partial sequences. Putative species inferred with the PTP/bPTP approach are marked as SC1-5, CW and W. 95% CI for each divergence time is shown in blue.

**Fig3. Bayesian unrooted tree** for *Urodacus yaschenkoi* based on the 28S partial sequences.

**Fig4. LDA for body proportions.** Individual scores for the first 3 axes of Linear Discriminant Analysis. 21 body-proportions were measure in *Urodacus yaschenkoi* adult females. Numbers (1,3,4) denote individuals belonging to one of the SC sublineages (SC1,3,4), and CW denotes individuals from the CW clade.
Fig 1.
Fig 2.
647 Fig3.

648

649 O Bayesian PP > 0.95
Fig 4.