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

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

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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*, 16*S*) and one nuclear locus (28*S*). 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.

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Title: Genetic and morphological analyses indicate that the Australian endemic scorpion *Urodacus yaschenkoi* (Scorpiones: Urodacidae) is a species complex

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19 Running title: Scorpion *Urodacus yaschenkoi* is a species complex

Abstract

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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*, 16*S*) and one nuclear locus (28*S*). 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.

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45 **Discussion.** Our results indicate that the taxonomic status of *U. yaschenkoi* requires revision, and
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47 extensive diversity highlights the importance of conserving *U. yaschenkoi* populations from
48 different Australian arid zones in order to preserve patterns of endemism and evolutionary
49 potential.

50

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 m^2) 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 mm^2 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 28*S* 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 *COX1* 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 *COX1* and *16S* genes respectively, and the final 28*S* alignment was 2076 bp in length. The final dataset contained 68 sequences for each of the mitochondrial genes and 27 sequences for the 28*S* 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 (28*S*) 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 *COX1* 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.

206 ***Delineation based on the analyses of morphological measurements***

207 Proportions of several characters that approximate body shape were assessed in 39 female adult
208 specimens that were keyed out as *U. yaschenkoi* (according to Koch, 1977) and were collected at
209 26 locations (Table 1, Fig1). Gender was determined by examining the genital opercula of adult
210 scorpions, with males having a small finger-like projection known as the genital papilla. Because
211 our collection contained only three males, the analyses were done only with females.

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

222 ***Divergence time estimation***

223 The mitochondrial gene tree was time calibrated with divergence times of nodes inferred from
224 95% highest posterior density (HPD) intervals. Scorpion-specific mutation rates of 0.007
225 substitutions/site/million years for *COXI* and 0.005 substitutions/site/million years for 16*S*
226 (Gantenbein et al. 2005a; Gantenbein & Largiadèr 2003) were used to calibrate the tree. These
227 estimates are derived from buthid scorpions and have been used to estimate divergence times
228 among various scorpion lineages including non-buthid taxa (Bryson et al. 2013a; Bryson et al.

2013b; Graham et al. 2012). 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 28*S* 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 28*S* 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.

292 ***Divergence dating***

293 Our time calibrated mitochondrial phylogeny suggested that the split between the major *U.*
294 *yaschenkoi* clades (SC, CW and W lineages) occurred during the late Miocene/early Pliocene (4–
295 7 MYA) (Fig2). Lineage diversifications within SC appear to have occurred during the Pliocene
296 and early Pleistocene (1.8–4 MYA), while finer-scale phylogeographic patterning within the sub-
297 lineages arose during the late Pleistocene (<1 Mya). Divergence time estimates should be
298 interpreted with some caution, as the nucleotide substitution rate was derived from a different
299 scorpion family (Buthidae) and there are large errors margins around 95% HPD estimates.

300 ***Biogeographic patterns***

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

307

308 ## Discussion

309 Our analyses reveal strong genetic and morphological diversification in *U. yaschenkoi* across its
310 range, pointing to the existence of a species complex with at least three putative species High
311 statistical support for the monophyly and the extent of genetic divergence between the main three
312 lineages (6.4–6.9%) exceeds estimates of interspecific divergence previously reported for other
313 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. yaschekoi* 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 sympatry.

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 chala 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. yaschekoi* putative species (SC and CW) show marked shape differences involving chela, further exploration of burrowing behavior or pray 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.

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593 **Table 1. *Urodacus yaschenkoi* specimen location and analyses made**. List of *Urodacus*
594 *yaschenkoi* collected from the field as live specimens (Field) or obtained from the Australian
595 museum collections (South Australian Museum - SA, Western Australian Museum - WA).
596 Geographic position (lat/log) and the geographic region details are reported for each sample. List
597 of haplotypes (mito, 28*S*) and GenBank Accession # scored in each individual. Morphological
598 variation scored (☞), Museum ID.

Sample	Source	Latitude	Longitude	Geographic Region	mito Haplotype	28S Haplotype	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			NA
BKB12	Field	-33.2242	141.3061	NSW	20	1	KP176775 / KP176743		NA
BK13	Field	-33.2283	141.3011	NSW	20	1			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 / KP176743		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

SAM1397	SAM	-30.7667	138.1767	SA	2	-	KP176757	⌲	NS1397
SAM1399	SAM	-27.1192	132.8300	SA	6	-	KP176761	⌲	NS1399
SAM1400	SAM	-27.1191	132.8300	SA	6	-	KP176761	⌲	NS1400
SAM1403	SAM	-26.6453	132.8858	SA	4	-	KP176759	⌲	NS1403
SAM1406	SAM	-31.2878	136.5831	SA	1	-	KP176756	⌲	NS1406
SAM1412	SAM	-26.2747	137.3269	SA	20	-	KP176775	⌲	NS1412
SAM1415	SAM	-33.8555	140.5361	SA	20	-	KP176775	⌲	NS1415
SAM1416	SAM	-34.0583	140.1500	SA	20	-	KP176775	⌲	NS1416
SAM1606	SAM	-26.6922	134.1722	SA	23	-	KP176778		NS1606
SAM1607	SAM	-26.5767	137.1933	SA	22	-	KP176777		NS1607
SAM1812	SAM	-33.3267	137.0931	SA	15	-	KP176770	⌲	NS1812
SAM1823	SAM	-33.7511	140.2747	SA	20	-	KP176775	⌲	NS1823
SAM1825	SAM	-33.7230	140.1238	SA	20	-	KP176775	⌲	NS1825
SAM1831	SAM	-33.7183	139.9300	SA	20	-	KP176775	⌲	NS1831
SAM1834	SAM	-33.7236	139.0438	SA	20	-	KP176775	⌲	NS1834
SAM1835	SAM	-33.7236	139.0438	SA	21	-	KP176776	⌲	NS1835
SAM1837	SAM	-33.7400	139.0816	SA	20	-	KP176775	⌲	NS1837
SAM1917	SAM	-32.6244	135.0322	SA	24	-	KP176779	⌲	NS1917
SAM1939	SAM	-33.1233	136.0214	SA	3	-	KP176758	⌲	NS1939
SAM2038	SAM	-33.1167	136.0000	SA	3	-	KP176758	⌲	NS2038
SAM2053	SAM	-24.4036	132.8886	NT	14	-	KP176769	⌲	NS2053
SAM2054	SAM	-28.4627	129.0102	SA	5	-	KP176760	⌲	NS2054
SAM2055	SAM	-28.4627	129.0102	SA	5	-	KP176770	⌲	NS2055
SAM2056	SAM	-28.4627	129.0102	SA	10	-	KP176765	⌲	NS2056
SAM2060	SAM	-28.4977	129.3205	SA	11	-	KP176766	⌲	NS2060
SAM2061	SAM	-28.4977	129.3205	SA	11	-	KP176766	⌲	NS2061
SAM2062	SAM	-24.5060	129.2619	NT	9	-	KP176764	⌲	NS2062
SAM2067	SAM	-32.0033	135.6558	SA	3	-	KP176758		NS2067
SAM2070	SAM	-28.8969	132.7575	SA	12	-	KP176767	⌲	NS2070
SAM2071	SAM	-28.8969	132.7575	SA	13	-	KP176768	⌲	NS2071
SAM2073	SAM	-28.5319	131.6903	SA	19	-	KP176774		NS2073

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 / KP176749	⚑	85020
WAM31	WAM	-27.4867	122.3119	WA	31	8	KP176786 / KP176750	⚑	85031
WAM32	WAM	-27.4867	122.3119	WA	30	8	KP176785 / KP176750	⚑	85032
WAM36	WAM	-27.3893	115.1847	WA	29	9	KP176784 / KP176751		78236
WAM37	WAM	-27.6145	121.9947	WA	17	10	KP176772 / KP176752		112637
WAM38	WAM	-26.4408	115.3661	WA	29	9	KP176784 / KP176751		78238
WAM46	WAM	-28.7333	123.8667	WA	16	11	KP176771 / KP176753		80246
WAM55	WAM	-27.4867	122.3119	WA	31	7	KP176786 / KP176749	⚑	83855
WAM56	WAM	-27.4867	122.3119	WA	30	7	KP176785 / KP176749	⚑	83856
WAM75	WAM	-27.4867	122.3119	WA	31	12	KP176786 / KP176754	⚑	83875
WAM88	WAM	-25.9307	128.4526	WA	8	13	KP176763 / KP176755		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.
601
602

603 **Table 2. List of primer sequences and corresponding amplicons sizes for the three**
604 ***Urodacus yaschenkoi* loci** (*COXI*, 16*S* rRNA, 28*S* rRNA).

Marker	Primer	Primer sequence	Size (bp)	Reference
COXI	F C1-J-2183	5'-CAACATTTATTTTGATTTTTTGG - 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'-CACCTTGGAGACCTGCTGCGGAT-3')		

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607 **Table 3. Species delineation analyses in *Urodacus yaschenkoi* based on 31 unique**
608 **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

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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* 16*S* 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 28*S* 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**.**

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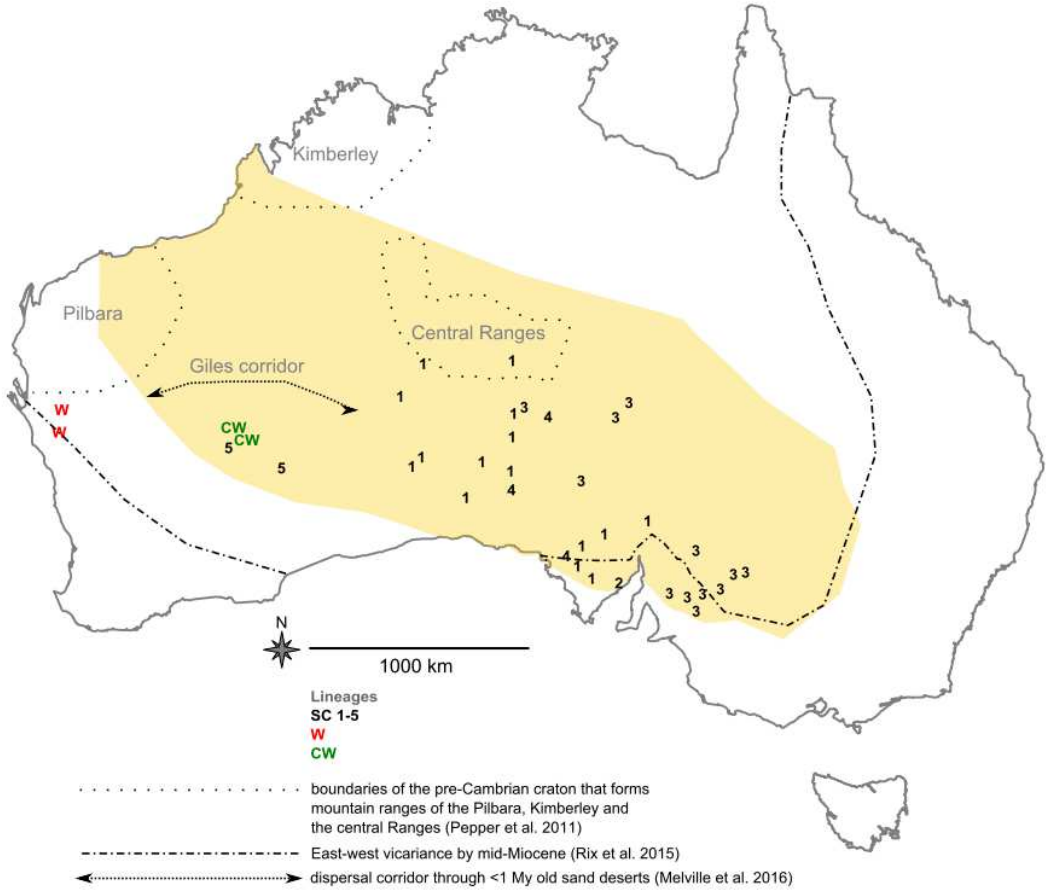
643 Fig1.



644 Fig2.

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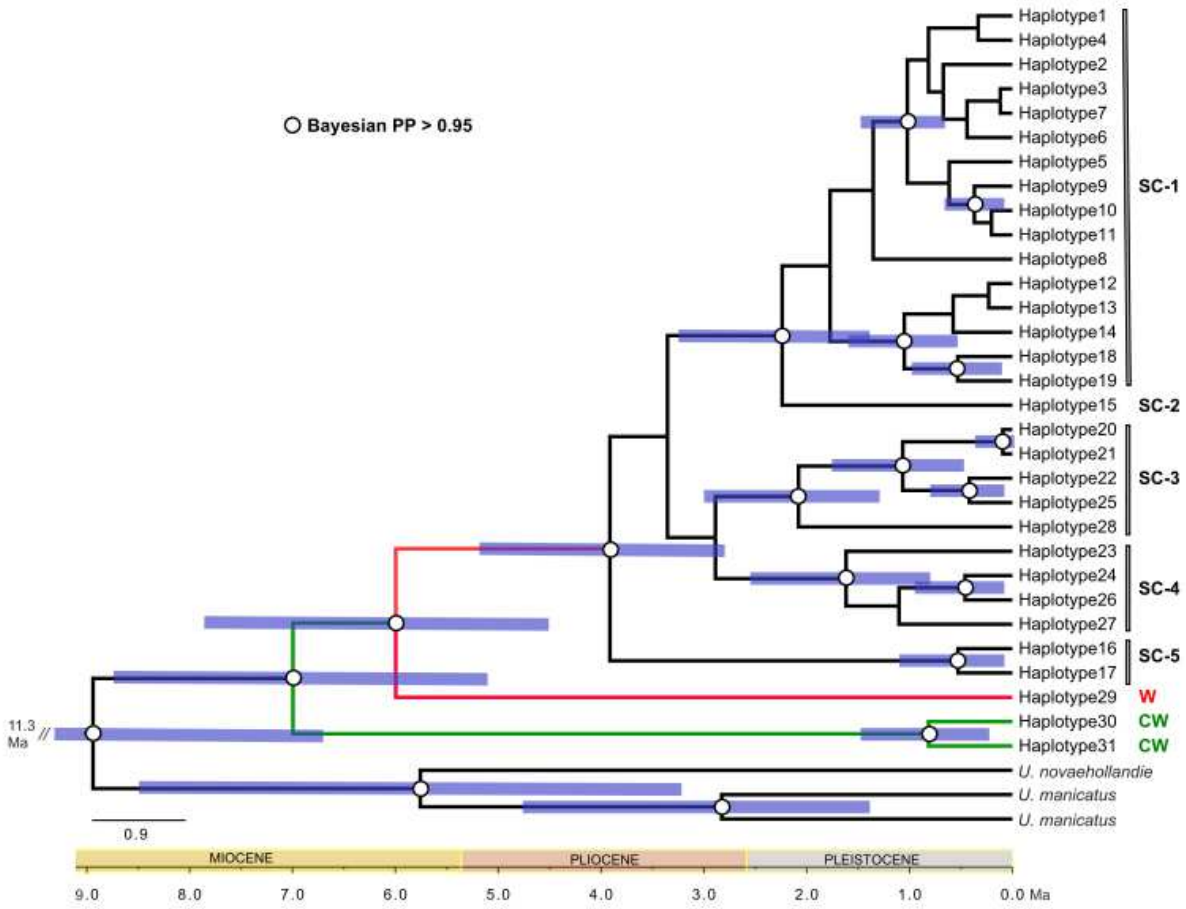

647 Fig3.

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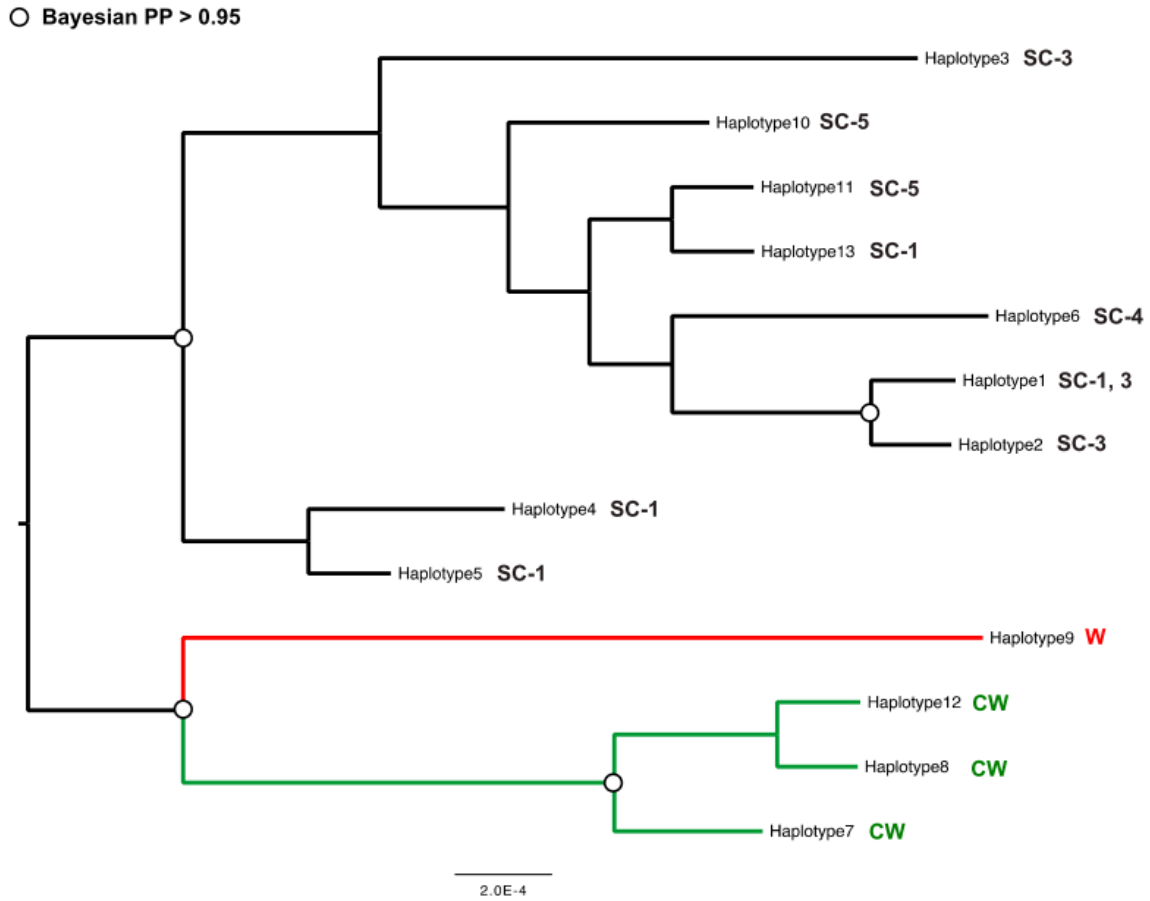

650 Fig4.

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