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# Islands in the desert: Species delimitation and evolutionary history of *Pseudotetracha* tiger beetles (Coleoptera: Cicindelidae: Megacephalini) from Australian salt lakes

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ABSTRACT

The Australian salt lakes are a natural archipelago-like laboratory for investigating evolutionary and population processes. Their environmental conditions have not undergone relevant changes since the aridification of Australia 10–5 million years ago. The genus *Pseudotetracha*, a group of nocturnal tiger beetles found on these remote salt lakes, includes 20 described species. Recent studies based on molecular markers and cytogenetics hinted at the existence of cryptic species within this group. Here we use various species delimitation algorithms to detect a high number of cryptic and undescribed taxa, and challenge the validity of the taxonomic characters traditionally used for discerning species in this group. Our analyses show that the divergence dates of the clades, between 10 and 5 million years ago, correspond to the period in which Australia was undergoing an aridification process that probably isolated the ancestral *Pseudotetracha* populations to individual lakes or palaeodrainage basins. This implies an important role of the isolation, produced by the aridification of Australia, in the speciation and divergence of *Pseudotetracha*, which underwent a remarkable radiation as the populations became geographically restricted.



## 1. Introduction

Australian salt lakes are distributed throughout the arid and semi-arid areas of the continent (De Deckker, 1983). They stay dry much of the time, only holding water after episodic rains which can occur once in a decade or even a century (Timms, 2005). The lakes are usually distributed in palaeodrainage basins, following the courses of palaeorivers (Morgan, 1993), and have remained relatively stable since the mid-Miocene (De Deckker, 1983; Morgan, 1993; Byrne et al., 2008), when Australia underwent an aridification process, which is considered to have begun about 15 million years ago. It wasn't until 10–6 million years before present that major changes to the landscape and vegetation reflected the termination of the warm, wet environments of the earlier Miocene. Subsequently, there was a temporary return (for about 2 million years) to warm wet conditions (from 5 to 3 million years ago) before the onset of the major glacial and interglacial oscillations of the Pleistocene (Byrne et al., 2008). The dry conditions were slightly perturbed afterward by glaciations (Bowler, 1981) or sporadic events (Etten and Vellekoop, 2009). Due to their geography and dynamics after the aridification, Australian salt lakes are an ideal natural archipelago-like laboratory for investigating evolutionary and population processes.

Indeed, molecular phylogenetic studies of a diverse array of Australian arid zone plants, invertebrates and vertebrates are beginning to accumulate (Byrne et al., 2008), exploring the effects of the climatic history of the continent on the current genetic structure and diversity of its organisms. While in some groups of organisms the phylogenetic structure is the result of their particular population history (Lanier et al., 2013; Dennison et al., 2015), some studies have shown how the aridification of Australia (10–5 million years ago) affected the evolution of particular groups (Pepper et al., 2011a). For example, a series of papers on Coleoptera have shown how the stygobiotic taxa originated (Cooper et al., 2002), having being driven underground by changes in the surface environmental conditions (Leijs et al., 2012), and how the speciation rates of other taxa increased during aridification (Toussaint et al., 2016a, 2016b). In tiger beetles (Vogler and Pearson, 2001) of the genus *Rivacindela*, these processes produced a noticeable coherence between the phylogenetic structure, the geographical range and the morphology (Pons et al., 2006).

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Previous works (López-López et al., 2012, 2013) on the Megacephalini tiger beetle genus *Pseudotetracha* Fleutiaux, 1874 hinted at a possible role of the aridification of Australia on their evolutionary history. This genus is constituted by 20 described species (McCairns et al., 1997; Sumlin, 1997; Häckel and Anichtchenko, 2015). They are nocturnal predators living mainly on salt lakes throughout the Australian arid zone (McCairns et al., 1997; Sumlin, 1997), where they hunt at night and dig burrows in which they spend the day. They are one of the two genera of Megacephalini that can be found in Australia, the other being *Australicapitona* Sumlin, 1992 in the northern tropical environments. Due to the remoteness of their habitats, the genus *Pseudotetracha* remains a relatively unknown group.

In order to have an accurate representation of the diversity in a group of living organisms and the processes that have contributed to its formation, it must be determined how many basic taxonomic units can be delimited within that group. This is especially important in understudied, rare or difficult to collect groups (Katz et al., 2015), like *Pseudotetracha*. The utility of mitochondrial markers for unveiling the diversity of organisms and their evolution, especially at genus/species/subspecies levels, has been tested in many groups of organisms, including beetles (Pons et al., 2006, 2011; Andújar et al., 2012; Li et al., 2015). Additionally, with the advent of the statistical analysis of molecular data, a new framework arose to assess the actual diversity of taxonomic groups, using the information provided by DNA sequences for delimiting species (Sites and Marshall, 2003). One of the first methods that did not require the samples to be ascribed *a priori* to particular species was the General Mixed Coalescent model (GMYC) (Pons et al., 2006; Fujisawa and Barraclough, 2013), which was tested using Australian Cicindelini. The GMYC and other algorithms have subsequently been used to test hypotheses on the identity and diversity of various beetle taxa (Pons et al., 2006; Monaghan et al., 2009; Ikeda et al., 2012; Soldati et al., 2014; Fujisawa et al., 2015; Li et al., 2015).

Species delimitation methods based on molecular data are useful in groups of organisms where the discrimination among species is problematic, due to difficulty acquiring a set of comparable characters (Powell et al., 2011). Recent studies on the genus *Pseudotetracha* have challenged the value of the characters traditionally used for discriminating species in this genus (McCairns et al., 1997; Sumlin, 1997), uncovering at least two cryptic species in the *blackburni/murchisona* species complex (López-López et al., 2012; Häckel and Anichtchenko, 2015) and predicting the existence of a high number of cryptic taxa (López-López et al., 2013). Thus, a comprehensive sampling program was required in order to (i) assess the actual diversity of this group using statistical species delimitation algorithms, and (ii) determine the phylogenetic relationships among its constituent taxa.

The aim of this work is to test the hypothesis that there is a large unknown cryptic diversity in *Pseudotetracha*. This diversity may have emerged during the aridification of Australia due to the isolation of lineages in geographically restricted archipelago-like lakes. The combination of molecular methods with geographical distribution will assist in clarifying the taxonomic identity of the poorly known species of this group and reveal putative unidentified taxa. Additionally, the use of phylogenetic and phylogeographic methods will provide a framework for tracing back their population history and comparing it to the sequence of aridification events in Australia.

## 2. Material and methods

Samples were collected from March to May 2012 in South Australia, Western Australia and the southern region of Northern Territory (Supplementary Table 1). For each sample, we sequenced mitochondrial fragments of the cytochrome oxidase III (cox3) and the large subunit of the ribosomal RNA (16S) using the protocols outlined in previous studies of this group (Zerm et al., 2007; López-López et al., 2012, 2013). We chose these fragments in order to be able to combine our data with the data available from those studies. The sequences obtained from the new samples sequenced in this work have been submitted to GenBank (accession codes KT969432–KT969670 for the cox3 fragment and KT969671–KT970055 for the 16S). We included in each alignment the sequences and outgroups used in those preceding studies. The sequences were aligned using MUSCLE (Edgar, 2004) in GENEIOUS (Drummond et al., 2011).

A concatenated matrix was built joining the two individual matrices. In cases where one of the two fragments could not be amplified for a given sample, it was encoded as missing data in the matrix. Generally, missing data do not affect the results of phylogenetic analyses (Wiens, 2006), although it can produce inaccurate results in some cases (Roure et al., 2013). The presence of missing data has a stronger effect in clades with long branches and high character substitution rates (Wiens, 2003), potentially affecting species delimitation methods, but mainly if the study group is narrow and undersampled (Ahrens et al., 2016).

Identical sequences were removed before the phylogenetic analyses, collapsing the matrix into haplotypes. The most appropriate partition scheme and the best nucleotide substitution model for each subsequent partition were determined in PARTITIONFINDER 1.1.1 (Lanfear et al., 2012), testing a partition for the 16S fragment and three for the cox3 fragment, corresponding to the three codon positions.

Four separate Bayesian Inference analyses were carried out in BEAST 1.8.3 (Drummond et al., 2012) in which we combined two different clock models and two tree priors (Table 1). The analyses were ran in the CIPRES Science Gateway (Miller et al., 2010). The best combination was selected according to the Bayes factors calculated in TRACER 1.6 (available from http://beast.bio.ed.ac.uk). The molecular clock was based on the rates obtained by Papadopoulou et al. (2010) for the 16S fragment and by Pons et al. (2010) for the cox3 fragment, which were cross-validated by running preliminary analyses in which one of them was fixed and the other estimated. The analyses ran for 50 million generations and the consensus tree for the best clock and tree prior was built using TREEANNOTATOR (distributed with BEAST).

This tree was used as the input for a GMYC species delimitation analysis using the R package "splits" (Pons et al., 2006; Fujisawa and Barraclough, 2013) including the supplementary functions by Powell et al. (2011) and considering both approaches: single and multiple rates along branches. The same tree was also the base for another species delimitation analysis using the Bayesian implementation of the PTP method (bPTP) (Zhang et al., 2013).

While phylogenetic trees help to understand the relationships among organisms at species or superior taxonomical levels, phylogeographic networks (Posada and Crandall, 2001) are more appropriate for depicting the population history within a species (Avise, 2000, 2009). In order to have a representation of the relationship among the genetic lineages and their geographical distribution, a phylogeographic approach was carried out by building haplotype networks for each main clade. Due to the different substitution rate of the two fragments (cox3 being more variable than the 16S), an independent network for each of the two fragments was built for each clade. Thus, a total of 12 uncollapsed matrices were made, one for each of the six main clades found in the tree and for each fragment (cox3 and 16S). Each of these matrices was processed with PopART (available at http://popart.otago.ac.nz) in order to build the corresponding phylogeographic networks using the Median Joining algorithm (Bandelt et al., 1999).

**Table 1**
Models compared by marginal likelihood (S.E. estimated from bootstrap replicates). Differences between log marginal likelihoods (specifically, log Bayes factors) are reported. Positive values indicate better relative model fit of the row's model compared to the column's model. Molecular clocks: REL (log-normal relaxed clock), STR (strict clock). Tree priors: COAL (Coalescent with constant population size), YULE (Yule model).

| Trace | ln*P*(data\|model) | S.E. | REL COAL | REL YULE | STR COAL | STR YULE |
|---|---|---|---|---|---|---|
| REL COAL | −7410.105 | ±0.323 | – | 4.595 | 359.494 | 403.989 |
| REL YULE | −7414.7 | ±0.104 | −4.595 | – | 354.899 | 399.394 |
| STR COAL | −7769.599 | ±0.173 | −359.494 | −354.899 | – | 44.496 |
| STR YULE | −7814.094 | ±0.223 | −403.989 | −399.394 | −44.496 | – |

## 3. Results

The 16S fragment (271 bp) was obtained for all the individuals, making up a total of 491 sequences: 393 from this study and 98 from the studies by Zerm et al. (2007) and López-López et al. (2013). The cox3 fragment (288 bp) had a lower amplification success rate, having been sequenced from 302 individuals: 239 from this study and 63 from the previous works.

The most appropriate partition scheme implied separate partitions for the 16S and for each of the cox3 codon positions. The best nucleotide model for each partition was: HKY+Γ for the 16S; and SYM+Γ, TIM+Γ and GTR+Γ for the respective cox3 positions. The log-normal relaxed clock performed better than the strict clock (Table 1). The Yule tree prior was only slightly worse than the Coalescent prior, but generated a bizarre topology and was therefore discarded. This was probably due to the fact that the Yule model (Yule, 1925; Gernhard, 2008) is modeled for representing branching depending on speciation events and thus did not accurately represent the intraspecific radiations that our samples are undergoing.

In the phylogenetic tree, our samples grouped into six main clades (Fig. 1). The first clade includes the samples identified as *P. whelani*. Its sister clade is formed by the samples corresponding to *P. oleadorsa* plus the sequences of *P. helmsi* and *P. ion* from Zerm et al. (2007). These entities form a clade that is sister to the rest of *Pseudotetracha* species. On that other clade, *P. australis* is most basal while the most derived clades correspond to the *blackburni*/*murchisona* species complex as described by Sumlin (1997). In this *blackburni*/*murchisona* species complex, three main clades could be distinguished: one corresponding to *P. corpulenta*/*cuprascens*, a second formed by *P. blackburni*, and a third one composed by *P. mendacia* and *P. pulchra*. The only morphological character used in previous works on this genus (McCairns et al., 1997; Sumlin, 1997), which correlates with the main clades obtained in this work is the presence and/or extension of a testaceous apex in the elytra. This apex exceeds 1/3 of the elytral length in *P. australis*, is narrower in *P. whelani*, is very narrow in *P. oleadorsa* and is absent in the *blackburni*/*murchisona* species complex (Fig. 2). Surprisingly, all the other characters traditionally used to discern species in *Pseudotetracha* have little correspondence with the phylogenetic lineages put forward in this work and even show a high degree of variation within the clades.

The GMYC analyses using the single method split our data into 43 clusters and 11 singletons, making a total of 54 entities. The multiple threshold approach produced 69 entities, of which 50 were clusters and 19 singletons. None of the two approaches was significantly better than the other (Chi square = 7.551546, 9 degrees of freedom). The bPTP algorithm divided the data into 37 clusters and 10 singletons, constituting 47 entities. Most of the obtained clusters had a high support value (Supplementary Tables 3–5). From these data, we delimited a total of 37 Consensus Clusters, defined as the groups that were separated in all three methods and by their geographic distribution (Fig. 1; see explanation below).

While the cox3 phylogeographic networks showed a higher diversity of haplotypes, the 16S included more samples and provided better resolution of the relationship between them (Supplementary Figs. 1–6). In general, the genetic structure of the networks showed a clear correspondence with the geography, with clusters of haplotypes isolated in groups of lakes or palaeodrainage basins (Fig. 3), with the exception of *P. australis* (Supplementary Fig. 1) which forms a unique interconnected population. Surprisingly, in the *P. corpulenta* clade (Supplementary Fig. 3) the haplotype variability of the 16S fragment was much higher than that of the cox3. The possibility of dealing with a pseudogene in this clade cannot be discarded, as Pons and Vogler (2005) found a similar situation in the Australian tiger beetle genus *Rivacindela*. Nevertheless, we consider this hypothesis unlikely as the structure and nucleotide composition of this fragment in members of this clade do not differ from of the other clades (results not shown).

## 4. Discussion

Our *Pseudotetracha* samples make up six main clades (Fig. 1), which correspond to six previously described taxa or groups of taxa: (a) *P. mendacia* (including *P. pulchra*), (b) *P. blackburni*, (c) *P. corpulenta* (including *P. cuprascens*), (d) *P. australis*, (e) *P. whelani* and (f) *P. oleadorsa* (including *P. spenceri*). These main clades diverged during the main aridification period of Australia (from 10 to 5 million years ago), and the clades included within them subsequently exhibit an accelerated radiation and diversification, presumably while the individual lineages were isolated in groups of lakes or palaeodrainage basins (from 5 million years ago to present, Fig. 1). Evidence obtained on the age of separation of lineages and the correspondence with Australian climatic and geological data suggest that isolation, due to the aridification of the continent, had a major role in speciation of the genus *Pseudotetracha*. Similar patterns of diversification in other groups of Australian arid zone biota have also been linked to aridification (Cooper et al., 2002; Pepper et al., 2011a; Leijs et al., 2012; Toussaint et al., 2016a, 2016b).

We observed that clusters obtained using the species delimitation methods seem to have a stronger correlation to geographic distribution than to morphology. The only character that correlates with the clades obtained in the tree is the presence and extension of a testaceous area in the elytral apex. This area exceeds 1/3 of the total length of the elytra in *P. australis*, is shorter in *P. whelani*, is narrow to virtually absent in *P. oleadorsa* and is completely absent in *P. corpulenta*, *P. mendacia* and *P. blackburni* (Fig. 2). All the other main morphological traits that have been traditionally applied to discern species in this group (elytral punctation, color, presence of cupreous reflections, extension and shape of the lateral carina of the pronotum, presence of small setae on the abdominal sternites and elytral shape) are heterogeneously distributed throughout the tree, and even exhibit variation within some of the clusters.

Generally, the GMYC and bPTP methods yielded similar results. Both of them split the samples into a multitude of clades. In some cases, clades considered as a single entity by one of the algorithms

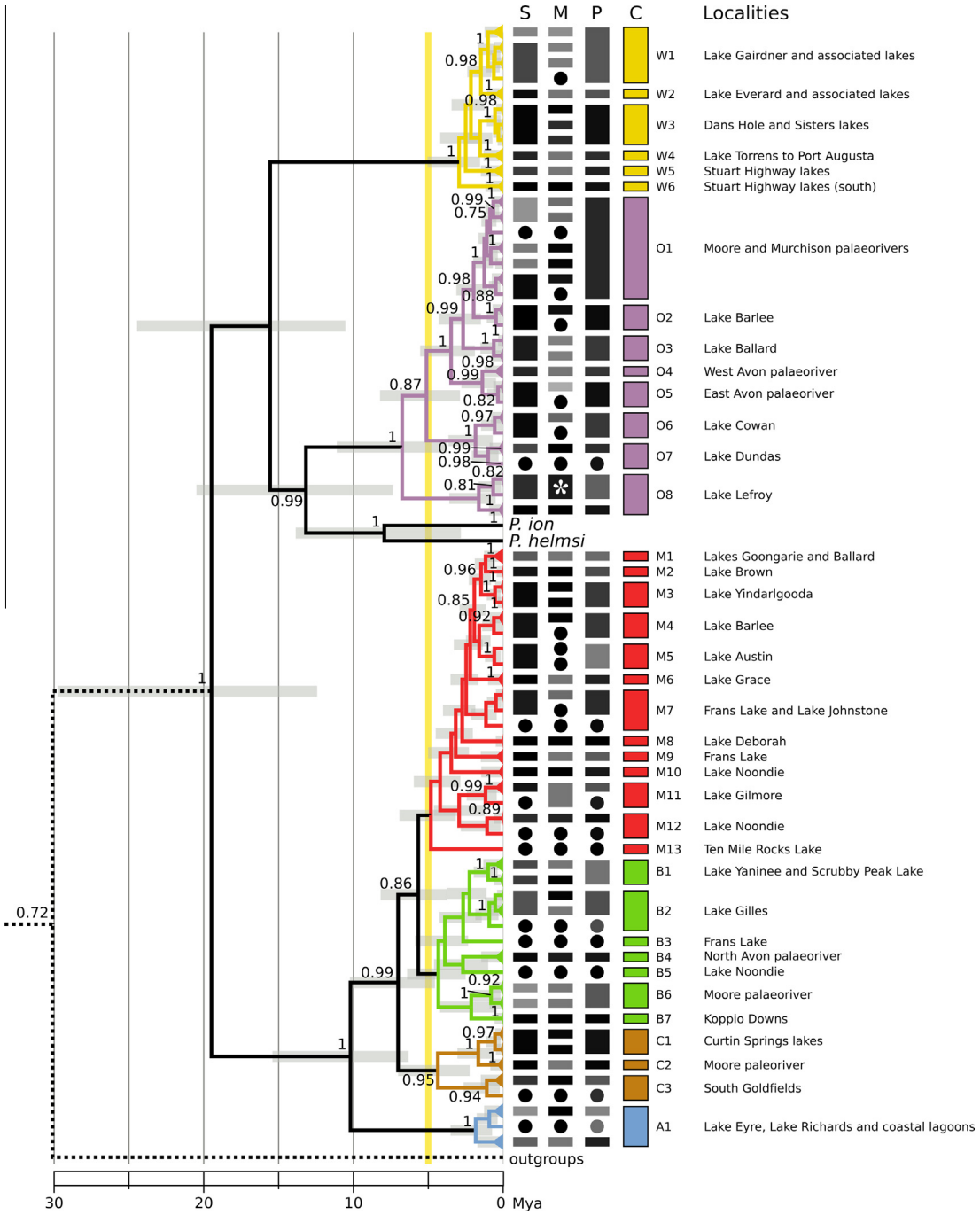


**Fig. 1.** Simplified chronogram obtained from the Bayesian Inference analysis of the concatenated matrix, using a log-normal relaxed clock model and a coalescent tree prior with constant population size. S: clusters delimited by the GMYC algorithm with a single threshold. M: clusters delimited by the GMYC algorithm with multiple thresholds. P: clusters delimited by the bPTP algorithm. C: consensus clusters delimited by considering the three previous alternative algorithms and the geographic distribution (code shown at the right of each cluster). Yellow: *P. whelani* clade, purple: *P. oleadorsa* clade, red: *P. mendacia* clade, green: *P. blackburni* clade, brown: *P. corpulenta* clade, blue: *P. australis*. Numbers in the nodes indicate the posterior probability where it has a value $\geqslant$0.75. The bars in the nodes indicate divergence time estimates with their 95% confidence intervals. The scale axis is the age in million years before present (Mya). The end of the Australian aridification 5 million years ago, according to Morgan (1993), is shown as a vertical yellow line. The asterisk (in cluster O8, multiple threshold) marks two clusters that were considered as a single one, due to their ancestor node having a higher support than each of them separately.

were split by one of the other methods (Fig. 1, Supplementary Table 2). The GMYC algorithm oversplit our samples into more clusters than the bPTP method: this difference has been widely reported in the literature (Zhang et al., 2013; Ahrens et al., 2016; Castelin et al., 2016). This oversplitting is probably caused by the well-defined genetic structure of *Pseudotetracha* species, with populations frequently isolated on lakes, a problem already observed in *Rivacindela* tiger beetles (Pons et al., 2006; Lohse, 2009) and other organisms (Hendrich et al., 2010; Talavera et al., 2013). In fact, according to some studies (Carstens et al., 2013; Talavera et al., 2013), these analyses should not be used as the only evidence for delimiting taxonomical units.

In order to mitigate this difficulty, we applied a methodology, similar to that used by Castelin et al. (2016), to delineate a series

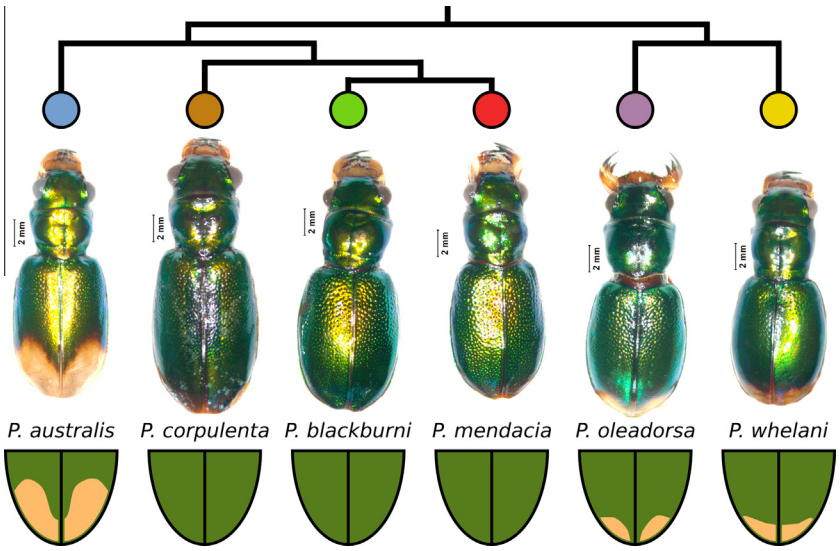


**Fig. 2.** External morphology of samples from each main clade, showing the phylogenetic relationships among them (above) and a schematic representation of the testaceous apex of the elytra (below). Samples, from left to right: 561, 544, 740, 656, 709 and 533.

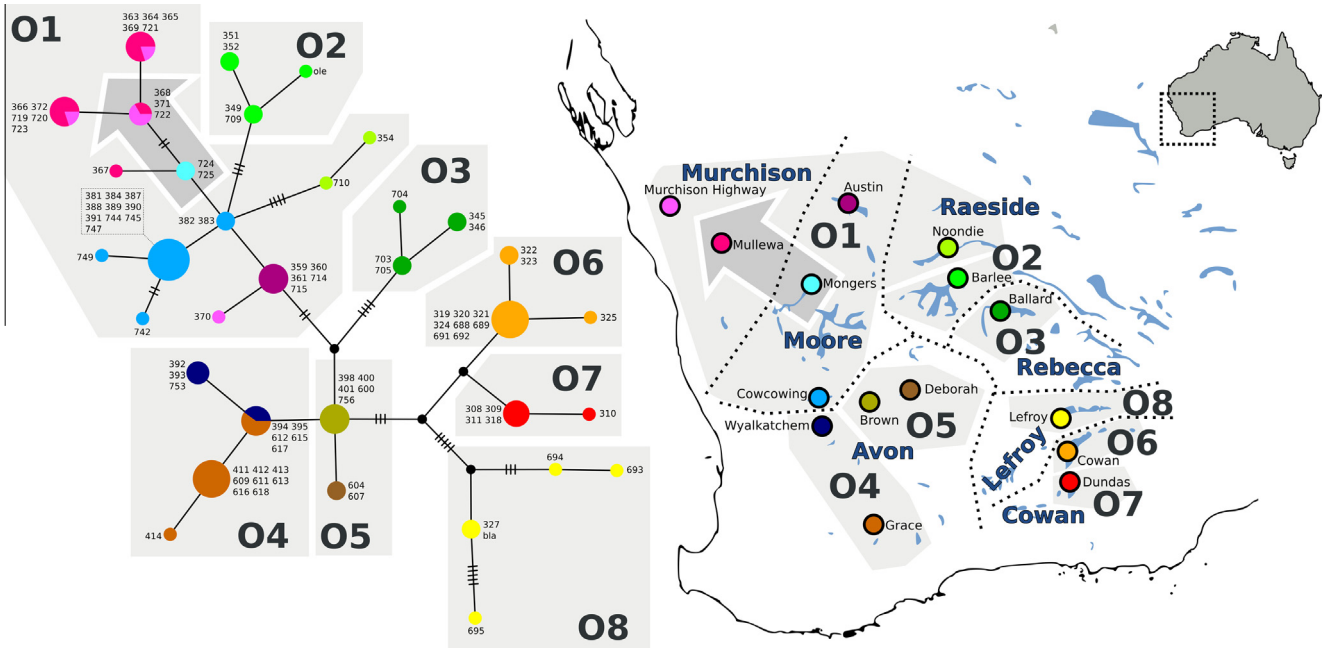


**Fig. 3.** Phylogeographic network for the 16S fragment of the *P. oleadorsa* clade (left) and geographic distribution of the localities in which the samples were collected (right). The haplotypes are colored after the localities, and their size is proportional to the number of samples that share each one. The black dots represent missing haplotypes, and the number of mutational steps is represented as hash marks when >1. The labels of the samples that share each haplotype are shown next to them. The consensus clusters defined after the species delimitation algorithms are labeled O1–O8, and their distribution range shown in the map. The limits (dotted lines) and names (blue text) of the palaeorivers according to the map by Morgan (1993) are shown in the map. The big gray arrow represents the expansion of the O1 cluster towards the Murchison palaeoriver. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of Consensus Clusters (Fig. 1), defined as the groups that were recognized by all the three methods. Additionally, where two sister Consensus Clusters shared the same geographic distribution, they were considered as a single Consensus Cluster. If we consider each Consensus Cluster as a putative species, our samples would made up a total of 38 species with a high degree of geographical coherence. These species would include the described or previously identified taxa *P. whelani* (cluster W4), *P. spenceri* (cluster O1), *P. oleadorsa* (cluster O6), *P. mendacia* (cluster M1), *P.* "blackburni-2" (cluster B1), *P.* "blackburni-1" (cluster B2), *P. blackburni* (cluster B7), *P. corpulenta* (cluster C1), *P. cuprascens* (cluster C2) and *P. australis* (cluster A1). Additionally, the cluster M4 could correspond to *P.* "blackburni-3", a possible species noted by Häckel and Anichtchenko (2015), according to its morphology and locality. It cannot be discarded that some clusters could correspond to *P. pulchra* and *P. castelnaui*, but their identification is uncertain due to the ineffectiveness of the available keys (McCairns et al., 1997; Sumlin, 1997) to accurately identify most of the samples. This study, based mainly on molecular data, highlights the necessity for an integrative taxonomic revision of this genus in order to clarify the taxonomic status of their species and ease their identification. The phylogeny presented in this paper and inclusion

of more data from these samples and species that are absent in this analysis could be used as a framework for identifying new characters that may correctly discern species in the genus *Pseudotetracha*.

The fact that the delimitation of these Consensus Clusters correctly separate the two cryptic *P. blackburni* species identified in previous works (López-López et al., 2012), located on separate lakes and having different karyotypes (López-López et al., 2013), which could operate as a reproductive barrier, supports the validity of this approach for discriminating species in *Pseudotetracha*. Surprisingly, none of these two entities (clusters B1 and B2), named "blackburni-1" and "blackburni-2" in those previous works, would correspond to the actual *P. blackburni* (cluster B7).

In general, our results show that there is a high level of cryptic speciation in *Pseudotetracha*, most likely favored by the aridification of the continent that isolated the populations of a few lineages in separate lakes or systems of lakes. This hidden diversity is similar to that found in other groups of Australian arid zone organisms (Pons et al., 2006; Schwentner et al., 2014). The role of isolation on speciation is more evident in clades like *P. whelani*, where the putative species found in this work are structured in groups of closely related lakes in the Gawler Ranges region, or *P. oleadorsa*, whose lineages are geographically delimited by the borders of ancient drainage basins (Fig. 3).

The directional distribution of haplotypes in the phylogeographic networks provides evidence of a limited dispersion capacity of these populations. Examples of this can be seen in the expansion of cluster O1 towards the Murchison palaeoriver (arrow in Fig. 3) and the colonization of Lake Harris by a sample from clade W4 (asterisk in Supplementary Fig. 6). Anatomical constraints might have reinforced their isolation as these species are only able to do short flights, or are anatomically unable to fly at all (*P. corpulenta*; personal observation). The rare movements observed might be favored by sporadic heavy rains, resulting in a more moderate environment and intermittent flow of water between adjacent lakes, following the paths of the extinct palaeorivers. *P. australis*, on the other hand, seems to form an extensive interconnected population (Supplementary Fig. 1), possibly due to tolerance of a wider salinity range and being able to survive in less saline habitats such as other wetlands, which will reduce the isolation and favor the movement of specimens between distant localities.

Our results show a correlation with previous studies on other groups of animals living in this area. The influence of the Miocene aridification on the origin of the current diversity, and the differentiation and diversification of lineages through isolation of populations on "islands", has been observed in other tiger beetles (Pons et al., 2006), other groups of insects (Cooper et al., 2002) and other components of the Australian arid zone biota (Byrne et al., 2008; Pepper et al., 2011b). The division of lineages according to palaeodrainage basins, which can be clearly observed in *P. oleadorsa*, is similar to that observed in other animals such as lizards (Pepper et al., 2011a). Other works on stygobiotic fauna also discuss the role of colonization and dispersion events in the formation of new lineages that contribute to the generation of a higher diversity (Leijs et al., 2012). This could also be the case of the recent events observed in *P. oleadorsa* or *P. whelani*. In conclusion, our work highlights the influence of the historical climatic changes on the current diversity of the Australian arid zone fauna. This study emphasizes the results of other works warning that this diversity could have been underestimated in previous reports dealing only with morphological characters. Therefore, the use of molecular data and species delimitation analyses is advisable in order to get an accurate representation of how many taxonomical units compose this fauna, and determine the extent to which the aridification affected the diversification of each group of organisms in this overlooked and understudied biome.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.05.017.

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