

PUBLISHED VERSION

Leijs, Remko; van Nes, Egbert H.; Watts, Chris H. S.; Cooper, Steven John Baynard; Humphreys, William Frank; Hogendoorn, Katja. Evolution of blind beetles in isolated aquifers: a test of alternative modes of speciation. PLoS ONE, 2012; 7(3):e34260

Copyright: © 2012 Leijs et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

PERMISSIONS

<http://www.plosone.org/static/policies.action#copyright>

3. Copyright and License Policies

Open access agreement. Upon submission of an article, its authors are asked to indicate their agreement to abide by an open access Creative Commons license ([CC-BY](#)). Under the terms of this license, authors retain ownership of the copyright of their articles. However, the license permits any user to download, print out, extract, reuse, archive, and distribute the article, so long as appropriate credit is given to the authors and source of the work. The license ensures that the authors' article will be available as widely as possible and that the article can be included in any scientific archive.

Open access agreement: US government authors. Papers authored by one or more US government employees are not copyrighted, but are licensed under a Creative Commons public domain license ([CC0](#)), which allows unlimited distribution and reuse of the article for any lawful purpose. Authors should read about [CC-BY](#) or [CC0](#) before submitting papers.

Archiving in PubMed Central. Upon publication, PLoS also deposits all articles in [PubMed Central](#). This complies with the policies of funding agencies, such as the [NIH](#) in the USA, the [Wellcome Trust](#), and the [Research Councils](#) in the UK, and the [Deutsche Forschungsgemeinschaft](#) in Germany, which request or require deposition of the published articles that they fund into publicly available databases.

<http://www.plos.org/about/open-access/license/>

LICENCE

The Public Library of Science (PLoS) applies the [Creative Commons Attribution License](#) (CC-BY) to works we publish (read the [human-readable summary](#) or the [full license legal code](#)). Under this license, authors retain ownership of the copyright for their content, but allow anyone to download, reuse, reprint, modify, distribute, and/or copy the content as long as the original authors and source are cited. **No permission is required from the authors or the publishers.**

Appropriate attribution can be provided by simply citing the original article (e.g., Kaltenbach LS et al. (2007) Huntingtin Interacting Proteins Are Genetic Modifiers of Neurodegeneration. PLoS Genet 3(5): e82. doi:10.1371/journal.pgen.0030082).

For any reuse or redistribution of a work, users must also make clear the license terms under which the work was published.

This broad license was developed to facilitate free access to, and unrestricted reuse of, original works of all types. Applying this standard license to your own work will ensure that it is freely and openly available in perpetuity.

If you have a question about the Creative Commons License please use this [contact form](#) and choose "General Questions."

3 September 2012

<http://hdl.handle.net/2440/72976>

Evolution of Blind Beetles in Isolated Aquifers: A Test of Alternative Modes of Speciation

Remko Leijs^{1,2,3*}, Egbert H. van Nes⁴, Chris H. Watts¹, Steven J. B. Cooper^{1,2}, William F. Humphreys^{2,5,6}, Katja Hogendoorn⁷

1 South Australian Museum, Adelaide, South Australia, Australia, **2** School of Earth and Environmental Sciences, University of Adelaide, Adelaide, South Australia, Australia, **3** School of Biological Sciences, Flinders University of South Australia, Adelaide, South Australia, Australia, **4** Department of Aquatic Ecology and Water Quality Management, Wageningen University, Wageningen, The Netherlands, **5** Western Australian Museum, Welshpool, Western Australia, Australia, **6** School of Animal Biology, University of Western Australia, Nedlands, Western Australia, Australia, **7** School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, South Australia, Australia

Abstract

Evidence is growing that not only allopatric but also sympatric speciation can be important in the evolution of species. Sympatric speciation has most convincingly been demonstrated in laboratory experiments with bacteria, but field-based evidence is limited to a few cases. The recently discovered plethora of subterranean diving beetle species in isolated aquifers in the arid interior of Australia offers a unique opportunity to evaluate alternative modes of speciation. This naturally replicated evolutionary experiment started 10–5 million years ago, when climate change forced the surface species to occupy geographically isolated subterranean aquifers. Using phylogenetic analysis, we determine the frequency of aquifers containing closely related sister species. By comparing observed frequencies with predictions from different statistical models, we show that it is very unlikely that the high number of sympatrically occurring sister species can be explained by a combination of allopatric evolution and repeated colonisations alone. Thus, diversification has occurred within the aquifers and likely involved sympatric, parapatric and/or microallopatric speciation.

Citation: Leijs R, van Nes EH, Watts CH, Cooper SJB, Humphreys WF, et al. (2012) Evolution of Blind Beetles in Isolated Aquifers: A Test of Alternative Modes of Speciation. PLoS ONE 7(3): e34260. doi:10.1371/journal.pone.0034260

Editor: Bengt Hansson, Lund University, Sweden

Received: November 11, 2011; **Accepted:** February 24, 2012; **Published:** March 30, 2012

Copyright: © 2012 Leijs et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This research was supported by Australian Research Council grants to SJB, CHW and WFH (A00106441 and DP0663675) and RL (DP0346583), and by Australian Biological Resources Study grants to CHW and WFH. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: remko.leijs@samuseum.sa.gov.au

Introduction

Strong evidence for sympatric speciation has recently been provided in vitro [1], and the concept is well supported by theoretical analyses [2–7]. Often cited examples from natural systems involve the evolution of new species in relatively closed systems such as crater lakes (e.g. cichlid fishes [7,8]), and islands (e.g. Anolis lizards [9], palms [6,10,11] Hawaiian spiders [12]) where there is evidence for colonisation by a single ancestral species and subsequent niche partitioning. However, even after the presence of sympatric, closely related sister species has been established, it remains uncertain whether the co-occurring species pairs have evolved in sympatry or whether the divergence of the species occurred in isolation and involved multiple invasions [13–16]. To distinguish between these modes of speciation a statistical approach is needed, which requires the presence of multiple sympatrically occurring species pairs. Such data sets have hitherto been lacking.

The recent discovery of communities of invertebrates in Australian subterranean aquifers that have evolved in isolation for millions of years provides a unique opportunity to evaluate the occurrence of sympatric versus allopatric modes of speciation in a natural environment.

In the Late Miocene – Pliocene, (10–5 million years ago, Mya), the interior of Australia underwent aridification [17]. During this

process hundreds of subterranean aquifers in calcrete limestone deposited along palaeo-drainage systems became biologically isolated [18–22] (Figure 1). Phylogenetic studies [21] revealed that surface species of diving beetles (Coleoptera, Dytiscidae) took refuge in these subterranean aquifers during one or more periods of extreme aridity. This resulted in the evolution of one to three species of blind, wingless (apterous), de-pigmented (stygobitic) endemic species per aquifer. The aquifers all provide similar, very stable ecological conditions [23], and thus the colonisation events can be viewed as a repeated natural speciation experiment.

The massive radiation generated by this natural experiment has only recently been uncovered. In the last 12 years 99 new stygobitic beetle species have been described from 52 isolated aquifers [24]. These species now represent, by far, the world's most diverse subterranean diving beetle fauna [25]. Each beetle species is restricted to a single aquifer, indicating the complete isolation of the system. The coexisting species all differ markedly in size and morphology ([24] and references therein), which points to the possibility that they occupy distinct niches. In most aquifers, the co-existing species appear to be descendants from distantly related ancestral lineages [21], suggesting an allopatric process of speciation. However, eleven of the studied aquifers contain sister species.

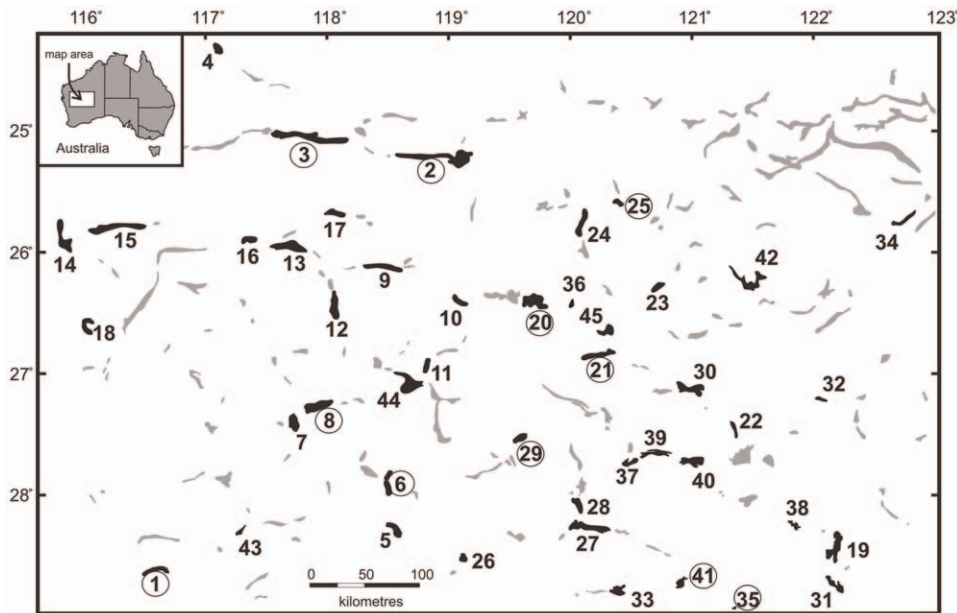


Figure 1. Distribution of calcrete aquifers in Western Australia. Black: aquifers where subterranean diving beetles were found; grey: aquifers not sampled or not containing diving beetles. The numbers denote aquifer localities; numbers in circles are aquifers containing sympatric sister clades. The coordinates and species composition of the aquifers are given in the supplemental information S1. doi:10.1371/journal.pone.0034260.g001

Here, as advocated by Fitzpatrick et al. [26], we investigate the biological processes that may have led to the divergence of the sympatrically occurring sister species. We consider two hypotheses to explain the presence of sister species in the same aquifer. Firstly, the same ancestral surface species may have colonised an aquifer repeatedly at different times, for instance, during different aridity maxima [27]. In this case, the first colonising species would evolve into a new stygobitic species prior to a second invasion by the same ancestral lineage. Secondly, speciation may have occurred *within* the aquifer, after invasion of the underground habitat by a single ancestral species.

We use a statistical model to test the repeated colonisation hypothesis and evaluate it against the within-aquifer speciation hypothesis. The model (Figure 2, methods) predicts the fraction of aquifers containing sympatric sister species, assuming two or three colonisation events. We show that it is unlikely that the observed high frequency of co-occurring sister species pairs and triplets is the result of repeated colonisations, and that the pattern is better explained by diversification within the aquifers.

Results

Phylogenetic analyses

Bayesian phylogenetic analyses (Figure 3a) demonstrate a well-supported topology of two tribes of diving beetles (Dytiscidae). These phylogenies also show nine cases of sympatric sister pairs, and two triplets of sympatric sister species. Nine of these sympatric sister clades are supported by high (1.00) posterior probability values, while the two clades that have lower support were also found with parsimony and neighbour joining analyses using PAUP* [28] and Bayesian analyses using MrBayes [29] (data not shown). Parameter estimates from the Bayesian analyses are available in the supporting information S2. A lineage-through-time (LTI) plot (Figure 3b) shows that the major radiation of subterranean beetles took place 3–7 million years ago.

Repeated colonisation model

We used a model (see methods) to test the repeated colonisation hypothesis in order to predict the fraction of aquifers with pairs and triplets of sympatric sister species. If we assume two distinct colonisation events, the probability of finding sympatric sister species is maximized when the initial colonization probabilities are 0.5 (supporting information S3 Figure 4). At this value, the model generally predicts a much lower number of sister pairs than observed and no triplets (Figure 4a). The observed fraction of sister pairs is within the 95 percentiles of the model outcomes if the ancestral species pool is assumed to contain less than 4 species. If we assume three colonisation events, the predicted number of pairs do not change substantially (Figure 4b), while the probability of finding triplets remains very small and only possible with very low numbers of ancestral species.

The phylogenetic analysis (Figure 3) shows that during the major radiation of subterranean species, the number of ancestral species was certainly larger than four (actually more than 20, Figure 3a and 3b). Therefore, we reject the repeated colonisation model as the only explanation for the evolution of sister species in aquifers.

Single colonisation model

Next, we explore the most extreme alternative, i.e. whether the number of sympatric sister pairs can be explained by single colonisations and subsequent speciation within the aquifers (see methods). Here, the predicted fraction of sister species does not depend on the number of ancestral species. Note that the model now generally predicts higher values than observed. However, if the initial colonization probability is between 0.2 and 0.85, the predicted number of pairs and triplets of sister species does not differ significantly from the observed values. An initial colonization probability of circa 0.78 provides a near-accurate prediction of the fraction of aquifers with sister taxa based on the observed 9 pairs and 2 triplets out of 45 aquifers (Figure 4c). Therefore, in contrast to the repeated colonisation model, the model of single

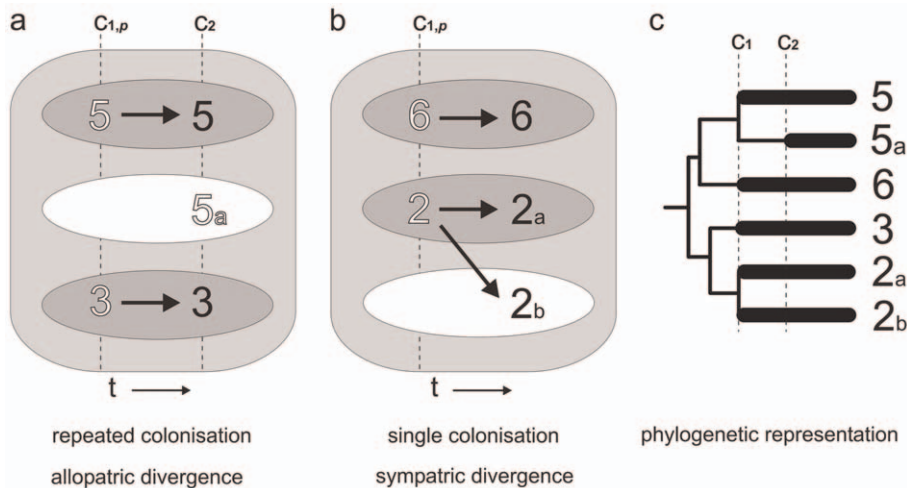


Figure 2. Schematic representation of the colonization models. (a and b): The outer box represents a calcrete aquifer, the ovals represent individual niches, which may get colonized with niche colonization probability p , C_1 and C_2 are colonization events. The numbers represent colonizing species that are randomly drawn out of a pool of n ancestral species. (c): Phylogenetic representation of the models; bold lineages evolve underground. Species 5 and 5a are sympatric sister species that evolved by repeated colonization; species 2a and 2b are sympatric sister species that diverged in the aquifer after the colonization of their ancestor species 2; species 3 and 6 independently colonized aquifers. doi:10.1371/journal.pone.0034260.g002

colonisation, which assumes subsequent diversification within the aquifers, is capable of predicting the observed number of sympatric sister species.

Discussion

This study provides strong support for speciation of blind water beetles within the isolated aquifers. Under the alternative model of allopatric speciation and repeated colonization by the same ancestral surface species, the frequency of sympatric sister species would be significantly lower than observed in the field. Thus, repeated colonization alone cannot explain the high frequency of pairs and triplets of sympatric sister species in the aquifers, and therefore, at least some speciation within the aquifer needs to be invoked to explain the high frequency of sister species pairs and triplets. Especially the probability of finding two triplets of species is extremely low in the model assuming repeated colonization. However, whilst the analyses demonstrate that a majority of the sister species can be explained by speciation within the aquifer, we cannot assert that this holds for *all* 11 sympatric sister groups. In some cases, speciation could have taken place by repeated colonisation, as suggested above.

The value of a model critically depends on its assumptions. The main assumption of our model of repeated colonisation is that the ancestral species have an equal chance to successfully colonize aquifers, else the ancestral species pool will be effectively smaller. To meet this assumption the ancestral species must have had widespread and largely overlapping geographical distributions. As already noted by Darwin [30], and confirmed by other studies [31–37], most dytiscid water beetles indeed have very widespread overlapping distributions, are able to fly large distances and are capable of rapidly colonizing newly available habitats, such as roadside ditches, ponds or temporary streams. Moreover, intrinsic factors of species, such as size or pre-adaptations to subterranean life, could make certain species more likely colonizers than others. Although diving beetle assemblages usually consists of a number of distinct size classes [38], in the system described here only species belonging to genera that exclusively fit in the smallest size class (2–5 mm) appear to have successfully colonized the aquifers. A

lineage-through-time (LTT) plot (Figure 3b) shows that prior to the major radiation of the subterranean species at least 30 ancestral species within these genera were present.

We further assumed that an aquifer can only contain a limited number of species, or niches. This is fully supported by the available data [21,24,39] showing that despite intensive survey work in the Yilgarn area over the last decade only up to 3 beetle species were found in each aquifer. A further assumption is that once a species has successfully colonised an aquifer it is very unlikely to subsequently colonise and diverge into another aquifer. The rationale for this is that suitable aquifers are isolated from each other by fine alluvial sediments that do not allow subterranean dispersals and above ground dispersal would be hampered by stygobiontic (eg. loss of wings, pigment, eyes) adaptations. The pattern of unique species per aquifer has also been found for several other taxa that live in these aquifers, such as Amphipoda [18] Isopoda [19] and Bathynellacea [20] and supports the long-term and near complete isolation between aquifers. Secondary divergence would only be possible when a single aquifer becomes physically fragmented.

Unlike several other speciation models [2–4,6,7], our model does not include assumptions about the genetic and ecological processes of diversification. We simply tested whether the occurrence of multiple independent sympatric sister pairs could be explained by repeated colonisation by the same ancestral lineages as a null-model, as an alternative to a process of speciation occurring within aquifers. We explored the behaviour of the repeated colonisation model by maximising the probability of finding sympatric pairs by using equal niche colonisation chances and by assuming that unoccupied niches will always be filled during the second colonisation. Even under such conservative parameter settings, allopatric speciation by repeated colonisations by ancestral lineages could not explain the observed number of sympatric sister species within aquifer.

Although our analysis provides strong support for speciation within the aquifer, we hesitate to classify these speciation events as sympatric speciation, for several reasons. First, as indicated by Butlin et al. [40] and Fitzpatrick et al. [26], [41], speciation processes should be viewed as a continuum in geographic modes

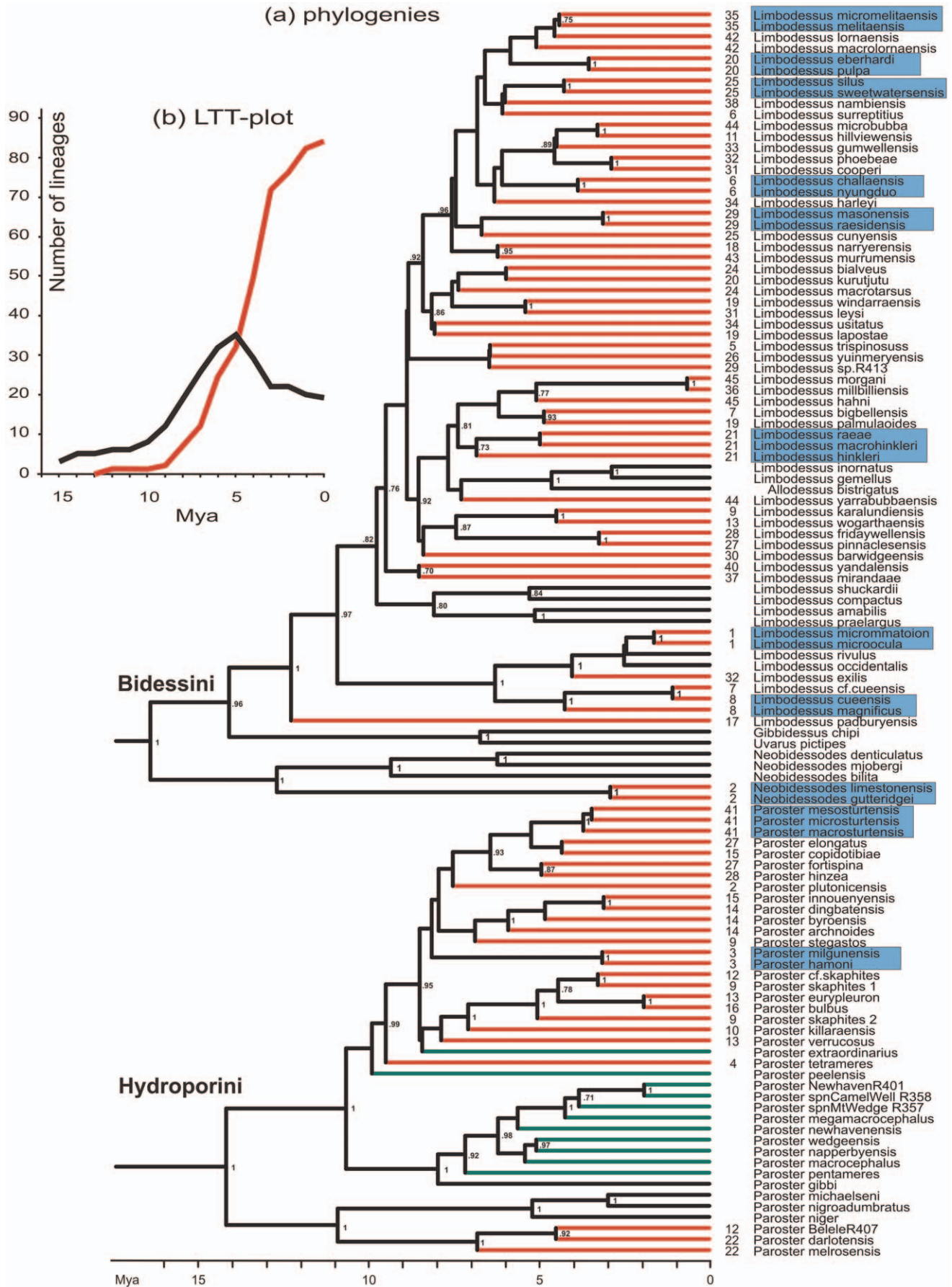


Figure 3. Molecular phylogeny and lineage trough time plot of dytiscid diving beetles. (a): Molecular phylogeny with sympatric sister pairs (blue boxes) shown. Red lines indicate terminal branches leading to a subterranean species. It is assumed that somewhere on the branch the colonization of the subterranean environment took place (see also Leys et al 2003). Black lines indicate surface lineages. Green lines indicate subterranean lineages from aquifers outside of the Yilgarn region. These were not used in the analyses. The numbers at the tips of the branches refer to the Yilgarn calcrete aquifer localities as indicated in Figure 1. Posterior probabilities >0.7 of the Bayesian analyses are indicated near the branches. (b): Lineage-through-time (LTT) plot for surface (black) and subterranean (red) lineages demonstrating the presence of twenty or more ancestral surface species during the major radiation of subterranean beetles 3–7 Mya. doi:10.1371/journal.pone.0034260.g003

with sympatric and allopatric speciation at the extremes. Second, identification of a mode of speciation for the sympatrically occurring sister species would require unwarranted speculation about the nature of reproductive barriers within the aquifers. Third, such a classification would ignore the possibility that, during the formation of the sister species groups, reproductive isolation varied over time. In this context, it is more informative to investigate the extant reproductive barriers within aquifers.

Conceivably, there are two scenarios for microallopatric/parapatric speciation. First, aquifers with a linear structure could become colonised by a single ancestral species at different localities simultaneously, while an overlap in their within-aquifer distributions is established only after a period of time has allowed the populations to become genetically and reproductively isolated. Second, some of these aquifers may become physically fragmented, e.g. due to fluctuations in water levels, and rejoin later allowing time for genetic isolation of beetle lineages. Recent comparative phylogeographic analyses of a sympatric sister triplet at Sturt Meadows (aquifer #41; Figure 3) and tree distantly related species at Laverton Downs (aquifer #19; Figure 3), provide some evidence for past population fragmentation events [42]. However, this does not imply that fragmentation is the basis of reproductive isolation between all sympatric sister species. One would expect such processes to occur more often in large or linear aquifers, but there was no difference between the surface area of aquifers containing sister species and aquifers without sister species ($t = 0.33$, $P = 0.74$). Sympatric sister species were found in very large, linear aquifers (e.g. Three Rivers aquifer #2: 240 km²), as well as in tiny aquifers (e.g. Sons of Gwalia aquifer #35: 2.51 km²).

In addition to parapatric/microallopatric speciation, our data do not exclude the possibility of sympatric speciation. Claessen et al. [2] propose sympatric speciation models where cannibalism and competition for food can result in size-structured populations, which can lead to ontogenetic niche shifts and ultimately to evolutionary branching [3]. Interestingly, in the diving beetles, all of the proposed ingredients for such ontogenetic niche shifts were present. The onset of aridity triggered the beetles to take refuge underground. During the transition from the surface to subterranean environment available food sources would have dramatically decreased, leading to fierce competition for food. Furthermore, the diving beetles are at the top of the subterranean food web, as especially larvae of the diving beetles are ferocious predators; and cannibalism among diving beetle larvae is common. In support of Claessen's [3] model, it is noteworthy that the sympatric subterranean species fall into different size categories [24], that are, in most of the localities, significantly non-overlapping (Vergnon et al., in preparation).

To date, studies of sympatric speciation of natural species have been hampered by small numbers of speciation events per taxon, which did not permit ruling out past involvement of geographic barriers to gene flow and repeated colonisation. The only occasions where inferences about geographical distributions of the ancestral species can be made more reliably are where organisms colonized islands [12,43], including crater lakes [8,44], or caves [45]. Our data substantially contribute to the study of sympatric evolution, as it demonstrates sympatric sister species in 11 rather than two or three

isolated communities, which is unique in that it allowed statistical analysis of the possible speciation modes.

In conclusion, using simple colonization models, we have shown that colonization of aquifers by ancestral diving beetles was largely a random process, and that the high occurrence of sympatric sister species within aquifers is best explained by a process of diversification within the aquifer. Our data thus suggests that within aquifer speciation is not rare in these systems. Due to the large number and variety of speciation events, this group offers considerable potential as a model system for further investigating the factors that promote divergence and speciation.

Methods

Taxon sampling and molecular analyses

This research is based on phylogenetic data of 114 diving beetle species belonging to the dytiscid tribes Bidessini and Hydroporini, including 84 subterranean diving beetle species from 45 aquifers in the Yilgarn region of Western Australia and almost all known surface species. We added DNA sequence data of 35 species (mainly from the Bidessini clade) to a mitochondrial DNA data set of 1655 base pairs, which was previously used to study the systematics and evolution of both tribes of diving beetles [21,39,46]. DNA methods used are described in Leys & Watts [46]. Uncorrelated lognormal molecular clock analyses with BEAST [47] using a mean rate of 0.0115 substitutions per site per million year [48] and a Yule process of speciation, were performed applying unlinked data partitions for each of the codons for the protein coding genes and separate partitions for stems and loops for RNA genes using a general time reversible model of sequence evolution with invariable sites and gamma distributed rates across sites (GTR+i+g). Tracer v1.4 [49] was used to make sure that the effective sample size (ESS) of the parameters during the BEAST runs were larger than 100. The GenBank accession numbers and estimated parameter values for the examined taxa are given in the supporting information S1 and S2.

We are aware of the potential problems with using a mtDNA tree as a representation of the species tree. However, phylogenetic analyses using the nuclear gene *cinnabar* [50] and unpublished data, concur with the mtDNA phylogeny presented here, with respect to sympatric sister species relationships. We therefore suggest that our mtDNA phylogeny provides an accurate assessment of the proportion of sympatric sister species and is suitable for testing the modes of colonisation in the subterranean habitats.

Models of repeated and single colonization of aquifers

The models are based on the assumption that temporary dried up pools in the drainage valleys may fill again after rain, and are recolonized randomly out of a suite of co-occurring diving beetle species. Colonization of the subterranean aquatic habitats would then have taken place at sites where these temporary pools dried out and were connected to calcrete aquifers. The first model was used to test the hypothesis that sympatric sister species may have evolved because colonization of the aquifers had taken place in at least two distinct periods (Figure 2a). The two periods must have been sufficiently far apart to allow for evolution of the first colonizers.

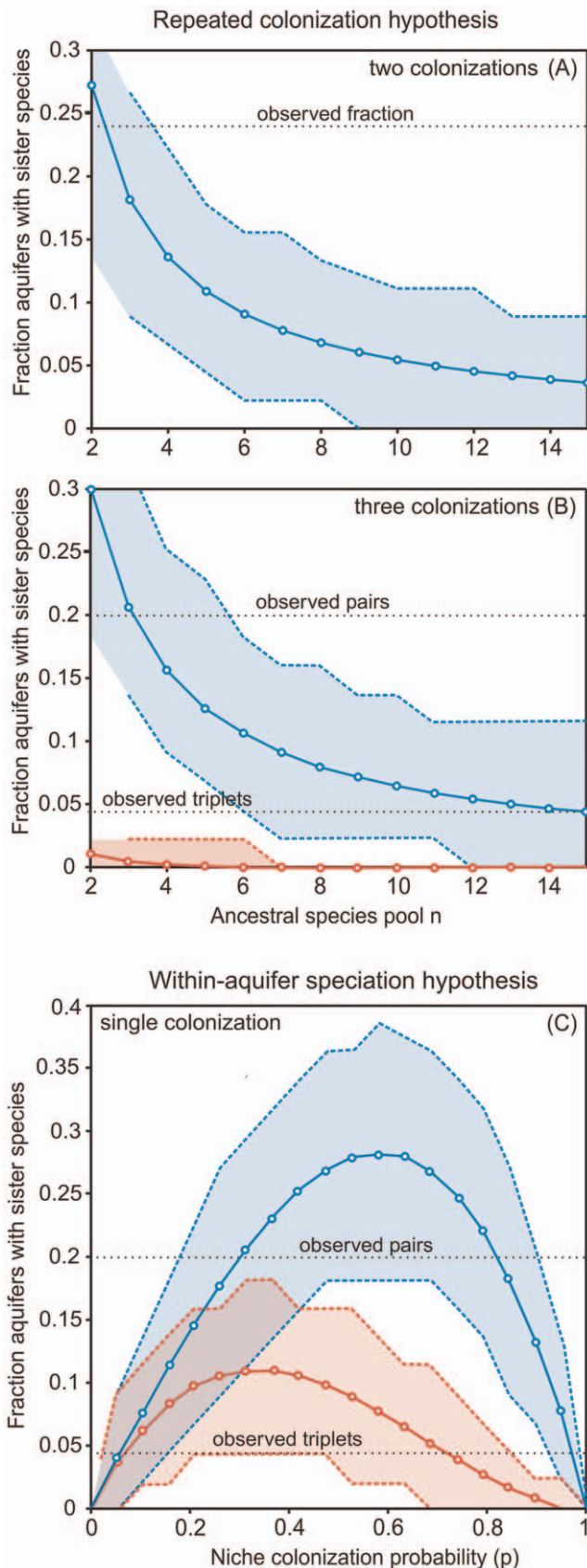


Figure 4. Results of colonization modeling. (a & b) Repeated colonization model. (a) The relationship of the size of the ancestral species pool and the fraction of the aquifers containing sister species

after two colonization events (formula 1). An initial niche colonisation probability (p_1) of 0.5 was used as this maximises the probability of sister pairs (see supporting information S3). The last colonisation probability (p_2) was set to 1. The observed fraction of aquifers with sympatric sister species (11/45) is also indicated. (b): The predicted fraction of aquifers containing sympatric sister pairs (blue; formula 2) and triplets (red; formula 3) calculated based on three colonization periods and a niche colonization probability ($p_1 = p_2 = 0.4$) that maximizes the probability of pairs and triplets (see supporting information S3). Horizontal lines indicate the observed fraction of aquifers with sister pairs and triplets. The last colonisation probability (p_3) was set to 1. (c): Within-aquifer speciation model. The relationship between the initial niche colonization probability and the predicted fraction of aquifers containing sympatric sister pairs (blue) and triplets (red) calculated with single colonizations and subsequent divergence within aquifers. Horizontal lines indicate the observed fraction of aquifers with sister pairs and triplets. The models are calculated using the observed number of aquifers with one (18 aquifers), two (16 aquifers) or three (11 aquifers) species. The shaded areas in (A–C) represent the 5% and 95% percentiles as confidence limits from 10000 randomizations. We assumed that all open niches were filled by speciation ($q = 1$).

doi:10.1371/journal.pone.0034260.g004

We assume that there are a few niches available in the aquifers (e.g. three in Figure 2a) and that each niche can be occupied by only one species because of competitive exclusion. In the first colonization period (C_1) each of these niches will be successfully colonized by a random species of a pool of n ancestral species with a probability p_1 . In a second colonization period (C_2) the remaining available niches (in Figure 2a represented by the white oval) will be colonized by species randomly drawn from the same species pool with a probability of p_2 . In aquifers with two niches, sympatric sister species can only occur through repeated colonization when during the first event only one niche is filled ($P = 2 p_1(1 - p_1)$) and during the second event the remaining niche is colonized by the same species ($P = p_2/n$). Hence, in aquifers with two niches, the probability of sister species through repeated colonization ($P_{2n, pair, 2c}$) is:

$$P_{2n, pair, 2c} = 2 p_1(1 - p_1) \frac{1}{n} p_2$$

Similarly, in aquifers with three niches, the probability of finding sister species after two colonization events is:

$$P_{3n, pair, 2c} = 3 p_1(1 - p_1)^2 p_2^2 \frac{2}{n} + 6 p_1(1 - p_1)^2 p_2(1 - p_2) \frac{1}{n} + 3 p_1^2(1 - p_1) p_2 \frac{2}{n}$$

For simplicity we only derive the probability of finding pairs for three colonization events if we assume $p = p_1 = p_2$ and $p_3 = 1$. The probability of finding pairs in two niches after three colonization events is then:

$$P_{2n, pair, 3c} = \frac{1}{n} (2p^2(1 - p) + 2p(1 - p)^2 + 2p(1 - p)^3)$$

The probability of finding pairs in aquifers with three niches after three events can be derived as:

$$P_{3n, pair, 3c} = 12 p^2(1 - p)^3 \frac{(n - 1)}{n^2} +$$

$$\frac{6}{n} (p(1 - p)^4 + p(1 - p)^5 + p^2(1 - p)^2 + p^2(1 - p)^4 + p^3(1 - p)^2 + p^3(1 - p))$$

Triplets can only occur through independent colonizations if we

assume that there are at least three successful colonization events. The probability of finding triplets through independent colonizations is then:

$$P_{3n,triplet,3c} = 6 p_1 (1-p_1)^2 p_2 (1-p_2) p_3 \frac{1}{n^2}$$

To calculate the maximum overall expected fraction of aquifers containing sister species pairs due to repeated colonization, we assume that the current number of species reflects the number of niches in an aquifer, and therefore, that all available niches become occupied during the last colonization. After two colonization periods, the maximum expected fraction of aquifers that contain sister pairs is then:

$$(a_1 P_{1n} + a_2 P_{2n,pair,2c} + a_3 P_{3n,pair,2c})/A \quad (1)$$

And for three colonization periods:

$$(a_1 P_{1n} + a_2 P_{2n,pair,3c} + a_3 P_{3n,pair,3c})/A \quad (2)$$

Where $P_1 = 0$, A is the total number of aquifers, and a_{1-3} are the number of aquifers with 1–3 species. The expected fraction of aquifers containing triplets is

$$a_3 P_{3n,triplet,3c}/A \quad (3)$$

In a second model we test the hypothesis that sympatric sister species are the result of a single colonization per aquifer. Here, after a colonization event with probability p , it is assumed that remaining empty niches are filled following diversification of a species that previously colonized a different niche in the aquifer with a probability of q (species 2 in Figure 2b). Thus, for single colonization events the probabilities of finding sister species pairs for aquifers with 2–3 niches are:

$$\begin{aligned} Q_{2n,pair} &= 2p(1-p)q, \quad Q_{3n,pair} \\ &= 3p^2(1-p)q + 3p(1-p)q(1-q) \text{ and } Q_{3n,triplet} \\ &= 3p(1-p)^2 q^2 \end{aligned}$$

Note that the probability of sympatric sister species occurring for both single colonization and within aquifer divergence does not depend on the number of ancestral species, and that it is possible for aquifers that have three niches to obtain a triplet of sympatric sister species when only one niche is colonized initially.

The overall expected fraction of sister species pairs arising by speciation within aquifers is:

$$(a_1 Q_{1n} + a_2 Q_{2n,pair} + a_3 Q_{3n,pair,})/A$$

where A is the total number of aquifers and a_{1-3} are the number of aquifers with 1–3 niches. We analyzed only the extreme case in which all remaining niches are filled following diversification ($q = 1$).

We based the number of niches a_{1-3} for each aquifer on the total number of recorded species per aquifer [24]. This approach assumes that after the last colonization there are no empty niches in the observed aquifers, while in the model this may occur. To be able to compare the modeled and observed values without this

bias, we assumed that in the last colonization event all niches were filled ($p_2 = 1$ respectively $p_3 = 1$), an assumption that leads to overestimation of the number of sister species (supporting information S3). We tested the behaviour of the model for different values of initial colonization probability and initial sizes of the ancestral species pool (supporting information S3). Based on this analysis, we chose our colonization probabilities to maximize the expected number of aquifers containing species pairs. We generated confidence limits by drawing the species of the 45 aquifers at random using the described models. The 5 and 95 percentiles of 10000 repetitions were used as the confidence limits. The randomisation program is available from the corresponding author.

Assumption of the models

Our repeated colonization model relies on the following assumptions:

(A) The ancestral species have an equal chance to make a successful transition into an aquifer. To meet this assumption the ancestral species must have had largely overlapping geographical distributions, which is supported by the available data, see discussion. (B) We take the number of species presently found in each aquifer to reflect the number of species that can colonise these aquifers. For this model we assume that each species occupies a single niche. (C) We consider the probability that a niche becomes occupied as the positive end-result of a range of processes that eventually leads to the occupation of a niche. We presume that when a niche is not occupied in a first colonization it will be in a second colonization period. These processes may also include initial colonization of a waterhole by surface species, surviving local competition, moving to the subsurface (interstitial) habitat during drying of the surface water and finally colonizing a subterranean niche.

Supporting Information

Supporting Information S1 List of examined taxa with details on sample localities, and GenBank accession numbers. (XLS)

Supporting Information S2 Parameter estimates of the BEAST analyses calculated using Tracer. (DOC)

Supporting Information S3 The relationship between the size of the ancestral species pool, niche colonization probabilities and the fraction of aquifers with sister species calculated with two and three colonization events. (DOC)

Acknowledgments

We thank Marten Scheffer, Remi Vergnon, Mike Lee, Roger Butlin and Gaynor Dolman and two anonymous reviewers for helpful comments that greatly improved the manuscript, Mike Eastwood for help with the probability model, and John 'Exo' Weilandt and Biometrics SA, Adelaide for assistance and advice.

Author Contributions

Conceived and designed the experiments: RL EN KH. Performed the experiments: RL EN. Analyzed the data: RL EN. Contributed reagents/materials/analysis tools: RL CW SC WFH. Wrote the paper: RL EN SC KH.

References

- Fukami T, Beaumont HJE, Zhang X-X, Rainey PB (2007) Immigration history controls diversification in experimental adaptive radiation. *Nature* 446: 436–439.
- Claessen D, de Roos AM, Persson L (2000) Dwarfs and giants: Cannibalism and competition in size-structured populations. *American Naturalist* 155: 219–237.
- Claessen D, Dieckmann U (2002) Ontogenetic niche shifts and evolutionary branching in size-structured populations. *Evolutionary Ecology Research* 4: 189–217.
- Doebeli M, Dieckmann U (2000) Evolutionary branching and sympatric speciation caused by different types of ecological interactions. *American Naturalist* 156: S77–S101.
- Gavrilets S (2003) Perspective: Models of speciation: What have we learned in 40 years? *Evolution* 57: 2197–2215.
- Gavrilets S, Vose A (2007) Case studies and mathematical models of ecological speciation. 2. Palms on an oceanic island. *Molecular Ecology* 16: 2910–2921.
- Gavrilets S, Vose A, Barluenga M, Salzburger W, Meyer A (2007) Case studies and mathematical models of ecological speciation. 1. Cichlids in a crater lake. *Molecular Ecology* 16: 2893–2909.
- Schlieven UK, Tautz D, Paabo S (1994) Sympatric speciation suggested by monophyly of crater lake cichlids. *Nature* 368: 629–632.
- Losos JB, Ricklefs RE (2009) Adaptation and diversification on islands. *Nature* 457: 830–836.
- Savolainen V, Anstett MC, Lexer C, Hutton I, Clarkson JJ, et al. (2006) Sympatric speciation in palms on an oceanic island. *Nature* 441: 210–213.
- Babik W, Butlin RK, Baker WJ, Papadopoulos AST, Boulesteix M, et al. (2009) How sympatric is speciation in the *Howea* palms of Lord Howe Island? *Molecular Ecology* 18: 3629–3638.
- Gillespie R (2004) Community assembly through adaptive radiation in Hawaiian spiders. *Science* 303: 356–359.
- Bolnick DI, Fitzpatrick BM (2007) Sympatric speciation: Models and empirical evidence. *Annual Review of Ecology and Systematics* 38: 459–487.
- Coyne JA, Orr HA (2004) Speciation. Sunderland, Massachusetts USA: Sinauer Associates, Inc. 545 p.
- Mims MC, Darrin HC, Fitzpatrick BM, Todd Streebman J (2010) Geography disentangles introgression from ancestral polymorphism in Lake Malawi cichlids. *Molecular Ecology* 19: 940–951.
- Schluter D, Boughman JW, Rundle HD (2001) Parallel speciation with allopatry. *Trends in Ecology & Evolution* 16: 283–284.
- Martin H (1998) Tertiary climatic evolution and the development of aridity in Australia. *Proceedings of the Linnean Society New South Wales* 119: 115–136.
- Cooper SJB, Bradbury JH, Saint KM, Leys R, Austin AD, et al. (2007) Subterranean archipelago in the Australian arid zone: mitochondrial DNA phylogeography of amphipods from central Western Australia. *Molecular Ecology* 16: 1533–1544.
- Cooper SJB, Saint KM, Taiti S, Austin AD, Humphreys WF (2008) Subterranean archipelago: mitochondrial DNA phylogeography of stygobitic isopods (Oniscidea: *Halomiscus*) from the Yilgarn region of Western Australia. *Invertebrate Systematics* 22: 195–203.
- Guzik MT, Abrams KM, Cooper SJB, Humphreys WF, Cho JL, et al. (2008) Phylogeography of the ancient Parabathynellidae (Crustacea: Bathynellacea) from the Yilgarn region of Western Australia. *Invertebrate Systematics* 22: 205–216.
- Leys R, Watts CHS, Cooper SJB, Humphreys WF (2003) Evolution of subterranean diving beetles (Coleoptera: Dytiscidae: Hydroporini, Bidessini) in the arid zone of Australia. *Evolution* 57: 2819–2834.
- Humphreys WF (2001) Groundwater calcrete aquifers in the Australian arid zone: the context to an unfolding plethora of stygal biodiversity. In: Humphreys WF, Harvey MS, eds. *Subterranean Biology in Australia 2000: Records of the Western Australian Museum Supplement*. pp 63–83.
- Humphreys WF, Watts CHS, Cooper SJB, Leijts R (2009) Groundwater estuaries of salt lakes: buried pools of endemic biodiversity on the western plateau, Australia. *Hydrobiologia* 626: 79–95.
- Watts CHS, Humphreys WF (2009) Fourteen new Dytiscidae (Coleoptera) of the genera *Limbodessus* Guignot and *Paroster* Sharp and *Exocelina* Broun from underground waters in Australia. *Transactions of the Royal Society South Australia* 133: 62–107.
- Balke M, Ribera I, Vogler AP (2004) MtDNA phylogeny and biogeography of Copelatinae, a highly diverse group of tropical diving beetles (Dytiscidae). *Molecular Phylogenetics and Evolution* 32: 866–880.
- Fitzpatrick B, Fordyce J, Gavrilets S (2008) What, if anything, is sympatric speciation? *Journal of Evolutionary Biology* 21: 1452–1459.
- Stein R, Robert C. Siliclastic (1986) sediments at sites 588, 590 and 591: Neogene and Paleogene evolution in the southwest Pacific and Australian climate. In: , Government US, editor (1986) 1986; Washington D.C. U.S. Government Printing Office. pp 1437–1445.
- Swofford DL (1998) PAUP* Phylogenetic analysis using parsimony (*and other methods). version 4. Sunderland, Massachusetts: Sinauer Associates.
- Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755.
- Darwin C (1859) On the origing of species by means of natural selection. London: Murray.
- Bistrom O (1996) Taxonomic revision of the genus *Hydrovatus* Motschulsky (Coleoptera, Dytiscidae). *Entomologica Basiliensia* 19: 57–584.
- Davy-Bowker J (2002) A mark and recapture study of water beetles (Coleoptera: Dytiscidae) in a group of semi-permanent and temporary ponds. *Aquatic Ecology* 36: 435–446.
- Fairchild GW, Faulds AM, Matta JF (2000) Beetle assemblages in ponds: effects of habitat and site age. *Freshwater Biology* 44: 523–534.
- Larson DJ, Alarie Y, Roughley RE (2000) Predaceous diving beetles (Coleoptera: Dytiscidae) of the Nearctic Region, with emphasis on the fauna of Canada and Alaska. Ottawa, Ontario, Canada: NRC Research Press.
- Watts CHS (2002) Checklist and guides to the identification, to genus, of adult and larval Australian water beetles of the families Dytiscidae, Noteridae, Hygrobiidae, Haliplidae, Gyrinidae, Hydraenidae and the superfamily Hydrophiloidea (Insecta: Coleoptera). Thurgoona, NSW, Australia Cooperative Research Centre for Freshwater Ecology. 110 p.
- Watts CHS (1985) A faunal assessment of Australian Hydradephaga. *Proceedings of the Academy of Natural Sciences of Philadelphia* 137: 22–28.
- Larson DJ (1997) Habitat and community patterns of tropical Australian hydradephagan water beetles (Coleoptera: Dytiscidae, Gyrinidae, Noteridae). *Australian Journal of Entomology* 36: 269–285.
- Vamosi J, Vamosi S (2007) Body size, rarity, and phylogenetic community structure: insights from diving beetle assemblages of Alberta. *Diversity Distribution* 13: 1–10.
- Cooper SJB, Hinze S, Leys R, Watts CHS, Humphreys WF (2002) Islands under the desert: molecular systematics and evolutionary origins of stygobitic water beetles (Coleoptera: Dytiscidae) from central Western Australia. *Invertebrate Systematics* 16: 589–598.
- Butlin RK, Galindo J, Grahame JW (2008) Sympatric, parapatric or allopatric: the most important way to classify speciation? *Philosophical Transactions of the Royal Society B-Biological Sciences* 363: 2997–3007.
- Fitzpatrick BM, Fordyce JA, Gavrilets S (2009) Pattern, process and geographic modes of speciation. *Journal of Evolutionary Biology* 22: 2342–2347.
- Guzik MT, Cooper SJB, Humphreys WF, Austin AD (2009) Fine-scale comparative phylogeography of a sympatric species triplet of subterranean diving beetles from a single calcrete aquifer in Western Australia. *Molecular Ecology* 18: 3683–3698.
- Losos JB, Jackman TR, Larson A, de Queiroz K, Rodriguez-Schettino L (1998) Contingency and determinism in replicated adaptive radiations of island lizards. *Science* 279: 2115–2118.
- Taylor EB, McPhail JD (1999) Evolutionary history of an adaptive radiation in species pairs of threespine sticklebacks (*Gasterosteus*): insights from mitochondrial DNA. *Biological Journal of the Linnean Society* 66: 271–291.
- Juan C, Guzik MT, Jaime D, Cooper SJB (2010) Evolution in caves: Darwin's 'wrecks of ancient life' in the molecular era. *Molecular Ecology* 19: 3865–3880.
- Leys R, Watts CHS (2008) Systematics and evolution of the Australian subterranean hydroporine diving beetles (Dytiscidae), with notes on *Carabhydrus*. *Invertebrate Systematics* 22: 217–225.
- Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 7.
- Brower A (1994) Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proc Natl Acad Sci USA* 91: 6491–6495.
- Rambaut A, Drummond AJ (2007) Tracer v1.4. <http://beast.bio.ed.ac.uk/Tracer>.
- Leys R, Cooper SJB, Strecker U, Wilkens H (2005) Regressive evolution of an eye pigment gene in independently evolved eyeless subterranean diving beetles. *Biology Letters* 1: 496–499.