Validation and description of two new north-western Australian Rainbow skinks with multispecies coalescent methods and morphology

Ana C. Afonso Silva 1, 2, Natali Santos 3, Huw A. Ogilvie 1, 4, Craig Moritz 1

1 Division of Ecology and Evolution, Research School of Biology and Centre for Biodiversity Analysis, Australian National University, Acton, ACT, Australia
2 cE3c - Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal
3 Universidade Federal do ABC, Santo André, SP, Brazil
4 Centre for Computational Evolution, University of Auckland, Auckland, New Zealand

Corresponding Author: Ana C. Afonso Silva
Email address: anacatarina.as@gmail.com

While methods for genetic species delimitation have noticeably improved in the last decade, this remains a work in progress. Ideally, model based approaches should be applied and considered jointly with other lines of evidence, primarily morphology and geography, in an integrative taxonomy framework. Deep phylogeographic divergences have been reported for several species of *Carlia* skinks, but only for some eastern taxa have species boundaries been formally tested. The present study does this and revises the taxonomy for two species from northern Australia, *Carlia johnstonei* and *C. triacantha*. We introduce an approach that is based on the recently published method StarBEAST2, which uses multilocus data to explore the support for alternative species delimitation hypotheses using Bayes Factors (BFD). We apply this method, jointly with two other multispecies coalescent methods, using an extensive (from 2163 exons) data set along with measures of 11 morphological characters. We use this integrated approach to evaluate two new candidate species previously revealed in phylogeographic analyses of rainbow skinks (genus *Carlia*) in Western Australia. The results based on BFD StarBEAST2, BFD* SNAPP and BPP genetic delimitation, together with morphology, support each of the four recently identified *Carlia* lineages as separate species. The BFD StarBEAST2 approach yielded results highly congruent with those from BFD* SNAPP and BPP. This supports use of the robust multilocus multispecies coalescent StarBEAST2 method for species delimitation, which does not require *a priori* resolved species or gene trees. Compared to the situation in *C. triacantha*, morphological divergence was greater between the two lineages within Kimberley endemic *C. johnstonei*, which also had deeper divergent histories. This congruence supports recognition of two species within *C. johnstonei*. Nevertheless, the combined evidence also supports recognition of two taxa within the more widespread *C. triacantha*. With this work, we describe two new species, *Carlia insularis* sp. nov and *Carlia*
isostriacantha sp. nov. in the northwest of Australia. This contributes to increasing recognition that this region of tropical Australia has a rich and unique fauna.
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Authors: Ana C. Afonso Silva¹,², Natali Santos³, Huw A. Ogilvie¹,⁴ and Craig Moritz¹

Affiliations
¹Division of Ecology and Evolution, Research School of Biology and Centre for Biodiversity Analysis, Australian National University, Acton, ACT, Australia.
²cE3c - Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal.
³Universidade Federal do ABC, Santo André, SP, Brazil.
⁴Centre for Computational Evolution, University of Auckland, Auckland, New Zealand.

Corresponding author: Ana C. Afonso Silva
e-mail: anacatarina.as@gmail.com
Abstract

While methods for genetic species delimitation have noticeably improved in the last decade, this remains a work in progress. Ideally, model based approaches should be applied and considered jointly with other lines of evidence, primarily morphology and geography, in an integrative taxonomy framework. Deep phylogeographic divergences have been reported for several species of *Carlia* skinks, but only for some eastern taxa have species boundaries been formally tested. The present study does this and revises the taxonomy for two species from northern Australia, *Carlia johnstonei* and *C. triacantha*.

We introduce an approach that is based on the recently published method StarBEAST2, which uses multilocus data to explore the support for alternative species delimitation hypotheses using Bayes Factors (BFD). We apply this method, jointly with two other multispecies coalescent methods, using an extensive (from 2163 exons) data set along with measures of 11 morphological characters. We use this integrated approach to evaluate two new candidate species previously revealed in phylogeographic analyses of rainbow skinks (genus *Carlia*) in Western Australia.

The results based on BFD StarBEAST2, BFD* SNAPP and BPP genetic delimitation, together with morphology, support each of the four recently identified *Carlia* lineages as separate species. The BFD StarBEAST2 approach yielded results highly congruent with those from BFD* SNAPP and BPP. This supports use of the robust multilocus multispecies coalescent StarBEAST2 method for species delimitation, which does not require *a priori* resolved species or gene trees.
Compared to the situation in *C. triacantha*, morphological divergence was greater between the two lineages within Kimberley endemic *C. johnstonei*, which also had deeper divergent histories. This congruence supports recognition of two species within *C. johnstonei*. Nevertheless, the combined evidence also supports recognition of two taxa within the more widespread *C. triacantha*.

With this work, we describe two new species, *Carlia insularis* sp. nov and *Carlia isostriacantha* sp. nov. in the northwest of Australia. This contributes to increasing recognition that this region of tropical Australia has a rich and unique fauna.

**Introduction**

Cryptic species – when two or more distinct species are inaccurately classified under one species name (Bickford et al., 2007) - present great challenges for taxonomy and species delimitation due to the desirability of validating candidate species using multiple lines of evidence (Fujita et al., 2012). But for biodiversity assessment and conservation reasons the need to properly describe species diversity is greater than ever (Bickford et al., 2007). In the same way, there is a concern that molecular data may promote taxonomic inflation by ‘over splitting’ divergent populations into candidate species (Isaac, Mallet & Mace, 2004; Hedin, Carlson & Coyle, 2015). The creation of more reliable and robust species delimitation approaches in the last decade has attempted to address this concern (Rannala, 2015).

To more robustly infer species boundaries, the use of integrative taxonomy is increasingly common (Bickford et al., 2007; Padial et al., 2010). The objective of this approach is to corroborate taxonomic validity with independent, distinct types of evidence. Given deep genetic divergence, fixed morphological differences are not necessary to diagnose species boundaries since speciation itself does not require phenotypic characters to evolve at the same rate as the
genome (Leaché & Fujita, 2010). Therefore, in taxa with inherently conservative morphology, it may be that the primary evidence for distinct species will come from genetic data.

Species delimitation consists of two potentially complementary approaches: discovery methods that do not require \textit{a priori} assignment of samples before analysis, and validation methods that test hypotheses based on samples already assigned to candidate species (Ence & Carstens, 2011). When candidate lineages are already identified, validation approaches are more robust because they explicitly model the process of lineage diversification (Carstens et al., 2013). This is especially so when there is a substantial number of informative genes, independent of those used to suggest candidate taxa. Model-based multilocus approaches that use the multispecies coalescent (MSC) are advantageous because they account for coalescent processes when estimating phylogenetic relationships (Edwards et al., 2016). And for species delimitation, objective and transparent model-based approaches are relevant, because they have the potential to reduce investigator-driven biases (Fujita et al., 2012). These methods can consider gene tree incongruence due to incomplete lineage sorting, variation in molecular sequences and variation in demographic parameters (Leaché & Fujita, 2010). With this in mind, Carstens \textit{et al.} (2013) recommend the best approach for species delimitation is to use multiple methods. Further, Rannala (2015) suggests that this should only be done when methods have algorithmically similar assumptions. However, we also note that MSC methods can over split – revealing high structured populations (or ephemeral species; Rosenblum et al., 2012) – rather than long isolated species, depending on the nature of the speciation process (Sukumaran & Knowles, 2017). Hence, species delimitation will always be more secure when taxa delimited using genetic methods are somehow corroborated by alternative sources of data (Oliver, Keogh & Moritz, 2014).
Previous work by Afonso Silva et al. (2017), which focused on understanding how phylogeographic structure and history differs between a climatic generalist and specialist, found two deeply divergent lineages within each of *Carlia johnstonei* Storr, 1974 and *C. triacantha* Mitchell, 1953 (Fig. 1). These sister taxa (Dolman & Hugall, 2008) have contrasting distributions, with the former being endemic to the Kimberley and the latter being widespread across northern Australia. The lineages within *C. johnstonei* are likely allopatric, with the nominal lineage (Johnstonei A) being found across the north and western Kimberley and the newly identified lineage (Johnstonei B) being endemic to islands off the coast of the northwest Kimberley (Fig. 1). Conversely, the two lineages of *C. triacantha* likely overlap geographically, with the nominal lineage being widespread across north and central Australia (Triacantha A) and the newly identified lineage (Triacantha B) found within the Kimberley and scattered locations in the central Northern Territory (Fig. 1).

Species of *Carlia* from the Australian tropics generally have deep phylogeographic structure for both mtDNA and large numbers of exons (e.g. Potter et al., 2016), and, where contact zones have been examined in detail, there is evidence of strong reproductive isolation between the more deeply divergent (but phenotypically cryptic) lineages (Phillips, Baird & Moritz, 2004; Singhal & Moritz, 2013). However, recent species delimitation and taxonomic revisions have focussed more on *Carlia* from the eastern woodlands and placed a greater emphasis on morphology (e.g. Hoskin & Couper, 2012; Hoskin, 2014). There is a need to re-examine the systematics of northern Australian *Carlia*, and here we have the opportunity to exploit large multilocus datasets (Bragg et al., 2015) to do that integrated with morphology. This is particularly relevant for Kimberley biodiversity, since there have been recent efforts to discover and describe new species (Köhler, 2011; Oliver et al., 2014, 2016; Andersen et al.,
2014; Ellis, 2016) in this still relatively unknown and remote region in the northwestern of Australia.

We reanalyse the extensive multilocus data used in Afonso Silva et al. (2017) using robust species delimitation methods, together with morphological analysis to validate species hypotheses. Following Rannala (2015), we use three algorithmically similar methods to validate potential new species. We apply BPP (Yang & Rannala, 2014) and two approaches using Bayes Factors to test species hypotheses: a SNP based approach, BFD* (Leaché et al., 2014) using SNAPP (Bryant et al., 2012) and a sequence-based approach, BFD with the recently developed StarBEAST2 method (Ogilvie, Bouckaert & Drummond, 2017). We consider three potential species hypotheses: (i) only the two currently defined species are separated; (ii) a three-species hypothesis - two species corresponding to the two more deeply divergent lineages of *C. johnstonei*, but collapsing the less divergent lineages within *C. triacantha*; and (iii) a four-species hypothesis - all four lineages correspond to different species. Using an integrative taxonomic approach, we present and analyse morphological data to test for congruent differences between all identified genetic lineages. Considering all lines of evidence, we then formally describe the new species and identify diagnostic traits, for both morphology and gene sequences. Genetic diagnostic traits include SNPs from available mtDNA ND4 gene sequences (Afonso Silva et al. 2017), following Renner’s (2016) suggestion to provide simple genetic diagnostics, particularly for morphologically similar species groups.

**Materials & Methods**

We used exon capture data to perform validation analyses and sequences of the mtDNA ND4 gene to identify diagnostic SNPs, and, also measured, analysed and identified diagnostic
morphological traits. We obtained sequences for the genetic data from Afonso Silva et al. (2017) (Dryad Digital Repository http://dx.doi.org/10.5061/dryad.jj1t). These included mtDNA sequence data of 101 *C. johnstonei* and 99 *C. triacantha* throughout both species’ distribution, for which we had specimens to do morphological analysis (Table S1, Fig. S1).

See Afonso Silva et al. (2017) for more detail about how the exon capture data was obtained. In summary, the data was retrieved from a custom set of loci designed from transcriptomes of *Carlia* and a couple of related genera (Bragg et al., 2015). After similar processing to Bragg et al. (2015), the final dataset contained a total of 51 samples with average of 40x coverage and approximately 2800 loci per sample. For the validation analyses, we retrieved data from the 20 geographically dispersed samples as used for species tree estimation in Afonso Silva et al. (2017) (Fig. 1, Table S1). These correspond to five individuals for each of the four lineages previously identified in Afonso Silva et al. (2017) (Fig. 1, Table 1), using the same *C. amax* samples as an outgroup (from Potter et al., 2016) that were used in that study. For these analyses, we required aligned haplotype sequences, for which we employed GATK (v3.3, McKenna et al., 2010) which was also used to identify heterozygous sites and mask sites with a low-quality genotype call (GQ<20). Here, we generated phased haplotypes using the individual overlapping sequencing reads to phase heterozygous sites within target loci and then used one haplotype per sample in later analyses.

We then used the EAPhy pipeline (Blom 2015 v.1.2; https://github.com/MozesBlom/EAPhy) to realign, filter and export alignments with complete data into NEXUS and PHYLIP format, as well as two sets of SNPs in FASTA format (using 0.2 as maximum proportion of Ns for each site, one SNP chosen randomly per gene and excluding singletons).
Genetic species validation

We applied three multispecies coalescent validation approaches to investigate species boundaries: Bayesian Phylogenetics and Phylogeography (BPP v3.3; Yang & Rannala, 2014), BFD (Bayes factor delimitation; Grummer et al. 2014) StarBEAST2 using multilocus data (Ogilvie, Bouckaert & Drummond, 2017), and BFD* SNAPP using SNP data (Leaché et al., 2014).

For the BPP analysis, we randomly selected two exon sets (to avoid unforeseen biases), each with 100 loci of between 250 bp and 1000 bp, to check for consistent results. The MSC assumes no recombination within loci, and free recombination among loci (Degnan et al., 2009). We are confident of satisfying the latter condition, as our exons are all derived from different genes (Bragg et al., 2015). Lanier & Knowles (2012) showed that intra-locus recombination had little effect in species-tree estimates under the MSC; however Potter et al. (2016) found that it can affect species delimitation. Hence, to further evaluate this effect, we used the program IMgc (Woerner, Cox & Hammer, 2007) to extract optimal recombination-filtered blocks (no four-gamete violations) and repeated BPP analysis for comparison. We performed joint Bayesian species delimitation and species tree estimation (method A11, Yang, 2015). This method uses the multispecies coalescent model to compare different models of species delimitation and species phylogeny in a Bayesian framework, accounting for incomplete lineage sorting due to ancestral polymorphism and gene tree species tree conflicts (Yang & Rannala, 2010, 2014; Rannala & Yang, 2013). Ancestral population size parameters (theta) were set to gamma prior $G(2, 1000)$, with mean $2/1000 = 0.002$ and the divergence time at the root of the species tree (tau) was assigned to $G(2, 2000)$, while the other divergence time parameters were assigned to the
Dirichlet prior (Yang & Rannala, 2010: equation 2). Preliminary analyses run using different combination of gamma priors, as suggested in Yang (2015), produced similar results, suggesting that our results are robust to the priors used. The phylogeny obtained in Afonso Silva et al. (2017) was used as a starting tree and all columns in the alignment were used in the likelihood calculation. Each exon set analysis was independently run twice to confirm consistency between runs, with a burn-in of 50,000 and a sampling frequency of five iterations for a total of 500,000 generations.

Bayes factor delimitation (BFD; Grummer, Bryson & Reeder, 2014) is an approach that compares the marginal likelihoods of competing species delimitation hypotheses using Bayes factors. To apply this approach, we ran two MSC methods to test our three potential hypotheses using C. amax as an outgroup: (i) a scenario with two species (C. johnstonei and C. triacantha), (ii) a scenario with three species (lineages Johnstonei A, Johnstonei B and C. triacantha) and (iii) a scenario with four species (with both lineages from C. triacantha and C. johnstonei as separate species).

StarBEAST2 v0.13.5 is a recently released sequence-based approach that reconstructs species trees with more flexibility than BPP (Ogilvie, Bouckaert & Drummond, 2017), and so provides an alternative MSC method to investigate species delimitation with Bayes factors (BFD). To verify consistency, we randomly selected another two sets of exons, each with 20 loci between 250 and 1000 bp. We then used jModelTest v2.1.10 (Guindon, Gascuel & Rannala, 2003; Darriba et al., 2012) to calculate nucleotide substitution model likelihood scores for each locus and to estimate optimal model using BIC (Supplemental Table S2). All BFD StarBEAST2 analyses were performed using a strict clock model, for 100,000,000 generations, with data sampled every 10,000 generations, the first 10% of each run was discarded as burn-in and priors
as in Table S3. For each analysis, two BFD StarBEAST2 replicates were conducted to ensure convergence and assessed using ESS values with Tracer v1.6 (Rambaut et al., 2015). We used stepping-stone sampling (Leaché et al., 2014) to determine the marginal likelihoods of four, three and two species (plus outgroup). All stepping-stone analyses used 16 steps with a beta distribution $\alpha$ parameter of 0.1 to optimise the power posterior discretization (Xie et al., 2010). The resulting marginal likelihoods were then used to compute Bayes factors (Kass & Raftery, 1995), quantifying the support for each species delimitation hypothesis against all others under consideration. The final tree was obtained by combining posterior replicates with LogCombiner (Drummond & Rambaut, 2007) and summarised using maximum clade credibility trees, after exclusion of 10% burn-in, with TreeAnnotator v1.7.2 (Drummond & Rambaut, 2007).

To use an approach that considers evidence from all available loci, we selected two independent SNP sets by sampling one SNP at random from each locus out of 2,163 total available loci and estimated species trees for each scenario using SNAPP (Bryant et al., 2012). We ran all analysis for 500,000 generations sampling every 500, with two replicates to ensure convergence and priors as in Table S3. After assessing convergence between runs and exon sets we proceeded to Bayes factor delimitation as described previously.

**Morphological data collection**

We analysed 200 specimens from the Museum and Art Gallery of the Northern Territory (MAGNT), Museum Victoria (MV), South Australian Museum (SAM), Western Australian Museum (WAM) and recently-collected specimens held at the Australian National University (with ANU ethical approval number A2012/14) (Fig. 1, Table S1). All analysed specimens were also sequenced for the mtDNA ND4 gene in Afonso Silva et al. (2017) (Fig. S1), with a total of
We examined five morphometric characters taken to the nearest 0.1 mm with Mitutoyo electronic callipers: snout-vent length (SVL), axilla-groin length (AGL), head length (HL) measured from anterior edge of tympanum to snout, head width (HW) measured at widest point of the head, and head depth (HD) measured at parietal scales. In order to minimize error, we used a dissecting microscope Leica MZ8 (equipped with camera Leica MC120 HD) for which forelimb (FLL) and hindlimb length (HLL) were measured through photographs using ImageJ (Abrámoff, Magalhães & Ram, 2004) (as in Fig. S3); as well as four additional smaller features: nasals separation (NS), ear aperture length (EAL), palpebral disc length (PDL) and eye to ear distance (EED) (as explained in Fig. S3).

We also assessed seven meristic characters using photographs: supralabials, infralabials, supraciliaries, lamellae under the 4th toe (from claw sheath to junction of 3rd and 4th toes), lamellae under the 3rd finger (from claw sheath to the junction of the 2nd and 3rd fingers), the mode of number of keels across the mid-dorsal line scales and the ear lobule numbers. These traits were counted as suggested by Cogger (2014) and similarly to Hoskin & Couper (2012).

Measurements and scales were generally analysed from the left side of the specimen, unless prevented by damage or poor preservation. All described measurements were collected in millimetres (mm).

For the ensuing species descriptions, we also measured the tail length and the distance between prefrontals if not in contact, but these traits were not used in the morphological analysis due to high level of missing data. For the designated holotypes, we additionally counted the number of midbody scale rows, vertebral (from the occiput to the edge of the hind limb along the
Morphological analyses

We investigated the relationship of each linear measurement with size (per mtDNA lineage), by plotting each variable against SVL and by comparing box plots of raw and size corrected measurements. After removing samples with missing data, all measurements were log-transformed to reduce their variance allowing a more conservative assessment of differences between mtDNA lineages. We then extracted size-corrected residuals from regressions between SVL and each measurement as a size-corrected log-transformed dataset. We investigated normality and heteroscedasticity after variable correction using density plots, Shapiro-Wilk test and Levene’s test. Multivariate normality was assessed with the Henze-Zirkler’s Multivariate Normality Test in the MVN package (Korkmaz, Goksuluk & Zararsiz, 2014).

In order to assess the morphometric distinctiveness of these lineages, we conducted Principal Component analyses on the log-transformed and on the size-corrected log-transformed datasets for each species. We used the `prcomp` function (stats package) with all measurement variables centred and plotted principal component 1 (PC1) against PC2, with a 75% confidence ellipse probability threshold (ggplot2 package, Wickham 2016).

To statistically evaluate whether the lineages are significantly different and which variables are contributing to this, we analysed log-transformed and size-corrected log-transformed measurements with a MANOVA, and confirmed the significance of non-normal variables with the non-parametric Wilcoxon test (stats package). Relevant meristic data was analysed independently with a generalized linear modelling with a Poisson distribution (stats package) since these are count data and not continuous variables.
Using the statistically significant measurement variables from the MANOVA, we tested
the accuracy in predicting assignment of lineage by applying a linear discriminant analysis
(LDA) with jackknife cross-validation implemented in the package MASS (Ripley et al., 2013).
Due to the presence of non-normal variables, we also applied a Random Forest (RF) analysis
using the package randomForest (Liaw & Wiener, 2002).
We investigated the effect of possible outliers in the data by calculating, for each of the
variables, interquartile range scores (function scores in outliers package, Komsta, 2011) to
identify samples with outliers and then perform a MANOVA with this dataset. Removing
outliers decreases 14% and 6% of analysed specimens for *C. johnstonei* and *C. triacantha*,
respectively. Since some of these outliers could represent expected phenotypic variation across
these species distribution and the overall results were similar, we present the analyses with all
individuals.
To account for the insufficient information on sex, we performed a linear model containing
sex and mtDNA lineage, using the available sexed individuals, which showed no difference in
SVL between males and females in either *C. johnstonei* or *C. triacantha*. This suggests sex
differences cannot explain our observed results, so we also present the analyses with all
individuals.
We performed all analyses in R v.3.3.1 (R Core Team, 2016) and all the data, input files,
code and morphological results are available at https://dx.doi.org/10.6084/m9.Figshare.4621963.

*Molecular diagnostics*

Following the recommendation of Renner (2016), we visually identified diagnostic SNPs
within the *ND4* mtDNA gene using all Afonso Silva et al. (2017) sequences with Genbank
accessions codes MF083173-MF083508 in Geneious v.7.1.9 (http://www.geneious.com, Kearse et al., 2012). Using as a reference an available skink mitogenome from *Scincella vandenburghi* (Park et al., 2016), we selected the available diagnostic SNPs per lineage within each species, where the nucleotide difference would correspond to an amino acid substitution.

The electronic version of this article in Portable Document Format (PDF) will represent a published work according to the International Commission on Zoological Nomenclature (ICZN), and hence the new names contained in the electronic version are effectively published under that Code from the electronic edition alone. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix http://zoobank.org/. The LSID for this publication is: urn:lsid:zoobank.org:pub:A7B29F16-079F-48BA-B4BE-3EC9A3D80D34. The online version of this work is archived and available from the following digital repositories: PeerJ, PubMed Central and CLOCKSS.

**Results**

**MSC Species delimitation**

All three MSC approaches assigned more support to the four-species hypothesis than either the two- or three-species hypotheses (Table 1).

Both BPP analyses, each with independent drawn sets of genes, yielded the same species tree (Fig. 2) and a posterior probability (PP) equal to 1 for five delimited species (all four lineages
plus the outgroup). The analyses processed with IMgc to exclude blocks with no four-gamete violations from within alignments, returned similar results with PP = 1 for four lineages plus outgroup. However, while topology for the original datasets was as expected by 99% of the models (Fig. 2), for each gene set without recombining blocks only 64% and 85% of the models supported the same topology.

For both BFD StarBEAST2 and BFD* SNAPP, Bayes Factors (BF) were obtained by subtracting the two-species hypothesis from both the three-species hypothesis as well the four-species hypothesis, and multiplying the difference of marginal likelihoods by a factor of two.

The BFs for both the BFD StarBEAST2 and BFD* SNAPP analyses were > 10 for the four-species hypothesis relative to the two- or three-species hypotheses (Table 1), which corresponds to decisive evidence for this model (Kass & Raftery, 1995). The marginal likelihood results were of similar magnitude across the two gene datasets for BFD StarBeast2 and across the two SNP datasets for BFD* SNAPP (Table 1, Fig. S2), although BFs were much higher for the latter.

The species tree topology with the main lineages was assessed in Afonso Silva et al. (2017) using the ASTRAL summary species tree method (Fig. 1), but here species trees were also estimated by BPP, StarBEAST2 and SNAPP. StarBEAST2 and SNAPP all returned majority support for the ASTRAL topology. For gene sets 1 and 2, StarBEAST2 support for the ASTRAL topology was 97% and 63%, respectively. Support was higher using SNAPP at 95% and >99% for SNP set 1 and 2, respectively.

### Morphological analysis

The morphological measurements suggest that snout-vent length (SVL) is an important differentiating trait between candidate species within each of *C. johnstonei* and *C. triacantha*.
Thus, further analyses were conducted also using size-corrected log-transformed variables (Fig. S5), so we could assess if the lineages were statistically different after accounting for SVL differences. For multivariate analyses, individuals with missing data were removed and after size correction some variables were still not normal (Table S4, 5), but were multivariate normal for both *C. johnstonei* (log-transformed $HZ p$-value $= 0.056$, size corrected $HZ p$-value $= 0.121$) and *C. triacantha* (log-transformed $HZ p$-value $= 0.104$, size corrected $HZ p$-value $= 0.272$).

In the PCA results for *C. johnstonei* with only log-transformed data (including SVL), the first axis (PC1) explained 74.4% of the total variation with all variables loading uniformly (and hence size-related) and the second axis (PC2) only explained 7.2% of variation (Fig. 3A, S6A). By contrast, in the PCA with the size corrected dataset (and excluding SVL), PC1 explains 26.2% and PC2 16.8% of the variation (Fig. 3C, S6C). The log-transformed PCA shows more evidence of clustering by lineage than does the size-corrected PCA. Together these observations point to a high similarity in shape, relative to divergence in body size. For *C. triacantha*, similar results were obtained (Fig. 3B, S6B). The proportions of variance explained for log transformed analysis were PC1 $= 74.3\%$ and PC2 $= 7.2\%$; whereas, for the size corrected analysis, PC1 $= 19.5\%$ and PC2 $= 15.4\%$ (Fig. 3D, S6D).

Using MANOVA, we assessed whether morphological measurements differences between lineages were significant (for more detail see Tables S4-5). For both species, the MANOVA confirmed that size (logSVL) differs between lineages in each species ($p = 1.05\times 10^{-6}$ in *C. johnstonei*; $p = 6.96\times 10^{-3}$ in *C. triacantha*). For size-corrected data, head depth ($p = 1.36\times 10^{-3}$), nasal separation ($p = 9.02\times 10^{-3}$), forelimb ($p = 7.89\times 10^{-3}$), and hindlimb ($p = 2.55\times 10^{-2}$) are important traits in distinguishing Johnstonei A from Johnstonei B; and head length ($p = 3.30\times 10^{-1}$)
and ear to eye distance \((p = 2.73 \times 10^{-2})\) for distinguishing Triacantha A from Triacantha B (Fig. 4). The significant non-normal variables within \(C. triacantha\) were confirmed with significant non-parametric test (Table S5).

The analysis of meristic data was based on three relevant characters (number of ear lobules, lamellae number under the 3\(^{rd}\) finger and under the 4\(^{th}\) toe) due to little or no variation in the other traits. Each of the three analysed characters was significantly different between Johnstonei A and B, but only ear lobule number showed a significant difference between Triacantha A and B (Fig.4, Table S6).

The prediction capacity of significant morphological data was investigated with a linear discriminant analysis (LDA) and a Random Forest analysis (RF). Jackknife results provided 85.87% accuracy for differentiating \(C. johnstonei\) lineages based on log-transformed morphological measurements (to include SVL as a variable) and 72.34% for \(C. triacantha\) lineages. While the accuracy estimated with a RF analysis was 81.52% for \(C. johnstonei\) and 68.09% for \(C. triacantha\).

The summary of each measured trait can be found in supplementary Table S7.

### Taxonomic assessment and species description

Considering the congruence across multiple genetic delimitation methods and of these with significant morphological divergence among lineages, we provide sufficient evidence for four species, two species within \(Carlia johnstonei\) and two species within \(C. triacantha\). Within \(C. johnstonei\), Johnstonei A is the nominal \(C. johnstonei\) species based on a holotype from the Mitchell Plateau, a region in which extensive sampling has shown that only Johnstonei A occurs.

For \(C. triacantha\) the holotype specimen is from Adelaide River, Northern Territory, a site close
(~ 15 km) to Triacantha A samples from Litchfield National Park (NTM R22162) – hence we suggest that Triacantha A should retain the species name. Accordingly, we here describe two new species - Johnstonei B as *Carlia insularis* sp. nov. and Triacantha B as *Carlia isostriacantha* sp. nov. In the following we provide diagnoses for the four species. Simple genetic diagnostics (mtDNA diagnostic SNPs; Table 2) are robust. For morphology alone, single traits mostly have overlapping ranges, but in combination with each other and geography, should be practical in the field.

*Carlia johnstonei* Storr, 1974 *Records of the Western Australian Museum*, Vol. 3, 151-165

Rough brown rainbow-skink

Holotype. WAM R43170, from Mitchell Plateau, Western Australia, in -14.866667 125.833333.

Diagnosis. Dark blackish *Carlia* morphologically distinguishable from geographically overlapping species with a combination of mid-dorsal scales bicarinate (two keels), more numerous supraciliares (usually 7 vs. 6 – *C. amax*, *C. munda*, *C. rufilatus*, *C. isostriacantha* sp. nov., or 5 – *C. gracilis*), larger ear aperture with numerous sharply pointed lobules (mean of 10 lobules), but typically less than in *C. insularis* sp. nov. (mean of 13 lobules). Further distinguished from the latter by smaller body size (mean 36.39 mm vs. 41.83 mm), reduced head depth (mean 3.59 mm vs. 4.48 mm), shorter limbs (forelimbs 9.51 mm vs. 11.45 and hindlimbs 14.82 mm vs. 17.77 mm) and less lamellae under longest finger (mean 16.75 mm vs. 19.69 mm) and toe (mean 22.83 mm vs. 26.31 mm).

Description. Snout-vent length (mm): 21.84 - 43.49 (N = 66, mean 36.39). Tail: 27.1 – 61.28 (N = 26, mean 46.04). Most specimens with separated prefrontal scales (93%) by an average of 0.32 mm (N = 50, 0.05 – 0.64). Ear aperture smaller (N = 62, mean 1.01, 0.50 - 1.44), than palpebral
disc (N = 62, mean 1.31, 1.05 – 1.59), with many small lobules (mean 10, 5 - 16). Lamellae under third finger 9 – 20 (N = 63 mean 16.75), fourth toe 15 – 27 (N = 63 mean 22.83) (Table S7). Most specimens are dorsally dark brown and ventrally yellow but with either a bright or dark blue gular.

Distribution. Distributed across the sub-humid area in the Kimberley, from the northeast Berkeley River region, to the southwest King Leopold Ranges (Fig. 1). Present in humid islands in the Kimberley, mostly the northern islands and those closer to the mainland. In drier environments, this species tends to be more restricted to mesic microhabitats in rocky gorges (Russell Barrett pers. comm.).

Remarks. The previous described paratype from East Montalivet Island (WAM R41462) in Storr (1974) by geographic location should belong to *C. insularis* sp. nov.

**Carlia insularis** sp. nov. (Figs. S7A, S8A, C and S9A) urn:lsid:zoobank.org:act:F058DFD2-799C-4242-8926-9F59AEC6FD44 Kimberley islands rainbow-skink

**Holotype.** WAM R158646, from North Maret island, Western Australia, in -14.3983 124.97750. Specimen collected in 2004 by Richard How (Fig. S7A).

**Paratypes.** Fenelon Island: WAM R117708, WAM R117709, WAM R117710; Corneille Island: WAM R117967; West Montalivet Island: WAM R158562, WAM R158571; Don Island: WAM R158610; North Maret Island: WAM R158647 (Table S1, Fig. S8A, C).

**Etymology.** *Insularis* is derived from the Latin word *insula*, for island, since this species is restricted to islands.

**Diagnosis.** Morphologically similar to *C. johnstonei* and distinguished from this species by the
presence of mid-dorsal body scales with a mix of two or three keels (Fig. 5), whereas *C. johnstonei* always has two keels. As mentioned previously, it is also distinguished from *C. johnstonei* by longer body size, higher relative head depth, longer relative limb length, more sharp lobules in the ear aperture (mean values of 13 vs. 10; Fig. 5) and more lamellae under longest finger and toe (average 3 more). Prefrontal scales are either narrowly separated or in contact, while *C. johnstonei* often has more widely separated prefrontals. From a genetic perspective, four sites that change amino acids in the mtDNA *ND4* sequence reliably distinguish *Carlia johnstonei* and *Carlia insularis* sp. nov. (Table 2). Geographically distinct from *C. johnstonei* in some of the most outer islands of the Bonaparte Archipelago (see below).

Comparison with congeners. Distinguished from remaining Australian *Carlia* species by a reduced upper preocular and well separated from posterior margin of second loreal scale (Hoskin & Couper, 2012); a distinct interparietal, usually seven supraciliaries, prefrontals usually separated; at least 34 mid-body scale rows, that are dorsally 6-sided, each scale with an angular free edge and strongly bicarinate, with the keels aligned to form continuous longitudinal lines; ear-opening surrounded by many small and pointed lobules (Cogger, 2014). It is endemic to Kimberley islands where *C. johnstonei* and *C. isostriacantha* sp. nov. also occur at a regional scale. See diagnosis to distinguish from *C. johnstonei*; and distinguishable from *C. isostriacantha* sp. nov. by the presence of two keeled-scales and usually seven supraciliaries instead of six.

Description of holotype. Individual with 42.01 mm as SVL, tail 69.33 mm, axilla-groin length 19.71 mm, head length 8.86 mm, head width 6.29 mm, head depth 3.85 mm, forelimb 12.47 and hindlimb 17.42 mm. Body with keeled dorsal scales, mostly two keels but some scales with three. Six supraciliaries, seven supralabials, six infralabials, 19 subdigital lamellae in 3rd finger,
26 subdigital lamellae in 4\textsuperscript{th} toe. Circular ear not smaller (1.37 mm) than palpebral disc (1.19 mm) with 12 sharp ear lobules. Prefrontals narrowly separately and nasals widely spaced (2.56 mm). Midbody scale rows 37, 43 vertebral scales and 62 ventral scales.

Description. Snout-vent length (mm): 27.93 - 51.44 (N = 35, mean 41.83). Tail: 29.05 – 69.98 (N = 18, mean 51.02). Most specimens with separated prefrontal scales (62\%) by an average of 0.18 mm (N = 21, 0.02 – 0.54). Ear aperture smaller (N = 32, mean 1.27, 0.85 – 2.16), than palpebral disc (N = 32, mean 1.44, 1.04 – 2.13), with many small lobules (up to 18). Lamelae under third finger 17 – 23 (N = 35 mean 19.69), fourth toe 21 – 30 (N = 35 mean 26.31) (Table S7).

Laterally and dorsally blackish brown while ventrally yellowish with sometimes a bright blue or a dark blue gular (Fig. S8C), where in breeding males (Fig. S9A) lateral midbody has a light brown almost orange colour.

Distribution. Across the northwest and outer islands of the Bonaparte Archipelago (northern Kimberley islands in Western Australia) with confirmed occurrence on the Fenelon, Coneille, East Montalivet, West Montalivet, Don, Berthier, North Maret and South Maret islands.

Remarks. Despite extensive sampling, there are no records of \textit{C. insularis} sp. nov. and \textit{C. johnstonei} occurring on the same islands. All islands where the former species is confirmed are either laterite or volcanic islands, whereas \textit{C. johnstonei} also occurs in sandstone islands (How et al., 2006). The individuals of \textit{C. insularis} sp. nov. were collected in vine thicket and deciduous vine forest habitats (Richard How; pers. comm.). Despite Descartes island being relatively close to Fenelon and Corneille islands, only \textit{C. johnstonei} was confirmed on this island.

\textit{Carlia triacantha} Mitchell, 1953, Records of the South Australian Museum, Vol. 11, 75-90

Desert rainbow-skink
Holotype. SAM R2697, from Adelaide River, Northern Territory, in -13.183 131.1.

Diagnosis. Species morphologically distinguished from congeners by having three strong keels in scales, prefrontals more often in contact or very narrowly separated and usually six supraciliaries. Although more work is still needed to find unambiguously diagnostic traits between this species and *C. isostricantha* sp. nov., *C. triacantha* are mostly smaller (mean 36.55 mm vs. 40.07 mm), with shorter relative head length (mean 7.24 mm vs. 8.25 mm) and fewer ear lobules (usually 6 vs. 9, Fig. 5B). Geographically diagnosis from *C. isostricantha* sp. nov., possible in the centre of Australia, particularly Pilbara and Macdonald ranges region.

Description. Snout-vent length (mm): 23.78 - 44.98 (N = 35, mean 36.55). Tail: 38.48 – 75.90 (N = 17, mean 60.80). Prefrontal in contact (63%) while the rest with separated prefrontals (N = 11) by an average of 0.26 mm (0.03 – 1.81). Ear aperture smaller (N = 30, mean 1.13, 0.64 - 1.73), than palpebral disc (N = 30, mean 1.41, 0.99 - 1.71), with often one larger anterior lobule and several small (up to 7). Lamellae under third finger 16 – 22 (N = 30 mean 18.83), fourth toe 23 – 28 (N = 29 mean 24.83) (Table S7). Dorsally brown and ventrally yellow blueish with sometimes whitish line under eye.

Distribution. Widely distributed from Pilbara in Western Australia to Northern Territory (Fig. 1). However, more sampling and genetic analyses are needed to investigate whether this species is continuously distributed from the mesic Top End to arid central Australia or if the central Top End is only occupied by *C. isostriciantha* sp. nov.

*Carlia isostriciantha* sp. nov. (Fig. S7B, S8B, D and S9B)

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Monsoonal three-keeled rainbow-skink
Holotype. WAM R171420, from Prince Regent Nature Reserve, Western Australia, in -15.98972 125.32944. Specimen collected in 2010 by Paul Doughty (Fig. S7B).

Paratypes. WAM R168173 (Boongaree Island), WAM R168675 (Katers Island), WAM R171211 (Darcy Island), WAM R171905 (Wargul Wargul Island), WAM R171906 (Molema Island), WAM R171908 (Sunday Island), WAM R171921 (Storr Island), WAM R171933 (Balami ridge) (Table S1, Fig. S8B, D).

Etymology. *Isostriacantha* is derived from equal in greek (*isos*) with *triacantha*, (three spines, referring to the three keels in scales) due to the difficulty of morphologically distinguishing from its sister species *C. triacantha*.

Diagnosis. As similar to *C. triacantha*, this species is morphologically distinguished from other *Carlia* species by having three strong keels in scales, prefrontals more often in contact or very narrowly separated and usually six supraciliaries. As above-mentioned, in contrast with it closest relative, *C. triacantha*, this species has longer body size, a relatively longer head and tends to have more ear lobules, on average nine very small lobules (Fig. 4 and 5, Table S7). Another possible trait to distinguish between these species is a white line that begins posterior to each hind limb and can extend to midway through the tail (Fig. S9B). This trait is more evident in freshly caught individuals, or photographs of them, than in long preserved specimens and needs to be further tested through more observations on genetically typed individuals. Genetically diagnosed from *C. triacantha*, by three ND4 mtDNA sites (Table 2) and geographically by occurring in the Kimberley, although geographic diagnoses in Northern Territory requires further work.

Comparison with congeners. This species can be separated from most Australian *Carlia* species by an upper preocular reduced and well separated from posterior margin of second loreal scale
(Hoskin & Couper, 2012); a distinct interparietal, with usually six supraciliaries, prefrontals usually in contact or narrowly separated; 28-36 rows of mid-body scales, that are dorsally 6-sided triscupid, each usually with an angular free edge and strongly keeled; often one larger anterior lobule with many small lobules in a round ear-opening that is smaller than palpebral disc, while the palpebral disc occupies much more than half of lower eyelid (Cogger 2014). Specifically with potentially sympatric species, *C. johnstonei*, *C. amax*, *C. rufilatus*, *C. gracilis* and *C. munda*, this species can be identified by the presence of three strong keels in scales, prefrontals usually in contact, six supraciliaries and absence of white lateral line anterior to the forelimbs. To distinguish from its sister species, *C. triacantha*, see Diagnosis above.

Description of holotype. Male individual with 43.22 mm as SVL, tail 63 mm, axilla-groin length 20.06 mm, head length 8.79 mm, head width 6.57 mm, head depth 3.83 mm, forelimb 13.06 mm and hindlimb 19.34 mm. Body with three keeled dorsal scales. Six supraciliaries, seven supralabials, six infralabials, 17 subdigital lamellae in third finger, 23 subdigital lamellae in fourth toe. Horizontal ear wider (1.83 mm) than palpebral disc (1.57 mm) with 13 small sharp ear lobules (one anterior larger). Prefrontals in contact and nasals widely spaced (2.34 mm). Midbody scale rows 35, 38 vertebral scales and 52 ventral scales.

Description. Snout-vent length (mm): 24.72 - 49.12 (N = 68, mean 40.07). Tail: 29.1 – 86.68 (N = 39, mean 61.55). Prefrontal in contact (73%) while the rest with separated prefrontals (N = 19) by an average of 0.12 mm (0.01 – 0.37). Ear aperture smaller (N = 67, mean 1.33, 0.69 - 1.96), than palpebral disc (N = 67, mean 1.46, 0.92 - 1.81), with often one larger anterior lobule, many small (up to 13) and sometimes one superior. Lamelae under third finger 11 – 24 (N = 62 mean 19.27), fourth toe 18 – 30 (N = 62 mean 24.82) (Table S7). Dorsally brown and ventrally yellow blueish, with a light line under eye to ear, and often with a very light whitish line in the back of
hindlimbs to tail if not regrown (Fig. S9B).

Distribution. Widespread across the Kimberley and adjacent (mostly southern Kimberley) islands in Western Australia, with isolated records in the western Gulf region, spanning the border of the Northern Territory and Queensland (Fig. 1).

Remarks. Afonso Silva et al. (2017) found one genetically discordant sample with mtDNA of *C. isostriacantha* sp. nov. and nuclear of *C. triacantha* from the Victoria River region (ABTC61613, Table S1). This suggests a need for further regional surveys and genetic studies, particularly in the Northern Territory where only a few specimens with tissues were detected, to define the boundaries of both species, at geographical and morphological level.

Discussion

We used extensive genetic and morphological data to identify two new species of Rainbow skinks, *Carlia insularis* sp. nov. (Johnstonei B lineage) and *Carlia isostriacantha* sp. nov., (Triacantha B lineage), in an understudied region of Australia, the Kimberley. We also redefined diagnoses and geographic distributions of *Carlia johnstonei* and *C. triacantha*. Our work takes advantage of recent progress in techniques for obtaining large-scale sequence data and in methods for species delimitation, as part of a broader integrative taxonomic approach. These advances are particularly important for identifying cryptic species, such as those described here, where morphological evidence alone is often insufficient for reliable species identification.

Evidence for new cryptic species

A previous phylogeographic study with >2000 loci Afonso Silva et al. (2017) revealed two new candidates species in the *Carlia* genus. The current work confirms these are new species.
using three robust hypothesis-driven validation methods based on several independent sets of genes from the larger exon dataset. The use of multiple different methods provides a robust test for the previous discovery in Afonso Silva et al. (2017), and further validates the proposed species delimitation.

Although the existence of *C. insularis* sp. nov. and *C. isostriacantha* sp. nov. is well supported in the genetic data, distinguishing these species morphologically is more difficult due to their cryptic nature. The genus *Carlia* generally has few diagnostic taxonomic characters that allow for the separation of species using morphology. Even for *C. johnstonei* and *C. triacantha* as currently recognised, there are only a few morphological characters that effectively distinguish between these species, mainly the number of keels on the dorsal scales and the arrangement of ear lobules (Storr, 1974). However, morphological measurements broadly overlap between both *C. johnstonei* and *C. triacantha* lineages. Despite these issues, we were able to find statistically significant differences in morphology across both measurements and meristic data, supporting the presence of these lineages as different species.

Differences in body size, head and limbs traits as well as ear lobule numbers help in distinguishing the lineages. Morphological variation across each pair of taxa is strongly affected by body size (SVL), with the newly described species being larger than their respective sister taxa. The same is observed for the other significant traits, even after accounting for size.

Although for both species, there are some overlap between morphological groups, there was more morphological similarity between the *C. triacantha* lineages than between *C. johnstonei* lineages (Fig. 3), likely reflecting the shallower divergence seen within *C. triacantha*.

Though we were able to identify a few distinct morphological traits, using morphology alone to identify individuals will remain a challenge without a reference to geography. For the
two lineages within *C. triacantha*, even geography is a poor guide for the central Northern Territory region. Therefore, for more reliable diagnosis, we follow the suggestion of Renner (2016) and include a set of diagnostic mtDNA SNPs to distinguish between *C. johnstonei* and *C. insularis* sp. nov., and between *C. triacantha* and *C. isostriacantha* sp. nov. These SNPs can be easily assessed by cheaper Sanger sequencing of the mtDNA gene *ND4* (primers and protocol in Afonso Silva et al., 2017).

*Biodiversity significance of the two new species*

*C. insularis* sp. nov. is an important addition to the known biodiversity of the Kimberley islands. This region has recently been the focus of several studies that have documented unique biodiversity communities, namely in terms of vegetation (Lyons et al., 2012), avifauna (Pearson & Caton, 2013) and herpetofauna (Doughty et al., 2012; Palmer et al., 2013). Studies to understand the biodiversity value in this region are also of importance to conservation, as this area is being considered as a biodiversity refuge for fauna vulnerable to the invasive Cane Toad (Palmer et al., 2013). Although the west Kimberley region has several endemic species, only a few are endemic just to the islands, namely a blindsnake (Ellis, 2016) and several land snails (Criscione & Köhler, 2013, 2014), making the discovery of *C. insularis* sp. nov. very significant. But more island-endemics reptiles are expected to be described, since Palmer et al. (2013) suggested the occurrence of a few potential new species (including samples that correspond to *C. insularis* sp. nov.) that have not yet been described.

Although our genetic data allows us to describe *C. isostriacantha* sp. nov. as a new species, further collecting and analyses are needed across central Northern Territory for this and other taxa (also suggested in Rosauer et al., 2016). Specifically, there is a need to identify the
geographic distributions of *C. triacantha* sensu stricto and *C. isostriacantha* sp. nov., as well as to examine morphological divergence in this poorly sampled region. In a group of *Ctenotus* skinks, Rabosky *et al.* (2014) highlight how intraspecific morphological variability and geographic sampling gaps caused an inadequate understanding of biological diversity. As with *Ctenotus*, we suspect that many other species in the *Carlia* genus may yet require taxonomic revision. Potter *et al.* (2016) have also suggested unknown lineage diversity in another *Carlia* species in the Australian Monsoonal Tropics, which may lead to the description of additional *Carlia* species, particularly on the islands off the northeast Top End.

**Advantages and issues of using MSC methods**

A key element of our analysis was the use of multispecies coalescent (MSC) methods, including pioneering the application of StarBEAST2 to Bayes Factor species delimitation (BFD). MSC models are a robust approach that better describes species formation by considering coalescent processes; however, methods based on the MSC are typically computationally intensive. To surpass this limitation, we subsetted independent smaller sets of loci from around 2,300 loci, which also has the advantage of producing multiple replicate results that may be compared to confirm that estimated parameter values are robust to the choice of loci.

BFD using SNAPP and StarBEAST2 requires sampling from different power posteriors, including sampling purely from the prior. We found that convergence was difficult to achieve for our data set when BFD StarBEAST2 was used to sample from the prior with more than 20 loci. Despite this limitation, BFD StarBEAST2 has advantages over existing methods for species delimitation. Compared to SNAPP which requires unlinked SNPs, StarBEAST2 can extract much more information from each locus. Compared to BPP, StarBEAST2 has many more
options for substitution models, population size models, and relaxed clock models.

Conclusions

As Oliver, Keogh & Moritz (2015) express, most genetically divergent lineages within species remain invisible to other scientific work, like conservation assessments and management planning. This reinforces the need to evaluate whether genetically distinct lineages within species should be formally described. Here we validate and describe two new species of rainbow skinks in the northwest of Australia, a highly biodiverse region of Australia that is still relatively understudied. Using an integrative taxonomic approach, we employ three MSC methods, including the application of a new approach to delimit species, as well as integrating morphological data to provide strong evidence for these two new species. This work brings the number of Australian *Carlia* to 26 species. However, further such work is needed across the Australian Monsoonal Tropics, since deeply divergent lineages within species of lizards are the norm in this region.

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References

Abràmoff MD., Magalhães PJ., Ram SJ. 2004. Image processing with ImageJ. Biophotonics international 11:36–42.

Afonso Silva AC., Bragg JG., Potter S., Fernandes C., Coelho MM., Moritz C. 2017. Tropical specialist vs. climate generalist: Diversification and demographic history of sister species of Carla skinks from northwestern Australia. Molecular Ecology 0:1–14. DOI: 10.1111/mec.14185.

Andersen AN., Bocciarelli D., Fairman R., Radford IJ. 2014. Conservation status of ants in an iconic region of monsoonal Australia: levels of endemism and responses to fire in the eastern Kimberley. Journal of Insect Conservation 18:137–146. DOI: 10.1007/s10841-014-9624-x.

Bickford D., Lohman DJ., Sodhi NS., Ng PKL., Meier R., Winker K., Ingram KK., Das I. 2007. Cryptic species as a window on diversity and conservation. Trends in Ecology and Evolution 22:148–155. DOI: 10.1016/j.tree.2006.11.004.

Blom MPK. 2015. EAPhy: A flexible tool for high-throughput quality filtering of exon-alignments and data processing for phylogenetic methods. PLOS Currents Tree of Life 1. DOI: doi:10.1371/ currents.tol. 75134257bd389c04bc1d26d42aa9089f.

Bragg JG., Potter S., Bi K., Moritz C. 2015. Exon capture phylogenomics: efficacy across scales of divergence. Molecular Ecology Resources 16:1059–1068. DOI: 10.1111/1755-0998.12449.
Bryant D., Bouckaert R., Felsenstein J., Rosenberg NA., RoyChoudhury A. 2012. Inferring species trees directly from biallelic genetic markers: bypassing gene trees in a full coalescent analysis. *Molecular Biology and Evolution* 29:1917–1932. DOI: 10.1093/molbev/mss086.

Carstens BC., Pelletier TA., Reid NM., Satler JD. 2013. How to fail at species delimitation. *Molecular Ecology* 22:4369–4383. DOI: 10.1111/mec.12413.

Cogger H. 2014. *Reptiles and amphibians of Australia*. New Holland, Sydney.

Criscone F., Köhler F. 2013. Six new species of *Australocosmica* Köhler, 2011 from the Kimberley Islands, Western Australia (Mollusca: Pulmonata: Camaenidae). *Zootaxa* 3608:101–15. DOI: 10.11646/zootaxa.3608.2.1.

Criscone F., Köhler F. 2014. Molecular phylogeny and taxonomic revision of the genera *Baudinella* Thiele, 1931, *Retroterra* Solem, 1985 and *Molema* Köhler, 2011 endemic to the coastal Kimberley, Western Australia (Gastropoda, Camaenidae). *Journal of Zoological Systematics and Evolutionary Research* 52:273–284. DOI: 10.1111/jzs.12065.

Darriba D., Taboada GL., Doallo R., Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* 9:772. DOI: 10.1038/nmeth.2109.

Degnan JH., DeGiorgio M., Bryant D., Rosenberg NA. 2009. Properties of Consensus Methods for Inferring Species Trees from Gene Trees. *Systematic Biology* 58:35–54. DOI: 10.1093/sysbio/syp008.

Dolman G., Hugall AF. 2008. Combined mitochondrial and nuclear data enhance resolution of a rapid radiation of Australian rainbow skinks (*Scincidae: Carlia*). *Molecular Phylogenetics and Evolution* 49:782–94. DOI: 10.1016/j.ympev.2008.09.021.

Doughty P., Palmer R., Cowan M., Pearson DJ. 2012. Biogeographic patterns of frogs of the
Kimberley islands, Western Australia. *Records of the Western Australian Museum* Supplement 81:109–124. DOI: 10.18195/issn.0313-122x.81.2012.109-124.

Drummond AJ., Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 7:214. DOI: 10.1186/1471-2148-7-214.

Edwards S V., Xi Z., Janke A., Faircloth BC., McCormack JE., Glenn TC., Zhong B., Wu S., Lemmon EM., Lemmon AR., Leaché AD., Liu L., Davis CC. 2016. Implementing and testing the multispecies coalescent model: a valuable paradigm for phylogenomics. *Molecular Phylogenetics and Evolution* 94:447–462. DOI: 10.1016/j.ympev.2015.10.027.

Ellis RJ. 2016. A New Species of Blindsnake (Scolecodphidia: Typhlopidae: *Anilios*) from the Kimberley Region of Western Australia. *Herpetologica* 72:271–278. DOI: 10.1655/Herpetologica-D-16-00007.1.

Ence DD., Carstens BC. 2011. SpedeSTEM: a rapid and accurate method for species delimitation. *Molecular Ecology Resources* 11:473–80. DOI: 10.1111/j.1755-0998.2010.02947.x.

Fujita MK., Leaché AD., Burbrink FT., McGuire JA., Moritz C. 2012. Coalescent-based species delimitation in an integrative taxonomy. *Trends in ecology & evolution* 27:480–8. DOI: 10.1016/j.tree.2012.04.012.

Grummer JA., Bryson RW., Reeder TW. 2014. Species delimitation using Bayes Factors: simulations and application to the *Sceloporus scalaris* species group (Squamata: Phrynosomatidae). *Systematic Biology* 63:119–133. DOI: 10.1093/sysbio/syt069.

Guindon S., Gascuel O., Rannala B. 2003. A Simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52:696. DOI: 10.1080/10635150390235520.
Hedin M., Carlson D., Coyle F. 2015. Sky island diversification meets the multispecies coalescent - divergence in the spruce-fir moss spider (Microhexura montivaga, Araneae, Mygalomorphae) on the highest peaks of southern Appalachia. *Molecular Ecology* 24:3467–3484. DOI: 10.1111/mec.13248.

Hoskin CJ. 2014. A new skink (Scincidae: Carlia) from the rainforest uplands of Cape Melville, north-east Australia. *Zootaxa* 3869:224–236. DOI: 10.11646/zootaxa.3869.3.2.

Hoskin CJ., Couper PJ. 2012. Description of two new Carlia species (Reptilia: Scincidae) from north-east Australia, elevation of Carlia pectoralis inconnexa Ingram & Covacevich 1989 to full species status, and redescriptions of Carlia pectoralis (de Vis 1884). *Zootaxa*:1–28.

How R., Schmitt L., Teale R., Cowan M. 2006. Appraising vertebrate diversity on Bonaparte Islands, Kimberley, Western Australia. *The Western Australian Naturalist* 25:92–110.

Isaac NJB., Mallet J., Mace GM. 2004. Taxonomic inflation: its influence on macroecology and conservation. *Trends in Ecology & Evolution* 19:464–469. DOI: 10.1016/j.tree.2004.06.004.

Kass RE., Raftery AE. 1995. Bayes Factors. *Journal of the American Statistical Association* 90:773–795. DOI: 10.1080/01621459.1995.10476572.

Kearse M., Moir R., Wilson A., Stones-Havas S., Cheung M., Sturrock S., Buxton S., Cooper A., Markowitz S., Duran C., Thierer T., Ashton B., Meintjes P., Drummond A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647–9. DOI: 10.1093/bioinformatics/bts199.

Köhler F. 2011. Descriptions of new species of the diverse and endemic land snail Amplirhagada iredale, 1933 from rainforest patches across the Kimberley, Western Australia (Pulmonata: Camaenidae). *Records of the Australian Museum* 63:167–202. DOI: 10.3853/j.0067-1975.63.2011.1581.
Komsta L. 2011. Outliers: Tests for outliers. R package version 0.14. https://CRAN.R-project.org/package=outliers.

Korkmaz S., Goksuluk D., Zararsiz G. 2014. MVN: An R package for assessing multivariate normality. The R Journal 6:151–162.

Lanier HC., Knowles LL. 2012. Is Recombination a problem for species-tree analyses? Systematic Biology 61:691. DOI: 10.1093/sysbio/syr128.

Leaché AD., Fujita MK. 2010. Bayesian species delimitation in West African forest geckos (Hemidactylus fasciatus). Proceedings of the Royal Society of London B: Biological Sciences 277:3071–3077. DOI: 10.1098/rspb.2010.0662.

Leaché AD., Fujita MK., Minin VN., Bouckaert RR. 2014. Species delimitation using genome-wide SNP data. Systematic Biology 63:534–42. DOI: 10.1093/sysbio/syu018.

Liaw A., Wiener M. 2002. Classification and regression by randomForest. R News 2:18–22.

Lyons MN., Keighery GJ., Gibson LA., Handasyde T. 2012. Flora and vegetation communities of selected islands off the Kimberley coast of Western Australia. Records of the Western Australian Museum Supplement 81:205–244. DOI: 10.18195/issn.0313-122x.81.2014.205-244.

McKenna A., Hanna M., Banks E., Sivachenko A., Cibulskis K., Kernytsky A., Garimella K., Altshuler D., Gabriel S., Daly M., DePristo MA. 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Research 20:1297–303. DOI: 10.1101/gr.107524.110.

Mitchell FJ. 1953. A brief revision of the four-fingered members of the genus Leiolopisma. Records of the South Australian Museum:75–90.

Ogilvie HA., Bouckaert R., Drummond AJ. 2017. StarBEAST2 brings faster species tree
inference and accurate estimates of substitution rates. *Molecular Biology and Evolution.*

DOI: 10.1093/molbev/msx126.

Oliver PM., Bourke G., Pratt RC., Doughty P., Moritz C. 2016. Systematics of small *Gehyra* (Squamata: Gekkonidae) of the southern Kimberley, Western Australia: Redescription of *G. kimberleyi* Börner & Schüttler, 1983 and description of a new restricted range species. *Zootaxa* 4107:49–64. DOI: 10.11646/zootaxa.4107.1.2.

Oliver P., Keogh JS., Moritz C. 2015. New approaches to cataloguing and understanding evolutionary diversity: a perspective from Australian herpetology. *Australian Journal of Zoology* 62:417–430. DOI: 10.1071/ZO14091.

Oliver PM., Laver RJ., Melville J., Doughty P. 2014. A new species of Velvet Gecko (*Oedura: Diplodactylidae*) from the limestone ranges of the southern Kimberley, Western Australia. *Zootaxa* 3873:49–61. DOI: 10.11646/zootaxa.3873.1.4

Padial J., Miralles A., Riva ID la., Vences M. 2010. The integrative future of taxonomy. *Frontiers in Zoology* 7:1–14. DOI: 10.1186/1742-9994-7-16

Palmer R., Pearson DJ., Cowan MA., Doughty P. 2013. Islands and scales: a biogeographic survey of reptiles on Kimberley islands, Western Australia. *Records of the Western Australian Museum* Supplement 81:183–204. DOI: 10.18195/issn.0313-122x.81.2013.183-204

Park J., Koo K-S., Kim I-H., Park D. 2016. Complete mitochondrial genomes of *Scincella vandenburghi* and *S. huanrenensis* (Squamata: Scincidae). *Mitochondrial DNA Part B* 1:237–238. DOI: 10.1080/23802359.2016.1156490.

Pearson DJ., Caton W. 2013. The avifauna of larger islands along the Kimberley coast, Western Australia. *Records of the Western Australian Museum* Supplement 81:125–144. DOI:
Phillips BL., Baird SJE., Moritz C. 2004. When vicars meet: a narrow contact zone between morphologically cryptic phylogeographic lineages of the rainforest skink, *Carlia rubrigularis*. *Evolution* 58:1536–1548. DOI: 10.1111/j.0014-3820.2004.tb01734.x.

Potter S., Bragg JG., Peter BM., Bi K., Moritz C. 2016. Phylogenomics at the tips: inferring lineages and their demographic history in a tropical lizard, *Carlia amax*. *Molecular Ecology* 25:1367–1380. DOI: 10.1111/mec.13546.

Rabosky DL., Hutchinson MN., Donnellan SC., Talaba AL., Lovette IJ. 2014. Phylogenetic disassembly of species boundaries in a widespread group of Australian skinks (Scincidae: *Ctenotus*). *Molecular Phylogenetics and Evolution* 77:71–82. DOI: 10.1016/j.ympev.2014.03.026.

R Core Team. 2016. A language and environment for statistical computing.

Rambaut A., Suchard MA., Xie D., Drummond AJ. 2015. Tracer v1.6., Available from http://beast.bio.ed.ac.uk/Tracer.

Rannala B. 2015. The art and science of species delimitation. *Current Zoology* 61:846–853. DOI: 10.1093/czoolo/61.5.846

Rannala B., Yang Z. 2013. Improved reversible jump algorithms for bayesian species delimitation. *Genetics* 194:245–253. DOI: 10.1534/genetics.112.149039.

Renner SS. 2016. A return to Linnaeus’s focus on diagnosis, not description: the use of DNA characters in the formal naming of species. *Systematic Biology* 65:1085–1095. DOI: 10.1093/sysbio/syw032.

Ripley B., Venables B., Bates DM., Hornik K., Gebhardt A., Firth D., Ripley MB. 2013. Package “MASS.” *Cran R*.
Rosauer DF., Blom MPK., Bourke G., Catalano S., Donnellan S., Gillespie G., Mulder E., Oliver PM., Potter S., Pratt RC., Rabosky DL., Skipwith PL., Moritz C. 2016. Phylogeography, hotspots and conservation priorities: an example from the Top End of Australia. *Biological Conservation* 204:83–93. DOI: 10.1016/j.biocon.2016.05.002.

Rosenblum EB., Sarver B a J., Brown JW., Des Roches S., Hardwick KM., Hether TD., Eastman JM., Pennell MW., Harmon LJ. 2012. Goldilocks meets Santa Rosalia: an ephemeral speciation model explains patterns of diversification across time scales. *Evolutionary Biology* 39:255–261. DOI: 10.1007/s11692-012-9171-x.

Singhal S., Moritz C. 2013. Reproductive isolation between phylogeographic lineages scales with divergence. *Proceedings of the Royal Society B: Biological Sciences* 280:20132246. DOI: 10.1098/rspb.2013.2246.

Storr GM. 1974. The genus *Carlia* (Lacertilia, Scincidae) in Western Australia and Northern Territory. *Records of the Western Australian Museum* 3:151–165.

Sukumaran J., Knowles LL. 2017. Multispecies coalescent delimits structure, not species. *Proceedings of the National Academy of Sciences* 2016:201607921. DOI: 10.1073/PNAS.1607921114.

Wickham H. 2016. *ggplot2: elegant graphics for data analysis*. Springer.

Woerner AE., Cox MP., Hammer MF. 2007. Recombination-filtered genomic datasets by information maximization. *Bioinformatics* 23:1851–1853. DOI: 10.1093/bioinformatics/btm253.

Xie W., Lewis PO., Fan Y., Kuo L., Chen M-H. 2010. Improving marginal likelihood estimation for bayesian phylogenetic model selection. *Systematic Biology* 60:150. DOI: 10.1093/sysbio/syq085.
Yang Z. 2015. The BPP program for species tree estimation and species delimitation. *Current Zoology* 61:854. DOI: 10.1093/czoolo/61.5.854.

Yang Z., Rannala B. 2010. Bayesian species delimitation using multilocus sequence data. *Proceedings of the National Academy of Sciences of the United States of America* 107:9264–9. DOI: 10.1073/pnas.0913022107.

Yang Z., Rannala B. 2014. Unguided species delimitation using DNA sequence data from multiple loci. *Molecular Biology and Evolution* 31:3125–3135. DOI: 10.1093/molbev/msu279.

**Figures**

Figure 1 Distribution map with used genetic samples and measured specimens for *C. johnstonei* (A) and for *C. triacantha* (B) lineages, and lineages relationships (C) as in Afonso Silva et al. (2017). Triangles correspond to the genetic samples used in this study while circles correspond
to specimens measured. Blue Johnstonei A, yellow Johnstonei B, green Triacantha A and purple Triacantha B. Tree obtained with 20 representative samples in ASTRAL and respective lineage bootstrap.

Figure 2 Species tree with topology from BFD StarBeast2 gene set1 presenting node posterior probabilities for the two sets of data used for all three MSC methods.
Figure 3 PCA with log transformed (A, B) and size corrected (C, D) morphological measurements for *C. johnstonei* and for *C. triacantha* with colours by mtDNA lineage.
Figure 4 Boxplots of significantly different traits between mtDNA lineages for *C. johnstonei* and *C. triacantha*. JA – Johnstonei A, JB – Johnstonei B, TA – Triacantha A and TB – Triacantha B.

Figure 5 Relevant diagnostic traits. Irregular keeling in dorsal body scales for *C. insularis* sp. nov. (A) and difference in ear lobules of *C. triacantha* (left) and *C. isostriacantha* sp. nov. (right). Illustrations by Erin Walsh.

Tables

Table 1 Species delimitation support. For BPP support is in posterior probabilities while for BFD
StarBeast2 and BFD* SNAPP is based in Bayes Factors calculated using the two species model as the null model (two species support by comparing with the four species model).

|                | BPP gene set1 | BPP gene set2 | StarBeast2 gene set1 | StarBeast2 gene set2 | SNAPP SNP set1 | SNAPP SNP set2 |
|----------------|---------------|---------------|-----------------------|----------------------|----------------|----------------|
| Two species    | 0             | 0             | -318.10               | -274.04              | -4517.80       | -4443.35       |
| Three species  | 0             | 0             | 223.35                | 203.79               | 3526.41        | 3370.49        |
| Four species   | 1             | 1             | 318.10                | 274.04               | 4517.80        | 4443.35        |

Table 2 ND4 mtDNA diagnostic SNPs for each lineage. The position of each SNP is aligned with *Scincella vandenburghi* mitochondrial genome (Park et al. 2016). For each nucleotide position is also presented the correspondent amino acid substitution. Grey background refers to which species the SNP is diagnostic for.

|                | 10851 | 10864 | 10992 | 11115 | 11218 | 11365 | 11413 |
|----------------|-------|-------|-------|-------|-------|-------|-------|
| *C. johnstonei* | T Ser  | A Thr  | A Tyr  | A Thr  | A Met  | C Thr  | A Asn  |
| *C. insularis* sp. nov. | A Thr  | G Cys  | A Thr  | A Met  | T Ile  | G Ser  | T* Ile* |
| *C. triacantha* | T Ser  | A Tyr  | G Ala  | C Leu  | C Thr  | G Ser  | C Thr  |
| *C. isostriacantha* sp. nov. | T Ser  | A Tyr  | A Thr  | A Met  | C Thr  | G Ser  | T Ile  |

* Substitution is not diagnostic for a few individuals