A Framework for Resolving Cryptic Species: A Case Study from the Lizards of the Australian Wet Tropics

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Abstract—As we collect range-wide genetic data for morphologically-defined species, we increasingly unearth evidence for cryptic diversity. Delimiting this cryptic diversity is challenging, both because the divergences span a continuum and because the lack of overt morphological differentiation suggests divergence has proceeded heterogeneously. Herein, we address these challenges as we diagnose and describe species in three co-occurring species groups of Australian lizards. By integrating genomic and morphological data with data on hybridization and introgression from contact zones, we explore several approaches—and their relative benefits and weaknesses—for testing the validity of cryptic lineages. More generally, we advocate that genetic delimitations of cryptic diversity must consider whether these lineages are likely to be durable and persistent through evolutionary time. [Exome capture; cryptic species; phylogeny; species delimitation; squamates; taxonomy.]

Cryptic species, or taxa that are morphologically similar but genetically divergent, exemplify the two major challenges of species delimitation. First, species form on a continuum (Darwin 1859; Mayr 1942; Mallet 1995; De Queiroz 2007). As populations differentiate across space and time, they gradually become more divergent. As reflected in the debate over defining operational taxonomic units via DNA barcoding (Moritz and Cicero 2004), deciding how much divergence is sufficient to name lineages as species can be arbitrary. As with morphologically distinct lineages, diagnosing cryptic lineages presents this challenge because they fall through the full range of the divergence continuum (Hedgecock and Ayala 1974; Gómez et al. 2002; McDaniel and Shaw 2003). Second, speciation proceeds heterogeneously across many axes. We typically recover correlations across axes—e.g., rates of trait evolution such as song and mitochondrial divergence (Winger and Bates 2015). However, when axes of differentiation are discordant—for example, when phenotypic disparity is high and genetic divergence is low—the status of lineages becomes ambiguous. By definition, cryptic lineages have diverged heterogeneously (Bickford et al. 2007)—they are genetically-distinct groups that exhibit little or no morphological divergence. The taxonomic process of naming a species is a binary exercise—either a lineage is recognized as a species or not—and accounting for heterogeneity in this binary framework remains a challenge.

Biodiversity researchers increasingly face these challenges because we are increasingly discovering new cryptic lineages (Bickford et al. 2007). A confluence in genetic advances and broader geographic sampling has led to rapid increases in the number of cryptic species, such that a quarter of articles published in Zoological Record Plus mention cryptic species (Bickford et al. 2007; see also de León and Poulin 2016). Cryptic species comprise a significant proportion of the diversity in some regions (e.g., tropics; Smith et al. 2008) and taxonomic groups (e.g., reptiles; Oliver et al. 2010), and recently, putative cryptic species have been identified in high-profile threatened species like orangutans (Nater et al. 2017). These findings, and their implications for evolutionary biology and conservation (Frankham et al. 2012), emphasize the need for a more rigorous framework to assess the taxonomic status of cryptic lineages (Adams et al. 2014; Struck et al. 2018).

In this work, we propose a framework that diagnoses those cryptic lineages that are expected to be sufficiently durable to contribute to build-up of diversity over time and space. Because speciation is a continuum, we expect that many nascent species are lost to hybridization and extinction as part of the protracted speciation process (Rosindell et al. 2010; Dynesius and Jansson 2014). As such, we adopt the biological species concept (BSC, (Mayr 1942)), which defines species as units that exhibit barriers to reproduction and are thus more likely to persist through time. Although some might find this definition overly restrictive, we apply it here in hopes of avoiding “taxonomic over-inflation” (Isaac et al. 2004). However, do we diagnose populations that are likely to have substantial reproductive isolation (RI)? In allopatry, the degree of morphological divergence is expected to correlate with the extent of RI (Mayr 1942; Bolnick et al. 2006; Funk et al. 2006). Thus, when genetic and phenotypic divergence concur, species delimitation is typically uncontroversial. For cryptic species, where we cannot use phenotypic divergence as a proxy for RI,
we must instead use multiple lines of evidence to assess likelihood of strong RI.

A popular approach to assess cryptic lineages is to apply statistical species delimitation to multilocus genetic data (Fujita et al. 2012; Carstens et al. 2013), which some argue makes species delimitation more objective (Rannala 2015). An illuminating line of research has explored the parameter space under which these coalescent-based methods are expected to return statistically robust results (Zhang et al. 2011; Olave et al. 2014). What remains to be seen is if these statistically robust lineages are also biologically robust (Sukumaran and Knowles 2017). That is, will newly delimited lineages remain distinct through changing geographies and environments, or will they be mere evolutionary ephemera lost to hybridization and/or extinction (Seehausen et al. 2008; Rosenblum et al. 2012; Dynesius and Jansson 2014)?

A stronger and more direct approach to delimitation is to test for strongly restricted gene flow in sympatry or parapatry (Richardson et al. 1986; Adams et al. 2014). This can be done either by sampling a modest number of individuals in sympatry or by assessing the extent of genetic introgression through intensive analysis of contact zones between parapatric taxa. However, when candidate taxa are allopatric, assessing the likelihood of strong RI is even more challenging, as long recognized under operational versions of the BSC. To the extent that RI is time-dependent (Coyne and Orr 1989; Sasa et al. 1998; Fitzpatrick 2002; Roux et al. 2016), another approach is to extrapolate from closely-related taxa where the relationship between divergence and extent of RI has already been determined. In contrast to a “bar-coding gap” (Hebert et al. 2004b), this approach uses genome-scale evidence to assess the likelihood of strong RI rather than patterns of genetic divergence across nominal species vs. populations.

We explore these multiple approaches to species delimitation through the study of morphologically cryptic, phylogeographic lineages in the lizards of the Australian wet tropics (AWT). The AWT is a narrow region of rainforest in northeast Queensland, Australia (Fig. 1). During repeated glacial cycles in the Quaternary (Graham et al. 2006), the rainforest and, accordingly, the species endemic to these rainforests, were split across two major refugia. Populations of these rainforest species diverged across these refugia; comparative data have recovered deep phylogeographic splits within species across more than twenty taxa (Moritz et al. 2009). Morphological analyses show limited phenotypic divergence among phylogeographic lineages (Schneider and Moritz 1999; Hoskin et al. 2005; Hoskin et al. 2011). Subsequent contact zone studies showed that some of these phylogeographic lineages are reproductively isolated (Phillips et al. 2004; Hoskin et al. 2005; Singhal and Moritz 2013).

Herein, we focus on three species groups—the ‘Carlia rubrigularis’, ‘Lampropholis coggeri’ and ‘Lampropholis robertsi’ groups—which are part of the broader radiation of Eugongylus lizards (family: Scincidae) (Skinner et al. 2011). These groups are ecologically-similar; all are small (30–55 mm) skinks of the leaf-litter in the rainforests of the AWT. Previous multilocus analyses revealed several phylogeographic lineages within each of these nominal taxa (Dolman and Moritz 2006; Bell et al. 2010). Like many phylogeographic
units, these lineages are mostly morphologically cryptic and geographically circumscribed, and their ranges are either geographically proximate or narrowly overlapping. With an eye to integrative taxonomy (Padiáal et al. 2010), we synthesize data on genetics, morphology, and reproductive isolation assessed in contact zones to resolve the species status of lineages within these three groups and to formally revise their taxonomy. More generally, we use these lizards as a data-rich case study to explore the challenges of delimiting species among cryptic lineages that are parapatric or allopatric.

**Methods**

**Sampling, Data Collection, and Data Processing**

In this study, we analyze genetic data for individuals across three species groups across five nominal species and 13 putative lineages. The *Carlia rubrigularis* group consists of five lineages: *C. rubrigularis*, northern Wet Tropics (N); *C. rubrigularis*, southern Wet Tropics (S); *C. rhomboidalis*, northern mid-east Queensland (N); *C. rhomboidalis*, southern mid-east Queensland (S); and *C. wundalathini* at Cape Melville (Fig. 1). The *Lampropeltis c Rogeri* group consists of four lineages: *L. c Rogeri*, northern Wet Tropics (N); *L. c Rogeri*, central Wet Tropics (C); *L. c Rogeri*, southern Wet Tropics (S); and *L. c Rogeri* in the Mt Elliot uplands (EU). The montane *Lampropeltis robertsi* group consists of four allopatric lineages: *L. robertsi*, Carbine Tableland uplands (CU); *L. robertsi*, Thornton Peak uplands (TU); *L. robertsi*, Mt Bellenden Ker (BK); and *L. robertsi*, Mt Bartle Frere and southern Atherton Tablelands (BF AU).

These lineages had been previously described through the sequencing of an average of 27 genetically dispersed individuals per lineage for mtDNA and 12 for dispersed nDNA (Dolman and Moritz 2006; Bell et al. 2010). Because these lineages were sampled extensively in previous genetic analyses, and because they are circumscribed geographically (Fig. 1), we limited our sampling to a few individuals per lineage (Fig. 1; Supplementary Tables S1 and S2 available on Dryad). We aligned the data using MAFFT (Katoh and Standley 2013). Like many phylogeographic studies, we first identified these lineages by sequencing mitochondrial loci. For these taxa, mtDNA and nDNA are highly congruent except within narrow contact zones, and mtDNA provides our most complete understanding of geographic limits (Dolman and Moritz 2006; Bell et al. 2010). Accordingly, we downloaded all gene-referenced NADH dehydrogenase subunit 4 (ND4) data for these populations from GenBank (Supplementary Table S3 available on Dryad). We aligned the data using MAFFT and identified the coding sequence using Exonerate.

**Phylogenetic Analyses**

We reconstructed the evolutionary history of 13 lineages and two outgroups by using STARBEAST v2.13.5,
Individuals were assigned to lineages following their ‘putative lineage’ designations (Supplementary Table S2 available on Dryad). We filtered loci to only include those that were >75% complete across samples and to remove loci ≥1500 bp because longer loci are more likely to capture recombination events. Because of the computational demands of running STARBEAST2, we generated three random subsamples of 200 loci each from the remaining loci. We ran STARBEAST2 on these random subsets for 5000 generations sampling every 100 generations. Each locus was assigned to its own partition, and a GTR model of molecular evolution was used. Because we lack robust age constraints for nodes in this species tree, we instead inferred branch lengths in units of substitutions per site.

For the mitochondrial phylogenetic analysis, we determined the best-fitting partitioning strategy using PARTITIONFINDER2 (Lanfear et al. 2016). We then inferred the mtDNA gene trees using MrBayes v3.2.6, running two runs of four chains each for 500000 steps (Ronquist et al. 2012). We set the branch length prior to exponential (100); two runs of four chains each for 500000 steps (Ronquist et al. 2012). We set the branch length prior to exponential (100); we removed any site that was missing or polymorphic for any lineage in a species group. For the remaining sites, we removed any site that was missing or polymorphic for any lineage in a species group. For the remaining sites, we calculated the D-statistic across all possible species comparisons. To assess significance, we calculated the standard deviation across 200 bootstraps and used a one-tailed z-test (Eaton and Ree 2013). For L. robertsi, we further tested for introgression using the DFOIL approach designed for five-taxon symmetric topologies (Pease and Hahn 2015); we could not apply this method to other groups because their topologies are asymmetric. This method confirmed our D-statistic results, so we do not discuss them further.

Finally, we collated previously-published data on reproductive isolation at three contact zones: L. coggeri N and C, L. coggeri C and S, and C. rubrigularis N and S (Phillips et al. 2004; Dolman 2008; Singhal and Bi 2017). These studies sampled densely through each contact zone to infer current rates of hybridization and to determine patterns of introgression across the genome and geography.

**Statistical Species Delimitation**

One of the most common ways to validate cryptic lineages is through multilocus coalescent-based (MSC) approaches (Fujita et al. 2012). Accordingly, and as recommended by Rannala (2015), we used two MSC approaches (BPP v3.3a and STACEY v1.2.4) to test species boundaries across these groups (Yang and Rannala 2014; Jones 2017). Using the same filtering as in our STARBEAST2 analyses, we selected three random samples of 100 loci per species group and generated input files for BPP.
and STACEY. We used the species tree inferred from the starbeast2 analyses (see Phylogenetic Analyses) as the guide tree for BPP. For the 'L. coggeri' group, we used two topologies of the species tree that reflected uncertainty in the placement of L. coggeri EU (Supplementary Fig. S1 available on Dryad). We then ran MCMC for 500,000 generations across three sets of priors to ensure our results were robust to prior specification. These priors were: 1) \( \theta = (2, 2000), t \sim (2, 2000), 2 \) \( \theta = (1, 10), t \sim (1, 10), \) and 3) \( \theta = (1, 10), t \sim (2, 2000) \). We ensured that we had 20–80% acceptance rate; having too high or too low of acceptance rates can affect results. We ran STACEY for 1e7 generations. Each locus was set to have its own clock rate and own substitution model under a lognormal (mean = 5, sd = 2); collapse weight \( \sim \) uniform (0, 1); population prior scale \( \sim \) lognormal (mean = 7, sd = 2), and relative death rate \( \sim \beta (u=1, \beta=8) \). Species delimitations were determined using speciesda with a burn-in of 10% and a collapse height of 0.0001 (Jones 2017). Analyses showed that results were robust across collapse heights from 0.0001 to 0.001.

**RESULTS**

**Analysis of Genetic Data**

Our exome capture approach worked well across all lineages. On average, we recovered an average of 2.29 Mb per individual across 2668 loci at an average coverage of 112× (Supplementary Table S2 available on Dryad).

The inferred topology is well-supported and is consistent with previous phylogenetic hypotheses based on mtDNA data (Fig. 2, Supplementary Fig. S2 available on Dryad; Bell et al. 2010) and other phylogenomic analyses (Bragg et al. 2018). Branching times and topologies were quantitatively and qualitatively similar across replicate analyses of starbeast2 (Supplementary Fig. S1 available on Dryad). The branching times between these lineages all occur within a narrow range and highlight that two lineages of C. rubrigularis as currently recognized are polyphyletic. Further, the splitting patterns generally agree with the biogeographic relationships between species. For example, L. robertsi BFAU is sister to L. robertsi BK, and the two lineages occur as adjacent montane isolates (Figs. 1 and 2). The exception to this congruence is a leaptogrid distribution of L. coggeri EU, which is sister to L. coggeri N and C rather than the geographically adjacent L. coggeri S (Figs. 1 and 2).

We inferred a 4× to 9× range of genetic divergences between lineages within nominal species, with nuclear \( d_{xy} \) at silent sites ranging from 0.5% to 1.95% and nuclear \( d_{st} \) ranging from 0.18% to 1.68% (Fig. 3, Supplementary Table S5 available on Dryad). Lineages associated with small ranges such as C. undination and the montane lineages of L. robertsi showed lower levels of within-population diversity. Measures of mtDNA and nuclear \( d_{xy} \) and \( d_{st} \) were correlated (\( d_{xy} = 0.61; d_{st} = 0.57, P\text{-value} = 0.016; d_{st} = 0.025; \) Fig. 3A, B). As for inferred branching times, divergences between some

_P. SINGHAL ET AL._—A FRAMEWORK FOR RESOLVING CRYPTIC SPECIES 1065

2018
FIGURE 3. Patterns of divergence between pairwise lineage-comparisons for (A) \( d_{xy} \) for silent sites in mitochondrial DNA (mtDNA) and nuclear DNA (nDNA), (B) \( d_{at} \) at silent sites in mtDNA and nDNA, and (C) branching times in units of substitutions per site per million years and \( d_{at} \) at mtDNA; branching times were inferred from the tree depicted in Fig. 1. Each pairwise comparison is coded as either being between 1) recognized: two lineages that were already recognized at the species-level, 2) elevated: two lineages that were elevated in the current study, or 3) population: lineages for which there is insufficient evidence to elevate them to species. Arrows identify the three pairwise comparisons for which we have data on reproductive isolation from contact zone studies (Fig. 4), the gray line indicates the transition point at which we first recover evidence for isolation between lineages (i.e., between \textit{Carlia rubrigularis} N and S). Many of the lineages that we propose to elevate are more diverged than recognised species.

FIGURE 4. Evidence for rates of hybridization and introgression at three contact zones between lineages included in this analysis: \textit{Carlia rubrigularis} N and S, \textit{Lampropholis coggeri} C and S, and \textit{L. coggeri} N and C. Contacts are listed in the legend in order of least to most divergent. A) Percent of individuals in the center of contact zones that were identified as hybrid. A hybrid individual was defined as individuals that had \( \geq 10\% \) membership in both parental species as determined by STRUCTURE. B) Distribution of cline widths in the contact zone across an average of 9.5K clines. C) The extent of linkage disequilibrium in each contact zone. Moran’s I measures the autocorrelation in cline widths across the genome, which serves as a proxy for linkage disequilibrium. These genetic estimates of reproductive isolation show evidence for selection against hybrids in \textit{C. rubrigularis} N and S and \textit{L. coggeri} C and S.

cryptic lineages were significantly greater than those between nominal species (Fig. 3C).

Our D-statistic tests for historical introgression among non-sister lineages recovered four likely cases of historical introgression between lineage-pairs: \textit{C. rubrigularis} N and S; \textit{C. rubrigularis} N and \textit{C. rhomboidalis}; \textit{L. coggeri} S and EU; and \textit{L. coggeri} C and S (Fig. 2, Supplementary Table S6 available on Dryad). No signature of historical introgression was recovered for strongly allopatric populations—e.g., among montane isolates of the ‘\textit{L. robertsi}’ species group, and \textit{C. wundalthini} vs. \textit{C. rubrigularis}. Our previous results from analyses of hybridization and introgression at contact zones show that \textit{C. rubrigularis} N and S and \textit{L. coggeri} C and S exhibit 1) a moderate proportion of hybrids and the center of the contact zones, 2) narrow cline widths across the genome, and 3) auto-correlation in cline widths across physical distances, all indicative of extensive disequilibrium in hybrids and substantial RI (Fig. 4). The less divergent lineage-pair, \textit{L. coggeri} N and C, shows none of the same patterns, with evidence of extensive introgression across the genome and geography.

Statistical species delimitation supported all lineages as species. BPP returned \( \geq 95\% \) probability for a speciation event at all nodes in our guide trees, and \textit{Stacey} inferred each species as a unique cluster (Fig. 2). This result was robust to priors and, for the ‘\textit{L. coggeri}’ group, uncertainty in the topology.

**Analysis of Morphological Data**

For all three species groups, PCAs resulted in a PC1 that accounted for most of the variation (67–84%). It was loaded highly and positively by all body measurements and indicated body size (Supplementary Tables S7–S9 available on Dryad). The remaining four PCAs in each species group represented variation in body shape (Supplementary Tables S7–S9 available on Dryad). PC2 accounted for 10–16% of the variation in the species groups and was loaded most heavily by relative AG length (positive) in ‘\textit{C. rubrigularis}’ and both AG length...
Body shape (PC2–PC5) 2.68 3 1 0.42
Body size (PC1) 3.89 1 28 0.06

S vs. EU 5.00 4 5 0.05 *
N/C vs. EU 3.35 4 5 0.11
Multivariate contrasts N/C vs. S 2.56 4 5 0.17

Body shape (PC2–PC5) 3.46 8 10 0.04 *
Body size (PC1) 2.46 2 58 0.09

Body shape (PC2–PC5) 0.45 4 5 0.77
Body size (PC1) 1.17 1 50 0.29

Variance was driven by CV1 (Roy's Greatest Root

No significant differences were detected between the

The three major lineages in the 'L. cogerri' group show no differences in body size ('lineage' effect on PC1, $F_{4,5}=2.46$, $P=0.09$), but they do differ in body shape ('lineage' effect on PC2–5, Wilks' Lambda $F_{4,5}=3.46$, $P=0.04$) (Fig. 5B, Table 1). This significant variance was driven by CV1 (Roy's Greatest Root $F_{4,5}=6.05$, $P=0.03$), which is loaded most heavily by PC2 (0.489; Supplementary Table S10 available on Dryad).

Multivariate contrasts revealed that only L. cogerri EU and S differ significantly in shape ($F_{4,5}=5.00$, $P=0.05$) (Table 1). Neither L. cogerri EU and N/C ($F_{4,5}=3.35$, $P=0.11$) nor L. cogerri N/C and S ($F_{4,5}=2.56$, $P=0.17$) differed in shape. Therefore, the only detectible difference was that L. cogerri EU has a relatively longer body and shorter legs than the geographically adjacent, but distantly related, L. cogerri S.

No morphological differences were detected between the N and S lineages of 'C. rubrigularis', for either body size ('lineage' effect on PC1, $F_{1,50}=1.17$, $P=0.29$) or body shape ('lineage' effect on PC2–5, Wilks' Lambda $F_{4,5}=0.45$, $P=0.77$) (Fig. 5A, Table 1). Similarly, no significant differences were detected between the TU/CU and BK/BFAU lineages of 'L. robertsi'. Body size was marginally non-significant ('lineage' effect on PC1, $F_{4,28}=3.89$, $P=0.06$), and body shape did not differ ('lineage' effect on PC2–5, Wilks' Lambda $F_{3,1}=2.68$, $P=0.42$) (Fig. 5C, Table 1).

**Discussion**

**Delimitation of Cryptic Species**

Initial phylogeographic explorations based on mtDNA revealed that each species group contained at least four to five lineages, most of which were deeply divergent. Subsequent sequencing of five to ten nuclear loci confirmed that these phylogeographic lineages were also diverged at the nuclear genome (Dolman and Moritz 2006; Bell et al. 2010). Now, genetic data based on over 2500 exons confirmed that these lineages exhibit genetic divergences of substantial but varying depths. These genetic divergences all fall within the range that comparative data suggest spans the transition from populations to isolated species (Roux et al. 2016). Although some of the lineages are far more divergent than some already recognized species, and although we focused on morphological traits standardly

| Table 1. Testing for morphological differences in body size and shape between the major lineages in each species group |
|---|---|---|---|
| 'C. rubrigularis' N vs. S | 1.17 | 1 | 50 | 0.29 |
| Body size (PC1) | | | | |
| Body shape (PC2–PC5) | 0.45 | 4 | 5 | 0.77 |
| 'L. robertsi' CU/TU vs. BK/BFAU | 3.89 | 1 | 28 | 0.06 |
| Body size (PC1) | | | | |
| Body shape (PC2–PC5) | 2.68 | 3 | 1 | 0.42 |
| 'L. cogerri' N/C vs. S vs EU | 2.46 | 2 | 58 | 0.09 |
| Body size (PC1) | | | | |
| Body shape (PC2–PC5) | 3.46 | 8 | 10 | 0.04 * |
| Multivariate contrasts N/C vs. S | 2.56 | 4 | 5 | 0.17 |
| N/C vs. EU | 3.35 | 4 | 5 | 0.11 |
| S vs. EU | 5.00 | 4 | 5 | 0.05 * |

Body size was tested using nested ANOVA. Body shape was tested using nested MANOVA ('lineage' overall effect and planned contrasts), and using the Wilks' Lambda as the $F$-statistic.
used in lizard taxonomy and eco-evolutionary studies (Ingram 1991; Losos 2011; Hoskin 2014), we found little or no morphological divergence between the major lineages within each of the three species groups. Accordingly, these can mostly be regarded as truly cryptic, rather than pseudo-cryptic, species.

Given morphologically cryptic lineages that span a range of divergences, and all of which are delimited using coalescent methods, we are thus faced with the two challenges of species delimitation: how to determine how much genetic divergence is sufficient when divergences are arrayed on a continuum; and how to reconcile when genetic and phenotypic data give conflicting perspectives. As a first step, we can directly assess levels of isolation between these lineages because three of the lineage-pairs in these groups meet in narrow zones of parapatry (Fig. 4).

Through these fine-scale contact zone analyses of isolation, we have two major findings. First, we find that, like genetic divergence, RI exists on a continuum, with lineages exhibiting varying degrees of isolation (Fig. 4A, B). For this set of lineages, divergence and isolation appear to scale, although non-linearly. The average cline width at the \( L. \) \textit{coggeri} \( N \) and \( S \) hybrid zone is about 5.5× less than that at the \( L. \) \textit{coggeri} \( N \) and contact. Yet, \( L. \) \textit{coggeri} \( C \) and \( S \) is only 1.6× more genetically divergent than \( L. \) \textit{coggeri} \( N \) and \( C \). Theory does not predict a linear scaling; the cline width of a locus is proportional to the inverse square root of selection on that locus (Barton scaling; the cline width of a locus is proportional to the inverse square root of selection on that locus (Barton and Gale 1993). Thus, as selection on a locus increases, cline width can sharpen narrowly and quickly, as seen here. Further evidence of this non-linear accumulation of RI can be seen in patterns of linkage disequilibrium in these contact zones. Data from \( L. \) \textit{coggeri} \( N \) and \( C \) show no evidence for disequilibrium at introgressing sites, whereas \( C. \) \textit{rubrigularis} \( N \) and \( S \) and \( L. \) \textit{coggeri} \( C \) and \( S \) exhibit extensive disequilibrium extending a few kilobases (Fig. 4C). These results confirm theoretical expectations that lineages can quickly transition from acting as populations (i.e., \( L. \) \textit{coggeri} \( N \) and \( C \)) to acting as genomically isolated species (i.e., \( L. \) \textit{coggeri} \( N \) and \( S \) and \( C. \) \textit{rubrigularis} \( N \) and \( S \)). (Turner 1967; Barton 1983).

Second, these data show that, despite being nearly identically morphologically, the more genetically divergent lineages have substantial RI. At least for \( C. \) \textit{rubrigularis} \( N \) and \( S \), these lineages are not isolated by premating isolation (Dolman 2008), but rather by post-mating selection against hybrids (Phillips et al. 2004). Based on estimates of dispersal length and cline width of the hybrid index, selection against hybrids is strong. Hybrids between \( C. \) \textit{rubrigularis} \( N \) and \( S \) and \( L. \) \textit{coggeri} \( C \) and \( S \) are estimated to be 50–70% and 10–65% less fit than their parents, respectively (Phillips et al. 2004; Singhal and Moritz 2012). Estimated selection on hybrids between \( L. \) \textit{coggeri} \( N \) and \( C \), on the other hand, is negligible. The selection against hybrids seen in \( C. \) \textit{rubrigularis} \( N \) and \( S \) and \( L. \) \textit{coggeri} \( C \) and \( S \) is comparable (if not greater) than that seen between morphologically distinct hybridizing taxon-pairs. (Barton and Gale 1993; Singhal and Moritz 2012). Such strong selection suggests that these lineages will remain evolutionary distinct in the future, despite the high potential for gene flow. As such, we propose to identify \( C. \) \textit{rubrigularis} \( N \) and \( S \) as separate species, and likewise for \( L. \) \textit{coggeri} \( S \) and \( C. \) \textit{rubrigularis} \( C \). Because we found no evidence for RI between \( L. \) \textit{coggeri} \( N \) and \( C \), we retain them as distinct populations within one species (\( L. \) \textit{coggeri} \( N/C \)).

However, how should we diagnose those lineages for which we cannot indirectly or directly assay RI? For example, \( L. \) \textit{coggeri} EU is geographically isolated from \( L. \) \textit{coggeri} \( N/C \) and \( S \), and the lineages in the ‘\( L. \) \textit{robertsi}’ group are isolated on different mountaintops. These lineages do not meet in contact zones, and because of both practical and ethical reasons, cannot be easily kept in the laboratory for experimental trials. Instead, we extrapolate our estimates of RI from the three lineage-pairs that do meet in parapathy (Fig. 4) to the species group as a whole. This extrapolation assumes that the tempo and mode at which RI evolves is similar across this clade. The few comparative data on the rate at which RI evolves suggests that it can vary across broad clades (Rabosky and Matute 2013). However, for a clade like this, which consists of broadly related, morphologically and ecologically-similar lizards found in a similar biogeographic context, we suspect there is likely to be less variation. Indeed, RI and divergence time correlate closely across five sister-species comparisons in \textit{Carlia}, \textit{Lampropholis}, and a closely-related genus, \textit{Saproscincus} (Singhal and Moritz 2013; Singhal and Bi 2017). Further, and importantly, these lineages likely resulted from similar speciation processes—i.e., these deep, cryptic lineages evolved due to very long periods of isolation in environmentally similar refugia (see below). Therefore, we believe we can sensibly extrapolate our results across other cryptic congenic lineages that diverged under similar processes.

To extrapolate, we use the divergence between \( C. \) \textit{rubrigularis} \( N \) and \( S \) as our cutoff because they are the youngest lineage-pair for which we have solid evidence of RI. Divergence estimates are highly correlated across the three metrics for genetic divergence (\( r = 0.7–9 \); Fig. 3). Still, we take a conservative approach and only elevate those lineages that show greater divergence than what is seen for \( C. \) \textit{rubrigularis} \( N \) and \( S \) across all metrics (with one exception, below). Notably, this cutoff is greater than that seen among several comparisons between nominal taxa (Fig. 3). Divergences between \( L. \) \textit{robertsi} CU and TU, \( L. \) \textit{robertsi} BK and BFAU, and \( C. \) \textit{rhomboidalis} \( N \) and \( S \) all fall below this cutoff (Supplementary Table S5 available on Dryad), so we recognize these as phylogeographic lineages within species. However, the divergence between \( L. \) \textit{robertsi} BK/BFAU and \( L. \) \textit{robertsi} CU/TU is greater than this cutoff. Accordingly, we propose to diagnose the BK/BFAU and CU/TU allopatric lineages as species; this deep divergence suggests they are likely to exhibit RI should they ever come into contact. We use these groupings for morphological comparisons, as well.

The case of \( L. \) \textit{coggeri} EU is more ambiguous, when considering genetic data alone. \textit{Lampropholis} \textit{coggeri} EU
is most closely related to L. coggeri N/C, and divergence falls just below our proposed cutoff for raw divergence and branching time (Fig. 3A, C) but just above the cutoff for net divergence (Fig. 3B). However, L. coggeri EU sits isolated off the far south end of the Wet Tropics, geographically closest to L. coggeri S. Therefore, it is much more likely to interact with L. coggeri S in future, with which it shows much greater divergence (Fig. 1, Fig. 3; Supplementary Table S5 available on Dryad). Further, alone among the comparisons made here, L. coggeri EU is morphologically (and perhaps ecologically; see below) distinct from L. coggeri S (Table 1). Because the L. coggeri S and EU comparison is more salient than the L. coggeri C and EU comparison, we further propose to elevate L. coggeri EU as a separate species.

**Taxonomic Outcomes**

The formal taxonomic revisions of these lineages are presented in Appendix 1 available on Dryad. To summarize, we revise the 'C. rubrigularis' group to retain C. wundalthini and C. rhomboidalis, retain C. rubrigularis S as C. rubrigularis, and elevate C. rubrigularis N to C. crypta sp. nov. For the 'L. coggeri' group, we retain L. coggeri N/C as L. coggeri, elevate L. coggeri S to L. similis sp. nov., and elevate L. coggeri EU to L. elliotensis sp. nov. For the 'L. robertsi' group, we retain L. robertsi CU/TU as L. robertsi BFAU/BK to L. bellendenkerensis sp. nov. The key components of the new species descriptions are as follows.

**Carlia crypta** sp. nov.

**Holotype:** QM J75457

**Diagnosis:** Carlia crypta sp. nov. is a small, dark-sided rainforest skink with pentaladactyl limbs (overlapping or very narrowly separated when adpressed) and a movable lower eyelid containing a transparent disc. It is reliably distinguished from its sibling species (L. similis sp. nov. and L. coggeri) by 17 nucleotide differences in the mitochondrial gene NADH dehydrogenase 4 that result in 15 amino acid differences among the species (Table A1).

**Lampropholis bellendenkerensis**

**Diagnosis:** A large Lampropholis with dark flanks and prominent spotting on the posterior ventral surfaces, a row of dark edged pale spots on underside of tail. This species is reliably distinguished from its closest congener (L. robertsi) by 13 nucleotide differences in the mitochondrial gene NADH dehydrogenase 4 that result in nine amino acid differences between the species (Table A2).

**Lampropholis elliotensis** sp. nov.

**Holotype:** QM J39855

**Diagnosis:** Lampropholis elliotensis sp. nov. is a small, dark-sided rainforest skink with pentaladactyl limbs (usually separated by several scales rows when adpressed) and a movable lower eyelid containing a transparent disc. It is reliably distinguished from its sibling species (L. similis sp. nov. and L. coggeri) by 17 nucleotide differences in the mitochondrial gene NADH dehydrogenase 4 that result in 15 amino acid differences among the species (Table A1).

**Lampropholis similis** sp. nov.

**Holotype:** QM J39855

**Diagnosis:** Lampropholis similis sp. nov. is a small, dark-sided rainforest skink with pentaladactyl limbs (overlapping or very narrowly separated when adpressed) and a movable lower eyelid containing a transparent disc. It is reliably distinguished from its sibling species (L. similis sp. nov. and L. coggeri) by 17 nucleotide differences in the mitochondrial gene NADH dehydrogenase 4 that result in 15 amino acid differences among the species (Table A1).
Yet, this statistical diagnosis contrasts to our biological diagnosis all putative lineages as species (Fig. 2). Similarly, our tests for introgression suggest there has been introgression between at least four lineage-pairs (Fig. 2), all of which are either previously-recognized or now elevated nominal species. Yet, for at least two of these lineage-pairs (C. rubrigularis N and S and L. coggeri C and S), our analysis of current patterns shows that hybridization occurs but is geographically highly-restricted (Fig. 4). This distinction between historical and current patterns illustrates the complicated relationship between gene flow and species borders.

The Practicality of Delimiting Cryptic Species

Genetic divergences for almost all our lineage comparisons fall in the so-called “gray zone” of speciation, in which lineages transition from behaving as populations to species (Roux et al. 2016). Defined by net silent divergence ($d_{S}$) at coding nuclear genes, this gray zone spans divergences from 0.5% to 2%. Given that many cryptic lineages originated during glacial cycles over the last few million years (Hewitt 2000), many of them should fall within this four-fold range of divergence. This underlines the challenge in delimiting cryptic lineages—many of them have a biogeographic and divergence history that places them in an ambiguous zone of divergence, where lineages are as likely to merge or remain distinct upon secondary contact.

Given this ambiguity, identifying strong phylogeographic structure within species should be just the first step in diagnosing species boundaries across cryptic boundaries (Fig. 6, Table 2). Additional validation is required, which we loosely group into four categories: 1) statistical species delimitation, 2) post hoc discovery of phenotypic differences that delimit lineages (i.e., integrative taxonomy; Padial et al. 2010), 3) indirect or direct estimates of evolutionary isolation between lineages, or 4) calibration-based approaches. Herein, we outline these approaches briefly and explain their conceptual and practical benefits and limitations. Note that after applying any of these approaches, researchers must still formally revise the taxonomy for these validations to be recognized. However, all too often, these taxonomic revisions are not done (Carstens et al. 2013; Pante et al. 2014).

First, perhaps the most common approach currently used is statistical species delimitation, which applies coalescent-based methods to determine which lineages are genetically unique (Ence and Carstens 2011; Yang and Rannala 2014). Often, statistical approaches are used as an early step to more quantitatively assess visual clusters, and in other studies, they are used as a final stage of analysis to diagnose species (Fig. 6). Across our three species groups, statistical species delimitation diagnosed all putative lineages as species (Fig. 2). Yet, this statistical diagnosis contrasts to our biological understanding of species boundaries. For example, although L. coggeri N and C are statistically distinct, introgression between them is widespread genomically and geographically (Fig. 4). This disconnect reflects the emerging consensus that, although statistical species delimitation methods can robustly identify populations, these populations are not always equivalent to species (Rosenblum et al. 2012; Dynesius and Jansson 2014; Sukumaran and Knowles 2017). In other words, the genetic distinctiveness of a population does not necessarily confer robust evolutionary distinctiveness as envisaged under the BSC. Thus, we take a deliberately conservative approach and refrain from elevating morphologically cryptic lineages whose sole support is from statistical analyses of genetic data (Oliver et al. 2015). However, statistical approaches to species delimitation are the most efficient and flexible method across taxonomic groups and are likely to remain an attractive option for many study systems, even if these approaches alone provide insufficient evidence to denote robust taxa.

Second, while mostly not true in the present study (Fig. 5), researchers often discover post-hoc phenotypic differences after further investigation of putative cryptic lineages, leading to so-called pseudo-cryptic species (Knowlton 1993). For example, cryptic lineages might vary in traits that facilitate RI between lineages (e.g., mating calls, (Barber 1951)) or ecological co-existence (e.g., divergence in life-history; Leys et al. 2017). Often, however, these phenotypic differences might only relate trivially to how distinctive a lineage is—i.e., minor differences in scale counts. In such cases, phenotypic differentiation alone might not necessarily confer evolutionary distinctiveness, and further validation would be required.
Third, researchers can assay strength of RI between lineages either through indirect studies of contact zones, observation of sympatry among cryptic lineages, or laboratory- or field-based tests of mate choice and hybrid fitness (Blair 1972; Hoskin et al. 2005; Dolman 2008). As shown in this work (Fig. 4), these approaches offer detailed data on how likely lineages are to remain distinct if and when they overlap with their congenerics. However, these approaches are often quite practically limited—generating these data can require contiguous ranges, high population densities, organisms amenable to experimentation, and substantial investment in both time and money. These practical limitations surfaced in the present work; we were unable to test for RI between allopatric lineages, nor could we bring them into a laboratory setting. Barring this, alternative approaches could be used to identify cases of abrupt genetic boundaries across dense sampling or test for geographically-extensive introgression across lineage boundaries using large numbers of markers (Melville et al. 2017).

For those lineages not amenable to indirect or direct testing for RI, we used a fourth general approach: using calibrations to determine how much divergence is sufficient to elevate a species. In the DNA barcoding literature, these calibrations are typically informed by patterns of divergence among nominal taxa, although they are used across broad swaths of the tree of life—i.e., all birds or all butterflies (Hebert et al. 2003). Another option is to use calibrations informed by data on the tempo at which RI evolves in closely-related taxa, as done in this study. While this approach still requires the expensive and time-consuming generation of data on isolation between lineages, the cutoff is more principled than barcode gaps. Unlike barcode gaps, which are applied widely across taxonomic groups, our cutoff is based on the observed isolation between lineages within a given clade, which likely share a common mode of lineage divergence and speciation. Such an approach allows us to tackle cryptic diversity while reflecting the variable nature of the speciation process. Moving forward, we will explore whether this cutoff could contribute to diagnosing species boundaries among phylogeographic lineages of other species of Carlia (Potter et al. 2016).

Across all these approaches, a potential flaw is insufficient sampling of species ranges. In particular, by sampling two ends of an array of populations, we can infer distinction between lineages where gene flow is actually continuous throughout (Pante et al. 2015). Because all approaches to cryptic species validation either have conceptual or practical weaknesses (Table 2), the ideal approach will likely vary across taxonomic groups. For example, in the Wet Tropics, phylogeographic studies have recovered deep splits in other taxa outside of lizards, including frogs and mammals (Moritz et al. 2009). For the frog lineages, many of which occur in dense numbers, meet in narrow contact zones, and are amenable to breeding experiments, data on hybridization patterns and mate choice have helped validate putative cryptic lineages and led to the formal revision of lineages (Hoskin et al. 2005; Hoskin 2007). However, for mammals, density is too low to allow indirect or direct tests of isolation, so other approaches will be required.

Importantly, the framework we applied here—generating initial descriptions of within-species phylogeographic diversity (Dolman and Moritz 2006; Bell et al. 2010), confirming these patterns with multilocus data sets, and then inferring fine-scale patterns of current RI (Phillips et al. 2004; Singhal and Moritz 2012, 2013; Singhal and Bi 2017)—represents a significant investment in time and money. For many systems, this approach is simply not tenable, and further, often too slow where decisions on species limits have immediate conservation consequences (Hedin 2015). That said, given the dangers of ‘taxonomic overinflation’ (Isaac et al. 2004; Frankham et al. 2012), we advocate that researchers validate putative cryptic lineages by both considering statistical delimitation approaches and other data on the biological reality of lineages, whether that be direct or indirect evidence for isolation.

CONCLUSION

Cryptic species challenge traditional notions of species, because the discrepancy between morphological and genetic axes of divergence can make them hard to categorize. Yet, other data suggest that many cryptic species are phenotypically divergent, but on axes of variation that are harder to measure (e.g., mating pheromones in lizards). In cases where we cannot identify phenotypic differences, like the lizards of the Wet Tropics, we can test the validity of these lineages through other means, such as looking at interactions between cryptic lineages in parapatry. Often, these richer, more integrative datasets complement genetic data and show that cryptic lineages are independently evolving units. However, as we also see in these taxa, despite marked genetic differentiation, some cryptic lineages might just be ephemera, destined to be lost to hybridization with congeneric lineages, if they meet in the future. These data remind us that species boundaries are hypotheses (Fujita et al. 2012), our best estimate of the fate of these lineages and a recognition of the ever-evolving nature of species (Darwin 1859; Mallet 1995).

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**Data accessibility**

- Raw sequencing data: PRJNA448788
- Pseudo-reference files and variant sets: DataDryad
- Raw sequencing data: PRJNA448788

### Table 2. A survey of approaches that can be used to validate putative cryptic lineages and the benefits and limitations of each approach

<table>
<thead>
<tr>
<th>Approaches to validate putative cryptic species</th>
<th>Benefits</th>
<th>Limitations</th>
<th>Examples from this study</th>
<th>Other examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statistical species delimitation</td>
<td>Efficient and affordable; can be applied to asexual and sexual organisms; many methods can handle ancestral polymorphism.</td>
<td>Populations cannot be easily distinguished from true-species (Sukumaran and Knowles 2017); different approaches often lead to differing results (Carstens et al. 2013); results can be deceiving in the presence of gene-flow overlap among cryptic lineages.</td>
<td>L. coggeri EU</td>
<td>Geckos (Leaché and Fujita 2010), carnivorous plants (Carstens and Satler 2013), cavefish (Niemiller et al. 2012), mouse lemurs (Hotaling et al. 2016).</td>
</tr>
<tr>
<td>Identification of morphological, behavioral, physiological differences among ‘pseudo’ cryptic lineages</td>
<td>Can lead to the identification of divergence in traits that are likely to keep lineages distinct (e.g., ecological differences, phenological shifts, mating calls).</td>
<td>Phenotypic differences do not guarantee that lineages will remain distinct if they interact, (e.g., Hoskin et al. 2015)</td>
<td></td>
<td>Lizards (Silva et al. 2017), bowerflies (Schouroury et al. 2002), mayflies (Leys et al. 2007), wasps (Smith et al. 2008), butterflies (Hebert et al. 2004a), salamanders (Ressler and Apodaca 2007), frogs (Hoskin et al. 2011).</td>
</tr>
<tr>
<td>Identification of range overlap among cryptic species</td>
<td>Offers robust evidence that lineages are not interbreeding.</td>
<td>Many cryptic species are parapatric or allopatric, so not applicable to many taxa.</td>
<td></td>
<td>Rottifers (Gómez et al. 2002), frogs (Stewart and Lougheed 2013), lizards (Sites et al. 1995), kelp (Tellier et al. 2011), corals (Laddner and Palumbi 2012).</td>
</tr>
<tr>
<td>Direct estimates of reproductive isolation through mate choice studies and crossing experiments</td>
<td>Offers robust evidence that lineages are not interbreeding.</td>
<td>Can only be used for sexually reproducing species that are amenable to lab husbandry; expensive and time-consuming; lab-based estimates of mate choice and hybrid fitness can differ from field-based estimates.</td>
<td></td>
<td>C. rubrigularis N, C. rubrigularis S, L. coggeri C, L. coggeri S</td>
</tr>
<tr>
<td>Indirect estimates of reproductive isolation from regions of parapary or sympathy</td>
<td>Allows indirect measure of factors structuring extent of gene flow between lineages such as extent of assortative mating, genetic incompatibilities, etc.</td>
<td>Can only be used for sexually reproducing species that co-occur and exist at sufficient density for sampling; indirect estimates can be influenced by often uncharacterized demographic factors.</td>
<td></td>
<td>C. rubrigularis N, C. rubrigularis S, L. coggeri C, L. coggeri S</td>
</tr>
<tr>
<td>Using calibrations of RI vs. genomic divergence based on data from closely-related species</td>
<td>Provides a well-informed guideline for species that are likely to evolve reproductive isolation at a similar tempo.</td>
<td>Still requires the extensive and expensive collection of data on reproductive isolation in closely-related species.</td>
<td></td>
<td>L. robertsi YU/CU, L. robertsi BK/ BFAU</td>
</tr>
<tr>
<td>Using calibrations based on sequence divergence</td>
<td>Efficient and affordable; can be applied to asexual and sexual organisms; can be more clade-specific if informed by patterns of divergence between nominal species in the clade.</td>
<td>Not conceptually well-grounded (but see Roux et al. 2004); what metric of divergence to use is unclear (Fregin et al. 2013); clades might vary in the rate at which they evolve reproductive barriers (Rakey and Matute 2013).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

We highlight, which approaches were used in this study and identify other examples from the broader literature; this list of examples is meant to be illustrative not exhaustive. Many of the studies that validated putative cryptic species did not also conduct a formal taxonomic revision.
• Code used to generate data: https://github.com/singhal/AWT_delimit and https://github.com/singhal/SqCL.

• Specimens used for morphological analyses & species descriptions: all are deposited at Queensland Museum for research use; accession numbers available in Supplementary Appendix 1 available on Dryad.

SUPPLEMENTARY MATERIAL

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.g7v1b.

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