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Speciation by monobrachial centric fusions: A test of the model using nuclear DNA sequences from the bat genus *Rhogeessa*

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ABSTRACT

Members of *Rhogeessa* are hypothesized to have undergone speciation via chromosomal rearrangements in a model termed speciation by monobrachial centric fusions. Recently, mitochondrial cytochrome-*b* sequence data tentatively supported this hypothesis but could not explicitly test the model's expectations regarding interbreeding among karyotypic forms. These data showed potential evidence for hybridization or incomplete lineage sorting between the karyotypically distinct *R. tumida* and *R. aeneus* and identified multiple lineages of karyotypically identical *R. tumida*. Here, we present a more comprehensive test of speciation by monobrachial centric fusions in *Rhogeessa*. Our analysis is based on sequence data from two nuclear loci: paternally inherited ZFY and autosomal MPI genes. These data provide results consistent either with incomplete lineage sorting or ancient hybridization to explain alleles shared at low frequency between *R. aeneus* and *R. tumida*. Recent and ongoing hybridization between any species can be ruled out. These data confirm the presence of multiple lineages of the $2n = 34$ karyotypic form ("*R. tumida*") that are not each other's closest relatives. These results are generally consistent with speciation by monobrachial centric fusions, although additional modes of speciation have also occurred in *Rhogeessa*. Phylogeographic analyses indicate habitat differences may be responsible for isolation and divergence between different lineages currently referred to as *R. tumida*.

1. Introduction

Chromosomal rearrangements have been proposed as a mechanism for speciation in a wide variety of taxa (King, 1993). One model of chromosomal speciation is speciation by monobrachial centric fusions (Baker and Bickham, 1986). Within mammals, this model has been hypothesized to have occurred in *Rattus* (Baverstock et al.,1983, 1986), *Castor* (Ward et al., 1991), the *Sorex araneus* species group (Searle, 1998), and the European house mouse, *Mus domesticus*. This phenomenon has been most extensively studied in *M. domesticus*, where breeding experiments have demonstrated that centric fusions result in reproductive isolation (Capanna et al., 1976; Gropp and Winking, 1981; White et al., 1978). It has also been proposed to explain the diversity in the bat genus *Rhogeessa* (Baker et al., 1985; Baker and Bickham, 1986).

Bats of the genus *Rhogeessa* (Chiroptera: Vespertilionidae), with their unusual karyotypic diversity, morphological similarity, and high species diversity represent an excellent system for studying chromosomal mechanisms of speciation. Much of the interest in this genus has focused on members of the "*R. tumida* complex", a group that inspired the model of speciation by monobrachial centric fusions (Baker and Bickham, 1986). Members of this species complex include *R. tumida* ($2n = 34$), *R. aeneus* ($2n = 32$), *R. io* ($2n = 30$), *R. velilla* ($2n = 42$), *R. genowaysi* ($2n = 42$) and *R. hussoni* ($2n = 52$; Bickham and Baker, 1977; Genoways and Baker, 1996). The karyotypes of *R. tumida* complex members have undergone extensive chromosomal rearrangements (all centric fusions) which, according to the hypothesized speciation model, led to reproductive isolation between populations containing different sets of fusions. All members of this group except *R. genowaysi* and *R. velilla* have karyotypes that differ monobrachially (i.e., contain biarmed chromosomes that have one arm but not the other in common) from other members of the group (Baker et al., 1985; Bickham and Baker, 1977).

Baird et al. (2008) showed, based on mitochondrial cytochrome-*b* (cyt-*b*) sequence data, that the *R. tumida* complex was a monophyletic group in which the relationships of many species were potentially consistent with a hypothesis of speciation by monobrachial centric fusions (Fig. 1). A few exceptions were noted: (1) two individuals of $2n = 34$ *R. tumida* occurred within a clade of $2n = 32$ *R. aeneus*; and (2) *R. tumida* includes multiple populations with $2n = 34$ that form three distinct lineages that are not all sister

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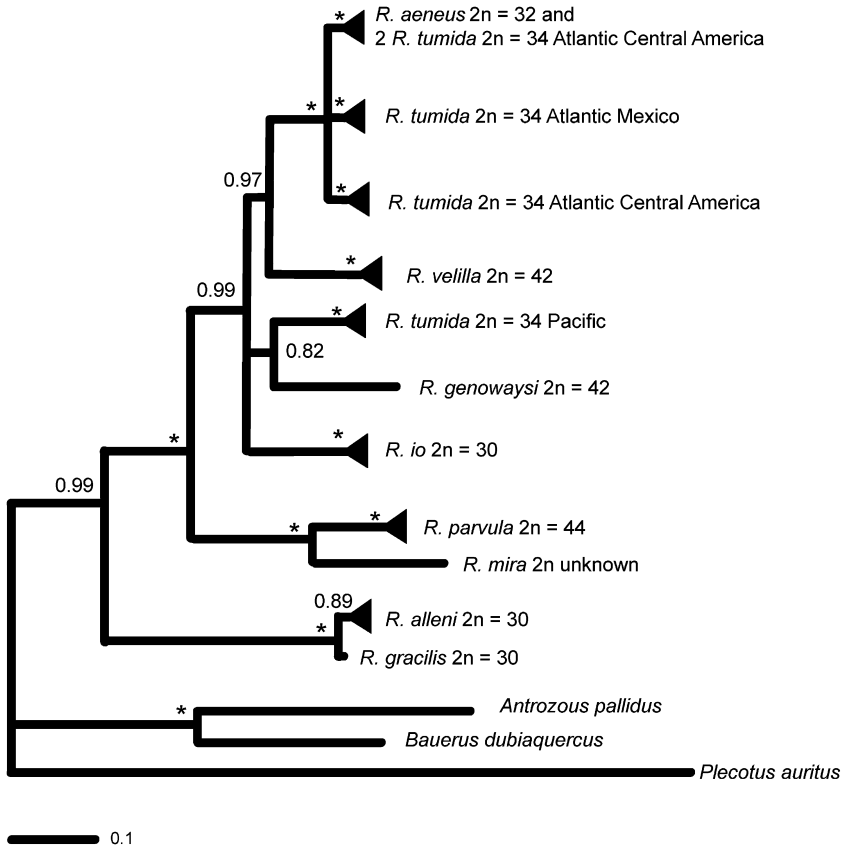

Fig. 1. Summary of phylogenetic relationships of *Rhogeessa* based on cytochrome-*b* sequences recovered in Baird et al. (2008). Branch lengths are to scale of the ML tree. Numbers at the nodes of major clades indicate Bayesian posterior probabilities (an asterisk represents p.p. = 1.0). Clades containing more than one individual and haplotype are represented by triangles.

taxa (one from the Pacific versant of Mexico and Central America, one from the Atlantic coast of Mexico and one from the Atlantic coast of Central America). According to this speciation model, individuals having karyotypes that differ by monobrachial fusions should be reproductively isolated, whereas those having no monobrachial differences should be able to interbreed (Baker and Bickham, 1986). Therefore, under the scenario of speciation by monobrachial centric fusions, one would expect monophyly of each karyotypic form and no evidence of hybridization between different karyotypic forms exhibiting monobrachial differences from one another. With respect to the observation of two *R. tumida* individuals within a clade of *R. aeneus* (two species that have monobrachial differences between their karyotypes), Baird et al. (2008) concluded that a possible explanation for the observed phylogenetic topology could be hybridization. However, they noted that mitochondrial sequence data alone are not sufficient to conclusively demonstrate hybridization. The results could also be accounted for by incomplete lineage sorting in the mtDNA gene sequenced (Avise, 2000). Therefore, nuclear markers must be examined to differentiate between these two alternative explanations.

We initiated this study to further investigate the possible occurrence of gene flow between *Rhogeessa* species having monobrachial differences in their karyotypes. We previously examined phylogenetic patterns from maternally-inherited markers (Baird et al., 2008), and will present here data from paternally and bi-parentally inherited markers. These three linkage groups should all have different lineage sorting periods due to their different modes of inheritance and effective population sizes (Chesser and Baker, 1996; Moore, 1995). Although not much is known about the behavior of *Rhogeessa*, other bats (including vespertilionids) have been shown to exhibit female philopatry and male-biased dispersal (Weyandt et al., 2005; Kerth et al., 2000; Wilkinson, 1985). If we assume the same happens in *Rhogeessa*, Y-chromosomal markers should have the shortest lineage sorting period, followed by mtDNA, and finally autosomal markers (Hoelzer, 1997; Moore, 1997). As summarized in Tosi et al. (2003; see citations within), the effective population sizes, and therefore lineage sorting periods, of mtDNA and Y-chromosomal loci are not equal. It has been shown that female philopatry can serve to preserve multiple mitochondrial lineages, whereas male migration has the opposite effect on Y-chromosome lineages. The effective population size of Y-chromosomal loci is further reduced with respect to that of mtDNA by variation in male reproductive success. Therefore, because they have the shortest lineage sorting period, Y-chromosomal markers should follow the overall species phylogeny more closely than do the other markers. Furthermore, lineage sorting of unlinked genes is not expected to produce congruent patterns across individual loci, other than those that are also consistent with species phylogeny.

Although they are now more widely available than in the past, Y-chromosomal and autosomal sequence data are still used relatively infrequently in combination with mtDNA to investigate species relationships (Tosi et al., 2003; Lim et al., 2008). Moreover, the majority of studies that have used autosomal sequences to construct phylogenies used the consensus sequence of the two alleles (i.e., a single sequence containing ambiguous bases to represent two different alleles at once) in phylogenetic analyses. This practice can be problematic in accurate phylogenetic reconstruction, and does not clearly depict hybridization events (Bradley et al.,

1993; Holloway et al., 2006). In our study, which seeks to test explicitly for hybridization and reproductive isolation, we use individual allele sequences to understand these processes better. We also selected nuclear loci that, in combination with the maternally-inherited mtDNA data already obtained (Baird et al., 2008), will give a more complete picture of the evolutionary history of the group. Included in this study are a paternally inherited Y-chromosome gene (Zinc Finger Y; ZFY) and a bi-parentally inherited autosomal gene (manose-6-phosphate isomerase; MPI).

The autosomal locus we examine in this paper (MPI) was previously used among other loci in an allozyme study of *Rhogeessa* species (Baker et al., 1985). All together, the allozyme data did not fully resolve the phylogenetic relationships of *Rhogeessa*, but they did succeed in grouping members of the *R. tumida* complex as monophyletic. The MPI locus itself showed unique alleles for each karyotypic form of *Rhogeessa*, with the exception that the $2n = 34$ form had one allele identical to the allele fixed in the $2n = 30$ form. The only two species to have multiple alleles at the MPI locus were *R. genowaysi* ($2n = 42$; 2 alleles) and *R. tumida* ($2n = 34$; 4 alleles). Therefore, we expected sequences of alleles from this locus to be informative about the relationships and hybridization (if any) between species.

The observation from Baird et al. (2008) that the $2n = 34$ form (*R. tumida*) does not constitute a monophyletic group based on mtDNA suggests that the status of this putative species should be tested using other data. LaVal (1973) studied the morphology of *Rhogeessa tumida* from throughout its range. He noted variation in several morphological characters but found no clear delineations along which to break this species up based on these differences. In fact, based on morphology, he still considered *R. io*, *R. velilla*, *R. aeneus* and *R. genowaysi* all to be *R. tumida*. Bickham and Baker (1977) and Baker et al. (1985) studied karyotypes of the $2n = 34$ form from throughout its range and consistently observed the same sets of centric fusions composing the $2n = 34$ karyotype. Baker et al. (1985) also studied allozyme variation in *Rhogeessa*. Based on these data they did not note any evidence to support splitting *R. tumida* into multiple species. Therefore, only mtDNA sequence data have supported the possibility of multiple species within the $2n = 34$ karyotypic form. In this study, we further investigated the possibility of multiple species within the $2n = 34$ karyotypic form using autosomal and Y-linked loci.

Given their identical karyotypes, it is unlikely that chromosomes played a role in creating diversification between the different lineages of *R. tumida* ($2n = 34$) recovered in Baird et al. (2008). The two Atlantic lineages differ by 2.5% K2P distance in cyt-*b* and the Pacific lineage differs from the Atlantic lineages by about 10% (Baird et al., 2008; Fig. 1). Two major geologic events correlate with current ranges of this species and have been shown to create phylogeographic structure in other species. The older of the two is the uplift of the various mountain ranges in Mexico and Central America. Because of the deeper split between the Pacific and Atlantic lineages, and the fact that their ranges roughly correspond to either side of these mountains, this vicariance hypothesis seems reasonable. Second, a seaway may have existed at the Isthmus of Tehuantepec during the Pliocene–Pleistocene (Mulcahy et al., 2006). This dates to roughly 2.5 mya, and may better correspond to the divergence of the two Atlantic lineages. In this paper we test whether these geologic events, as well as habitat differences, may have contributed to diversification in different lineages of *R. tumida*.

Recently, Baker and Bradley (2006) reviewed the importance of genetic data in describing species of mammals. They viewed these data in the context of genetic divergence as it relates to established species boundaries. They considered a species as "a group of genetically compatible interbreeding natural populations that is genetically isolated from other such groups" (Baker and Bradley, 2006). They established criteria that should be useful for estimating the boundaries of reproductive isolation across mammalian lineages. This is critical in species groups such as the *R. tumida* complex where there is little morphological variation and mating behaviors are as yet unstudied. It is in this context that we examine additional genetic data from *Rhogeessa* to estimate the limits of species boundaries.

The goals of this study include further examination of the phylogenetic relationships among *Rhogeessa* species using nuclear DNA sequence markers. Specifically, we test the hypotheses of relationships from mtDNA data presented in Baird et al. (2008) and use nuclear data to examine the potential evidence for hybridization between *R. tumida* and *R. aeneus*. We also explicitly test the expectations of the speciation by monobrachial centric fusions model regarding interbreeding between karyotypic forms outlined above. Additionally, we test alternative geographic hypotheses to explain the divergence between the different genetic lineages of "*R. tumida*" (the $2n = 34$ forms).

2. Methods

2.1. Taxon sampling

A total of 31 male *Rhogeessa* were sequenced for the ZFY dataset and 63 *Rhogeessa* for MPI (Appendix 1). All samples were taken either from frozen museum tissue or tissues from animals captured in Guatemala. A map of sampling localities is shown in Fig. 2. For the ZFY dataset, *Myotis tricolor* and *Bauerus dubiaquercus* were used as outgroups. For the MPI dataset, we compared (using BLAST; Altschul et al., 1990) a *Rhogeessa* MPI sequence against the *Myotis lucifugus* genome sequence in GenBank and used the matching sequence as an outgroup in addition to the sequence from *B. dubiaquercus*. All *Rhogeessa* species represented in Baird et al. (2008) are included in the ZFY dataset, excluding *R. genowaysi* for which we only have a single female specimen. *Rhogeessa parvula* and *R. alleni* were excluded from the MPI dataset because of our inability to amplify clean samples of this gene in these taxa.

2.2. DNA extraction and sequencing

DNA was extracted from frozen tissue samples using a Qiagen DNEasy Kit (Qiagen, Valencia, CA). For the ZFY gene, DNA was amplified using the primers LGL335F (5′-AGA CCT GAT TCC AGA CAG TAC CA-3′) and LGL331R (5′-CAA ATC ATG CAA GGA TAG AC-3′; Cathey et al., 1998). The resulting amplification was not chromosome-specific, thus resulting in the homologous region being amplified from both the X (Zinc Finger X gene) and Y-chromosomes. The amplified fragment corresponded to the last intron in the ZFY cistron with some exon sequence flanking each side. These products were purified using a Viogene Gel Extraction Kit (Viogene, Sunnyvale, CA). Purified products were then amplified with the primers Las335YF (5′-CCA AAC AGG TGA GGG CAC ATA-3′) and LGL331R (same as above) to obtain a Y-specific fragment. This fragment was then sequenced with the Las335YF primer.

For the MPI gene, DNA was amplified using the primers MPIEX4F (5′-TGC CAA CCA CAA GCC AGA RAT GG-3′) and MPIEX5R (5′-GGG AGA TCC GYT TCA CCA ACA GG-3′). The resulting amplification contained the 3′ end of MPI exon 4 and the 5′ end of exon 5, with an intron in between. These products were cleaned using the same methods described above. An initial sequencing reaction was performed using the same primers as in the PCRs. Because individuals of *Rhogeessa* are diploid for the MPI locus, this sequencing step results in a consensus of the two alleles. In the case of heterozygous individuals, polymorphic sites were identified by a double

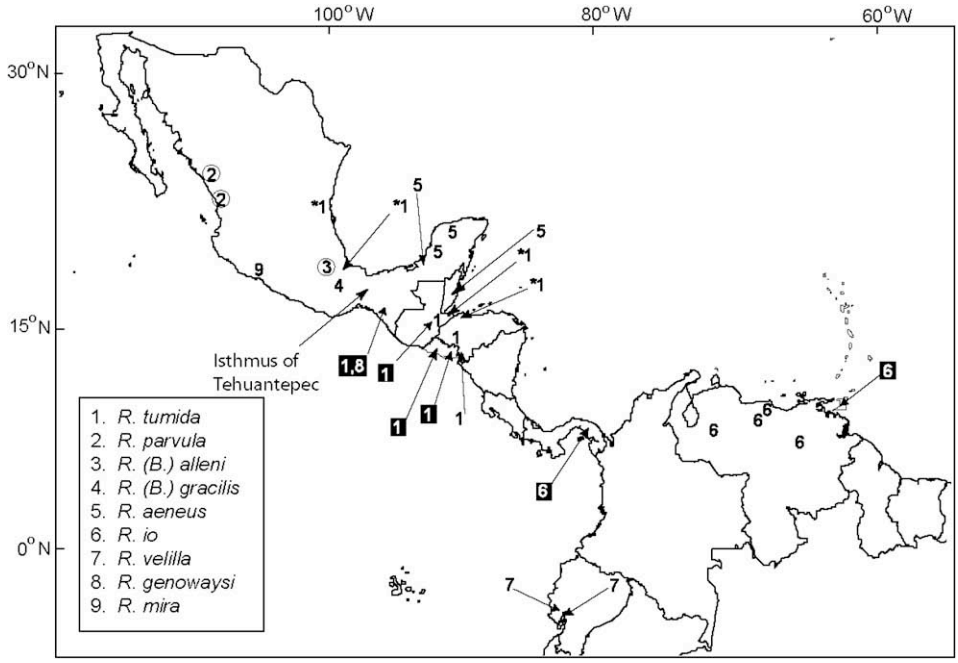

Fig. 2. Sampling localities for individuals of *Rhogeessa* examined at the ZFY and MPI loci. Localities in close proximity are omitted. Circled numbers represent localities only in ZFY dataset; numbers in black boxes represent samples only in MPI dataset. All other numbers represent localities in both datasets. *R. tumida* localities (1) marked with an asterisk (∗) represent populations that fall phylogenetically within the Atlantic *R. tumida* lineages. The Isthmus of Tehuantepec, where a seaway is proposed to have been in place during the Pliocene–Pleistocene which was tested as a phylogeographic barrier in this study, is also indicated.

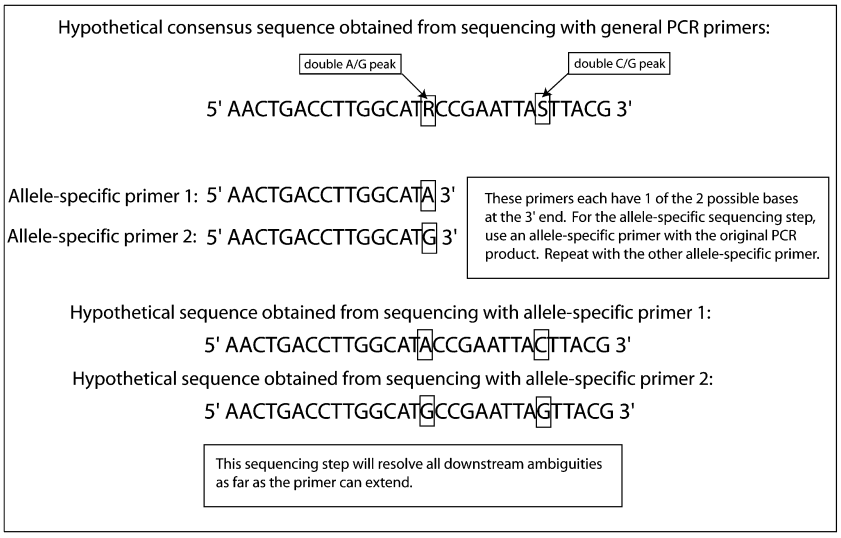

Fig. 3. Allele-specific primer design process.

peak in the initial sequencing step, and allele-specific primers were then designed (Fig. 3). A list of all allele-specific primers used in this study is given in Table 1. Allele-specific primers were made by making the nucleotide at the 3′ end of the primer specific for one of the polymorphic bases. A second primer was made in the same way for the other base. Sequencing for each individual allele was then performed using template from the original PCR (from the MPIEX4/MPIEX5 primer set) plus each allele-specific primer in separate reactions. This step resulted in separate sequences for each individual allele. Allele-specific priming was not done in the case of homozygotes or a heterozygote for only one position in the gene. In these cases, alleles could be deduced without the need for additional sequencing.

Table 1
Allele-specific primers used for sequencing the MPI locus.

Primer name	Primer sequence
MPI156TF	5′-GGCTAGAATACATGGGCAAT-3′
MPI156CF	5′-GGCTAGAATACATGGGCAAC-3′
MPI349GF	5′-GCCTGACTTCTTGGTTAGGG-3′
MPI349AF	5′-GCCTGACTTCTTGGTTAGGA-3′
MPI374GR	5′-GGAGCCTACAGAAGTGGGAAG-3′
MPI374TR	5′-GGAGCCTACAGAAGTGGGAAT-3′
MPI486GR	5′-TGGCTTAGGCTCTGCTTTAG-3′
MPI486AR	5′-TGGCTTAGGCTCTGCTTTAA-3′
MPI157CF	5′-GCTAGAATACATGGGCAACC-3′
MPI157GF	5′-GCTAGAATACATGGGCAACG-3′
MPI290AF	5′-TTAGTGTGCTTGCTGAGGA-3′
MPI290GF	5′-TTAGTGTGCTTGCTGAGGG-3′

2.3. Phylogenetic analysis

We used ModelTest version 3.06 (Posada and Crandall, 1998) to determine the appropriate model of evolution for each dataset under the Akaike Information Criterion. The appropriate model (TVM for ZFY and K80 + I + Γ for MPI) was implemented in a Bayesian analysis using the MrBayes version 3.1.2 program (Ronquist and Huelsenbeck, 2003) for each dataset. For MPI, 3 million generations were run and 70,000 of these were discarded as burn-in. The same number of generations was run for ZFY and 60,000 were discarded as burn-in. The models of evolution described above were also used in a maximum likelihood analysis using GARLI version 0.951 (http://www.zo.utexas.edu/faculty/antisense/garli/Garli.html). GARLI was also used to find ML bootstrap support values for clades (based on 100 replicates) for ZFY and MPI. For both datasets, all indels were discarded prior to phylogenetic analyses. With the MPI locus, each individual allele was used as an OTU in the phylogenetic analysis. Trees were visualized using TreeView version 1.6.6 (Page, 1996). A total of 561 base pairs was used in phylogenetic analyses for MPI and 602 for ZFY.

2.4. Hypothesis testing

We used parametric bootstrapping to test for monophyly of the Pacific *Rhogeessa tumida* clade in the MPI dataset (Huelsenbeck et al., 1996; Van Den Bussche et al., 1998). This was done using PAUP 4.0b10 (Swofford, 2002) to obtain parsimony scores for an initial unconstrained tree, as well as a constraint tree for monophyly of all Pacific *R. tumida* individuals. Each of these heuristic searches was done using 100 addition-sequence-replicates and TBR branch-swapping. Mesquite version 1.12 (Maddison and Maddison, 2006) was used to simulate 100 datasets under the model indicated by ModelTest for the constrained tree. These simulated datasets were used to find an expected distribution of differences in tree scores between constrained and unconstrained trees.

2.5. Phylogeographic analysis

We implemented Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992) using Kimura 2-parameter (K2P) distances of cyt-*b* sequences from a previous study (Baird et al., 2008) to test several alternative geographic hypotheses for diversification of multiple *R. tumida* lineages. We tested for phylogeographic structure based on: (1) mountain uplifts in Mexico and Central America; (2) historical seaway across the Isthmus of Tehuantepec; and (3) habitat differences. These analyses were performed using Arlequin version 2.001 (Schneider et al., 2000).

3. Results

3.1. ZFY

The ZFY tree (Fig. 4) supports the monophyly of the *R. tumida* complex (posterior probability = 1.00). Although we were unable to resolve many interspecific relationships using this slow-evolving gene, we could distinguish many of the major clades recognized in the mtDNA phylogeny (Baird et al., 2008). Both *R. io* and *R. velilla* form distinct clades. Another clade consists of all *R. aeneus* samples, the Atlantic Mexican *R. tumida* samples and the Pacific *R. tumida* samples. All of the members of this clade share a single haplotype. Slightly different from that haplotype is the haplotype shared by all Atlantic Central American *R. tumida* individuals. As in the mtDNA phylogeny, the ZFY phylogeny shows *R. parvula* and *R. mira* as sister to the *R. tumida* complex, although they are not supported as being sister to one another as they are in the mtDNA dataset. Also similar to the mtDNA results, *R. gracilis* and *R. alleni* (both tentatively called members of the genus *Baeodon* by Baird et al., 2008) are very closely related to one another, and distantly related to the remaining *Rhogeessa* species.

3.2. MPI

Thirty-four alleles were identified from members of the *R. tumida* complex at the MPI locus (Fig. 5), although we were unable to amplify this locus cleanly from individuals of *R. parvula* and *R. alleni*. Several major clades are fixed for a single MPI allele, whereas the Pacific *R. tumida* group and *R. aeneus* are highly variable at this locus. Interspecific relationships among members of the *R. tumida* complex are not well-resolved using this locus. However, MPI data are sufficient to confirm monophyly of most of the major clades. As in mtDNA and ZFY, the *R. tumida* complex is resolved as a monophyletic group (posterior probability = 1.00). *Rhogeessa mira* is sister to the *R. tumida* complex. *Rhogeessa io* is very distinct from the other members of the *R. tumida* complex, and only two alleles were observed in that species. The single individual of *R. io* from Panama was heterozygous for a private allele and an allele shared with specimens from Venezuela. Similar to the mtDNA results (Baird et al., 2008), both *R. velilla* and the Atlantic Central American *R. tumida* form highly supported monophyletic clades, and at the MPI locus each are fixed for a single allele. The *R. genowaysi* specimen is homozygous for a unique MPI allele but is otherwise not significantly supported as being different from the Pacific *R. tumida* at this locus. The Pacific *R. tumida* alleles are highly variable and do not provide strong support for monophyly of this taxon. We performed parametric bootstrapping to further test whether we can reject the hypothesis of monophyly of the Pacific *R. tumida*. The unconstrained tree and the tree that was constrained for monophyly of the Pacific *R. tumida* individuals differed by only one step. The threshold for rejection of the monophyly hypothesis at $p < 0.05$ was found (through parametric bootstrapping) to be a difference in tree scores of greater than or equal to 10. Therefore, we cannot reject monophyly of the Pacific *R. tumid*a individuals based on these data. Moreover, *R. aeneus* is not monophyletic, with one allele occurring within the clade of Atlantic Mexican *R. tumida*, and two other alleles also occurring outside of the main *R. aeneus* clade, but not clustering with any other major clade. Like the Pacific *R. tumida* group, our samples of *R. aeneus* included few homozygous individuals.

3.3. Phylogeography

The results of the three AMOVAs are given in Table 2. The only significant result obtained was based on groups that were defined by habitat type. In this case, individuals captured in dry, semi-arid environments were significantly differentiated from those captured in humid environments. These results indicate that 86.3% of the genetic variation in cyt-*b* sequences for these individuals is between those inhabiting dry areas and those inhabiting humid areas.

4. Discussion

4.1. Phylogenetic relationships

All three *Rhogeessa* DNA sequence datasets—mtDNA (Baird et al., 2008; Fig. 1), ZFY (Fig. 4) and MPI (Fig. 5)—agree on several important issues: (1) the *R. tumida* complex is monophyletic; (2) in most cases, species that are karyotypically distinct form monophyletic lineages; (3) there is evidence for either hybridization or

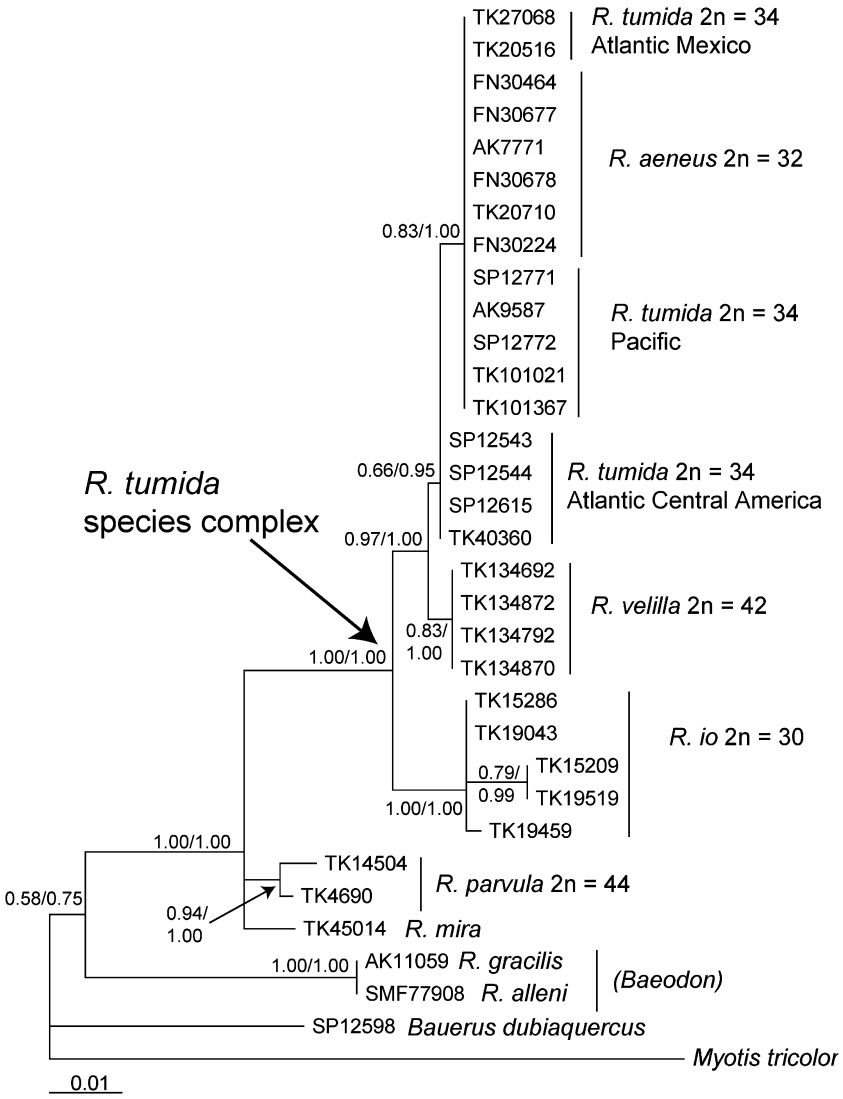

Fig. 4. ZFY tree. Numbers at nodes represent ML bootstrap proportions followed by Bayesian posterior probabilities. Sample names correspond to those found in Appendix 1.

lineage sorting between karyotypic forms having monobrachial differences from one another (i.e., between *R. tumida* and *R. aeneus*); (4) the karyotypic form with $2n = 34$ (currently known as *R. tumida*) contains several distinct genetic lineages; (5) the two $2n = 42$ species, *R. genowaysi* and *R. velilla*, are genetically distinct and phylogenetically distant from one another; and (6) *R. alleni* and *R. gracilis* are only distantly related to other *Rhogeessa* species.

The main differences among our analyses include the way particular relationships within the *R. tumida* complex are resolved. In most cases this is a result of insufficient phylogenetic signal in a particular dataset, rather than significant differences among datasets. For example, in the ZFY tree (Fig. 4), *R. aeneus*, Pacific *R. tumida* and Atlantic Mexican *R. tumida* share one haplotype. Differences among these lineages could probably be revealed by examining longer sequences. This observation does, however, highlight the close relationship between Pacific and Atlantic Mexican *R. tumida* among ZFY alleles, whereas with mtDNA the Pacific *R. tumida* lineage is highly divergent from the other two lineages that have a $2n = 34$ karyotype. Furthermore, the MPI phylogeny recognizes the Atlantic Mexican and Central American *R. tumida* lineages as each being monophyletic, but fails to group the Pacific *R. tumida* individuals together in a monophyletic group. The parametric bootstrapping analysis shows, however, that monophyly of the Pacific individuals cannot be rejected. This result is not surprising, as only one branch in the ML tree disrupts the monophyly of the Pacific $2n = 34$ individuals.

Data from mtDNA (Baird et al., 2008), ZFY, and MPI all confirm that the group currently recognized as *R. tumida* contains multiple distinct genetic lineages. DNA sequence data distinguish three different $2n = 34$ lineages: an Atlantic Mexican group, an Atlantic Central American group, and a Pacific group. Cytochrome-*b* (Baird et al., 2008) and MPI are able to distinguish all three as distinct clades and ZFY shows the Atlantic Central American form as distinct from the other two (which share a common haplotype). *Rhogeessa tumida* has been studied using morphological, karyotypic, allozyme and now DNA sequence data. The morphological study of LaVal (1973) shows some variation in several morphological characters throughout the range of *R. tumida*. He did not believe there was enough difference in these characters to distinguish what are now recognized as species distinct from *R. tumida*, including *R. aeneus*, *R. io*, *R. genowaysi*, and *R. velilla*. Bickham and Baker (1977) examined banding patterns of karyotypes from individuals throughout the range of *R. tumida*. They found no differences between individuals along the Atlantic versant of Mexico/Central America or those along the Pacific versant. All $2n = 34$ karyotypes were found to be composed of the same set of centric fusions.

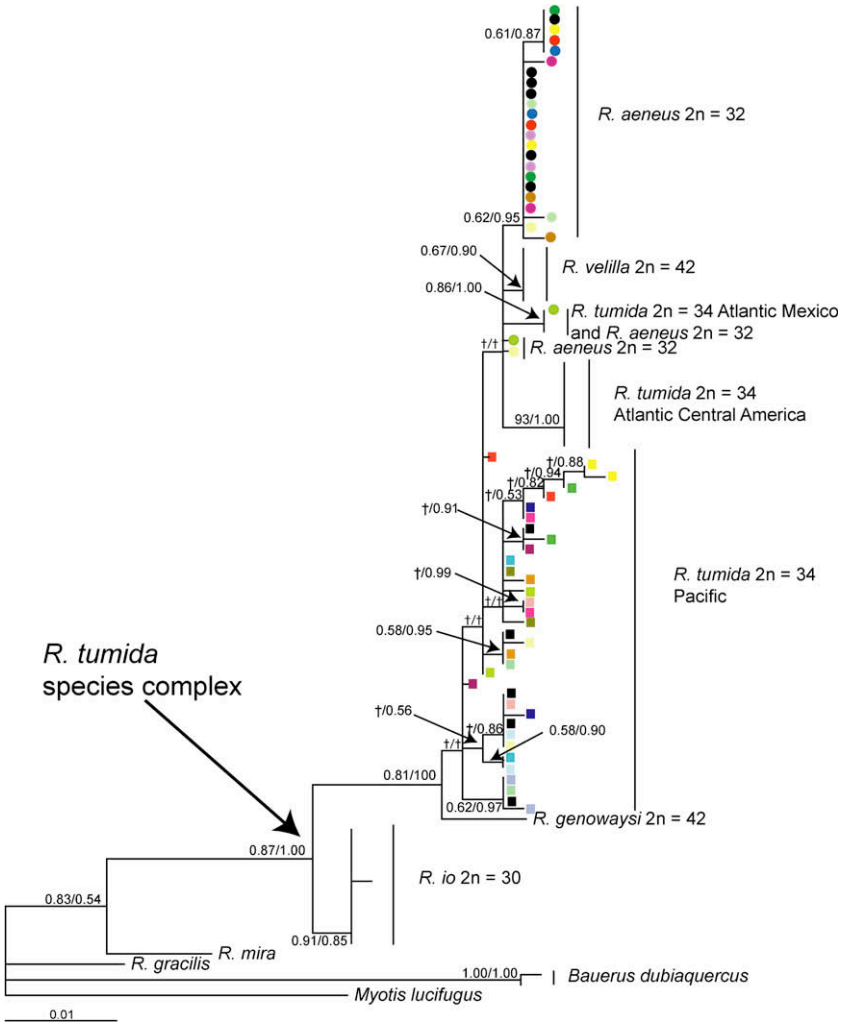

Fig. 5. MPI Maximum likelihood tree. Numbers at nodes represent ML bootstrap proportions followed by Bayesian posterior probabilities. Circles represent *R. aeneus* alleles (circles of the same color represent alleles from the same individuals; a black circle represents a homozygous individual), squares represent Pacific *R. tumida* alleles (same coloring scheme as circles). Daggers (†) represent nodes with bootstrap support <0.50 or posterior probability <0.50.

Table 2
Results of AMOVA analyses based on cyt-*b* sequences in *R. tumida*. Asterisk indicates significance at $p < 0.05$.

Hypothesis	Groups	Proportion of variance in haplotype diversity among groups	*p*-Value
Mountain uplifts in Mexico and Central America as isolating mechanism	1. Individuals from Atlantic Mexican *R. tumida* clade, Atlantic Central American *R. tumida* clade, and Guatemalan samples from Atlantic side of Sierra Madres that phylogenetically group with Pacific *R. tumida* clade 2. Pacific *R. tumida* except Guatemalan samples	49.12	0.0699
Isthmus of Tehuantepec as isolating mechanism	1. Atlantic Mexican *R. tumida* (all from west side of Isthmus) 2. All other *R. tumida* (all from east side of Isthmus)	27.59	0.2227
Habitat differences as isolating mechanism	1. Atlantic Mexican and Central American *R. tumida* (represent humid environment) 2. Pacific *R. tumida* (represent semi-arid environment)	87.33	0.0121*

Baker et al. (1985) showed that allozymes exhibited variability within the $2n = 34$ karyotypic form (*R. tumida*), but they did not discern any geographic patterns to this variation. Therefore, our DNA sequence data (Baird et al., 2008; this paper) are the only evidence that these three groups of *R. tumida* are independently evolving lineages. No dataset we have examined shows any clear evidence of recent interbreeding among the three different lineages of "*R. tumida*".

4.2. Taxonomy

Given that no clear-cut morphological differences among the three genetic lineages of *R. tumida* are as yet known, and that they are chromosomally identical, deciding their proper taxonomic status is difficult. We attempted to perform a detailed genetic study of whether the Pacific and Atlantic Central American *R. tumida* forms interbreed along a potential contact zone in Guatemala. We were

successful at collecting individuals belonging to the Pacific and Atlantic clades within 100 km of one another and did not detect any evidence of interbreeding between the two lineages. Our sample size, however, was too low to exclude the possibility of gene flow between the different lineages. Therefore, it is still unknown whether some degree of interbreeding is occurring between different genetic lineages of the $2n = 34$ form, if they ever occur sympatrically. We do know that the Pacific lineage is highly divergent from the others based on cyt-*b* and MPI and in most areas is separated geographically by mountain ranges. Although the sampling for the Atlantic Mexican form is sparse, it seems to be separated from the Atlantic Central American form by *R. aeneus* on the Yucatan peninsula. Thus, it is likely that the three different *R. tumida* lineages are allopatric throughout most of their ranges. Considering this potential allopatry and the fact that they are apparently good "Genetic Species" (Baker and Bradley, 2006) based on all markers sequenced, we are currently conducting more detailed morphological analyses prior to modifying the taxonomic status of these lineages.

The nuclear data also confirm the distant relationship of both *R. alleni* and *R. gracilis* to the remaining *Rhogeessa* species shown in Baird et al. (2008), who tentatively supported placing both of these species in the genus *Baeodon* following Hoofer and Van Den Bussche (2003). Although we were unable to sequence MPI alleles from *R. alleni*, results from that locus show *R. gracilis* to be genetically distant to the remaining *Rhogeessa*. Our unsuccessful amplifications of the MPI locus in *R. alleni* using primers that performed well in other *Rhogeessa* species could indicate sequence differences at priming sites in *R. alleni* as compared to the remaining species, further supporting the distant relationship of *R. alleni* to the rest of *Rhogeessa*. *R. alleni* and *R. gracilis* share the same allele at the ZFY locus and were weakly supported as the sister group to the remaining species of *Rhogeessa*. Based on these data, it is likely that placing both of these species in the genus *Baeodon* is appropriate. We recommend further study of the genetic relationships of *R. alleni* and *R. gracilis* relative to *Rhogeessa* before formally changing their taxonomy.

4.3. Genetic variation

The MPI dataset exhibits high allelic variability in the Pacific *R. tumida* lineage compared to the other species and lineages in the *R. tumida* complex. Almost all Pacific *R. tumida* individuals are heterozygous (homozygotes are indicated by black squares in Fig. 5), and in many cases the two alleles within an individual are very different from one another. Many allele pairs are variable at more than two sites (in *R. aeneus*, the other clade with many heterozygotes, the alleles usually only vary at one or two sites). This stands in stark contrast to the other species in the complex, most of which are fixed for a single allele. This observation also contrasts with the results from the cyt-*b* sequences, where within-species diversity was very similar for all three *R. tumida* lineages (Baird et al., 2008). There are several possible explanations for this observation. Individuals from the Pacific clade span a broader geographic range than most other species in the *R. tumida* complex. This could result in more isolated populations within the species and therefore greater genetic diversity. This observation could also indicate female philopatry and greater male dispersal of Pacific *R. tumida*, although our ZFY data provide no support for this hypothesis. This pattern is also expected in taxa from a hybrid origin (Holloway et al., 2006). The use of individual allele sequencing has revealed patterns of genetic variation that would not have been detected using consensus sequences in phylogenetic analyses. We encourage the practice of sequencing alleles when possible to uncover evolutionary processes that might otherwise go undetected.

4.4. Hybridization vs. lineage sorting

One similarity in all sequence datasets mentioned previously is potential evidence for hybridization or incomplete lineage sorting among the three *R. tumida* clades and *R. aeneus*. In the cyt-*b* phylogeny, two individuals of Atlantic Central American *R. tumida* fall within the *R. aeneus* clade (Baird et al., 2008; Fig. 1). In the ZFY phylogeny, *R. aeneus* shares a single haplotype with all Pacific and Atlantic Mexican *R. tumida*. In MPI, one *R. aeneus* individual is heterozygous for an allele otherwise restricted to the Atlantic Mexican *R. tumida* clade and a unique allele that clusters separately from the main *R. aeneus* clade (Fig. 5). In analyses of both the MPI and ZFY loci, we could sequence only one of the potential *R. tumida* hybrids from the mtDNA dataset, and it was homozygous for an MPI allele that falls within the Atlantic Central American *R. tumida* clade. This individual also falls in the Atlantic Central American *R. tumida* clade in the ZFY tree.

As previously mentioned, a Y-chromosomal tree, due to its shorter lineage sorting period, should follow the true species phylogeny most closely, followed by mtDNA, and finally autosomal markers. Unfortunately, our ZFY marker does not provide much resolution between some members of the *R. tumida* complex. The cyt-*b* phylogeny presented in Baird et al. (2008) is the most well-resolved tree and has a lineage sorting period less than that of the MPI tree presented here. The fact that the cyt-*b* tree, with its shorter lineage sorting period, does not show the same topology as the MPI tree is striking. The MPI and cyt-*b* datasets both show individuals from *R. tumida* and *R. aeneus* occurring in the same clade; however, this pattern does not involve members from the same lineage of *R. tumida*. The ZFY tree is in agreement with the MPI tree in that no Atlantic Central American *R. tumida* are involved in lineage sorting/hybridization events with *R. aeneus*. However, based on ZFY data alone we cannot rule out lineage sorting/hybridization events between *R. aeneus* and Atlantic Mexican or Pacific *R. tumida*.

Both incomplete lineage sorting and ancient hybridization can account for the observed phylogenetic pattern. Individual loci, especially those that are haploid, cannot by themselves support or refute hypotheses of lineage sorting or hybridization. Conclusions regarding the presence of lineage sorting/hybridization are most robust when derived from a comparison of phylogenetic patterns derived from loci inherited in different manners (as in our data, described below). The strongest evidence for rejecting a recent hybridization hypothesis lies in comparing the cyt-*b* phylogeny to the MPI phylogeny. Incomplete lineage sorting will result in nonconcordant patterns between loci due to the fact that it is a random process and independent of the lineage sorting in different loci. Our data exhibit this lack of concordance in phylogenetic patterns with respect to the relationship between *R. tumida* and *R. aeneus*. Any recent hybridization events are expected to show the same topological patterns across loci. On the other hand, an ancient hybridization event can produce the nonconcordant patterns expected of lineage sorting if sufficient time has elapsed since the event. In the case of our data, the two Atlantic Central American *R. tumida* that group with *R. aeneus* for cyt-*b* are homozygous for Atlantic Central American *R. tumida* MPI alleles. If the mitochondrial capture that produced the cyt-*b* pattern happened long ago (and divergence values in our data suggest that it did), many generations of back-crossing to the parental *R. tumida* would erase a similar pattern in MPI. The occurrence in the MPI tree of one *R. aeneus* allele within a clade of Mexican *R. tumida* can also be explained by lineage sorting. It is evident from all of the datasets that *R. aeneus* is probably the most recently evolved species (among the currently recognized species of the *R. tumida* complex) and that the two Atlantic *R. tumida* lineages are its closest sister taxa. This is where one expects lineage sorting to be the most problematic, but also where hybridization is most likely if a complete isolating

mechanism has not been established. Because the patterns expected to be produced by ancient hybridization and lineage sorting are the same, we cannot rule out either hypothesis outright. At a minimum, our data as well as the karyotypic data show no evidence of any regular, recent, or ongoing hybridization among major lineages of the *R. tumida* complex including the three genetic lineages of *R. tumida*.

4.5. Chromosomal speciation

With regard to the speciation by monobrachial centric fusions model we are testing, the nuclear sequence data reported here are able to provide stronger evidence of general support for this model than cyt-*b* sequences alone (Baird et al., 2008). Our data demonstrate that there has been no recent gene flow between species that differ from one another by monobrachial fusions. These data indicate that a scenario of speciation by monobrachial centric fusions should not be rejected, and the expectations of the model are supported. Baker and Bickham (1986) indicated in their description of the model that the presence of monobrachial differences between karyotypes should result in instantaneous reproductive isolation and that even a single difference should be sufficient for complete reproductive isolation. Our data suggest that these expectations are met in *Rhogeessa*, with the possible exception of an ancient hybridization event between *R. tumida* and *R. aeneus*. On the other hand, if monobrachial centric fusions were the only force driving reproductive isolation in *Rhogeessa*, we would expect to see evidence of gene flow between populations that do not have monobrachial differences between their karyotypes. We do not see this condition met based on the presence of various genetically distinct lineages with the $2n = 34$ karyotype (“*R. tumida*”). In this case, there are three distinct genetic lineages that share the same set of chromosomal fusions (Baker et al., 1985). All of these observations lead us to conclude that although our data are generally consistent with a hypothesis of speciation by monobrachial centric fusions this speciation mechanism is not the only mechanism that has led to diversification within *Rhogeessa*.

Because we have repeatedly seen multiple lineages with the $2n = 34$ karyotype, between which reproductive isolation could not have been caused by chromosomal differentiation, we tested geographic alternatives to explain these patterns. The results from our AMOVAs indicate that habitat differences may have created isolation leading to diversification between the Atlantic and Pacific lineages of “*R. tumida*”. The two Atlantic clades, from Mexico and Central America, occupy moist or humid habitats whereas the Pacific clade occurs in dry habitats. Although our samples from Guatemala (which group with the Pacific clade) were captured on the Atlantic side of the Sierra Madre mountain range through Central America (all others from the Pacific clade were captured in the Pacific drainage), they were found on the semi-arid western side of the Motagua Valley. All other samples from Guatemala were collected near the coast where the habitat is humid. We were only able to capture one individual from “transitional” habitat of the Motagua Valley and it grouped with the Atlantic Central American clade in all of our phylogenetic analyses. The habitat in which we captured this individual was more humid than many other areas in the transition zone between habitats.

Our results of monobrachial differences creating complete reproductive isolation are similar to those in studies of shrews that also exhibit potential speciation via this mechanism. Results from hybrid zones in shrews exhibiting monobrachial differences show increased genetic structure of microsatellites on chromosomal arms involved in fusions compared to markers on arms not involved in fusions. These results show that rearrangements affect the barrier to gene flow between different karyotypic forms (Basset et al., 2006). On the other hand, Britton-Davidian et al. (2002) showed that allozymes showed no structure with respect to different chromosomal forms in house mice, indicating the presence of gene flow between populations which differ by monobrachial fusions. Although some of these data, including ours, support the model of speciation by monobrachial centric fusions, some results show that the process may not be as simple as that outlined in Baker and Bickham (1986).

The presence of reciprocally monophyletic and karyotypically distinct lineages in the *R. tumida* complex in all three DNA sequence datasets is consistent with an important role of reproductive isolation from monobrachial fusions in generating speciation events in this group. The very short branch lengths observed at the base of the *R. tumida* complex clade, and the difficulty in resolving relationships at that level in all datasets indicate that speciation and diversification at that time period was rapid. This observation is consistent with the expectations of the speciation by monobrachial centric fusions model, which states that speciation should happen virtually instantaneously with fixation of the chromosomal rearrangements (Baker and Bickham, 1986). Alternatively, the lack of resolution at this position on the tree could be due to insufficient change in the sequence examined. Nonetheless, we argue that rapid speciation is a possibility based on a qualitative evaluation of relative branch lengths at the base compared to the rest of the tree. Our observed phylogenetic patterns are consistent with rapid chromosomal change followed by rapid reproductive isolation. In fact, this expected rapid bout of chromosomal rearrangements has been observed in *Mus domesticus* by Nachman and Searle (1995) who estimated that the fixation rate of centric fusions in that species was 2.25×10^{-4} fixations per generation. If many chromosomal rearrangements were occurring during the time period corresponding to the base of the *R. tumida* complex on our tree, and the rearrangements resulted in rapid reproductive isolation, this process could lead to the phylogenetic patterns we have consistently seen in all three molecular datasets.

The cyt-*b* phylogeny (Baird et al., 2008) is the most well-resolved of the three datasets and shows the occurrence of two clades within the *R. tumida* complex that are both composed (at least in part) of a $2n = 34$ lineage and a $2n = 42$ lineage. Although not resolved at the same level, the nuclear datasets agree that there are multiple lineages of $2n = 34$ and $2n = 42$ karyotypes. This raises the question as to whether there may be some sort of selective advantage to possessing these particular karyotypic arrangements or whether multiple lineages which happen to have the same rearrangements have arisen by random processes. A somewhat similar observation has been made in the karyotypically variable species *Mus domesticus*, where some chromosomes are often involved in fusions, while others have been observed rarely in fusions (Nachman and Searle, 1995; Gazave et al., 2003). The question of why certain chromosomes tend to pair up in fusions has been examined by other authors (Gazave et al., 2003), but explanations are rare and usually specific to certain scenarios. Our findings in *Rhogeessa* of apparently parallel origins of the same karyotype suggest that there may be a limited number of stable configurations of the chromosomes. If true, this suggests caution should be applied in using karyotypic data for inferring phylogenetic relationships.

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Appendix A

Specimens examined. TK = Natural Science Research Laboratories, Texas Tech University; AK = Texas Cooperative Wildlife Collection, Texas A&M University; ASNHC = Angelo State Natural History Collection, Angelo State University; FN = Royal Ontario Museum; SP = Carnegie Museum; SMF = Senckenberg-Museum, Frankfurt/Main. For MPI locus, individuals with two sequences reported were heterozygous (each allele was submitted separately to GenBank), those with only one sequence reported were homozygous.

Appendix (*continued*)

Species	Voucher number	Locality	ZFY GenBank accession number	MPI GenBank accession number
Baeodon alleni	SMF77908	Puebla, Mexico	EU185125	
Rhogeessa aeneus	TK20704	Belize dist., Belize		EU220301 EU220302
	TK20706	Belize dist., Belize		EU220303
	TK20707	Belize dist., Belize		EU220304 EU220305
	TK20710	Belize dist., Belize	EU185108	EU220306 EU220307
	TK20711	Belize dist., Belize		EU220325
	TK20712	Belize dist., Belize		EU220308 EU220309
	AK7771	Orange Walk, Belize	EU185118	EU220310 EU220311
	FN30223	Campeche, Mexico		EU220312 EU220313
	FN30224	Campeche, Mexico	EU185109	EU220314
	FN30225	Campeche, Mexico		EU220315 EU220316
	FN30462	Yucatan, Mexico		EU220317 EU220318
	FN30463	Yucatan, Mexico		EU220319 EU220320
	FN30464	Yucatan, Mexico	EU185119	EU220321
	FN30677	Campeche, Mexico	EU185107	EU220323 EU220324
	FN30678	Campeche, Mexico	EU185126	
	ASNHC1414	Campeche, Mexico		EU220326
Rhogeessa genowaysi	TK20597	Chiapas, Mexico		EU220390
Rhogeessa gracilis	AK11059	Oaxaca, Mexico	EU185103	EU220392
Rhogeessa io	TK15164	Guarico, Venezuela		EU220335
	TK15209	Guarico, Venezuela	EU185130	EU220336
	TK15286	Guatopo, Venezuela	EU185124	EU220337
	TK19004	Bolivar, Venezuela		EU220338
	TK19005	Bolivar, Venezuela		EU220339
	TK19043	Bolivar, Venezuela	EU185127	EU220340
	TK19450	Barinas, Venezuela		EU220341
	TK 19458	Barinas, Venezuela		EU220342
	TK19459	Barinas, Venezuela	EU185100	
	TK22536	Darien, Panama		EU220345 EU220346
	TK25079	Trinidad Nariva, Trinidad		EU220344
	TK19519	Barinas, Venezuela	EU185131	EU220343
Rhogeessa mira	TK45014	Michoacan, Mexico	EU185106	EU220391
Rhogeessa parvula	TK4690	Sinaloa, Mexico	EU185132	
	TK14504	Sinaloa, Mexico	EU185114	
Rhogeessa tumida	TK20516	Oaxaca, Mexico	EU185115	EU220327
	TK20594	Chiapas, Mexico		EU220356 EU220357
	TK20596	Chiapas, Mexico		EU220358
	TK27068	Tamaulipas, Mexico	EU185116	EU220328
	TK34866	San Salvador, El Salvador		EU220359 EU220360
	TK34980	La Paz, El Salvador		EU220361 EU220362
	TK40186	Valle, Honduras		EU220363 EU220364
	TK40345	Atlantida, Honduras		EU220347
	TK40360	Atlantida, Honduras	EU185117	EU220348
	TK101020	Valle, Honduras		EU220365 EU220366
	TK101021	Valle, Honduras	EU185113	EU220367 EU220368
	TK101044	Valle, Honduras		EU220369

(*continued on next page*)

Appendix (*continued*)

Species	Voucher number	Locality	ZFY GenBank accession number	MPI GenBank accession number
Species	Voucher number	Locality	ZFY GenBank accession number	MPI GenBank accession number
	TK101052	Valle, Honduras		EU220370 EU220371
	TK101266	Valle, Honduras		EU220372 EU220373
	TK101367	Comayagua, Honduras	EU185128	EU220374 EU220375
	TK101370			EU220389
	AK9587	Valle, Honduras	EU185111	EU220376 EU220377
	AK9613	Valle, Honduras		EU220378 EU220379
	SP12543	Izabal, Guatemala	EU185104	EU220349
	SP12544	Izabal, Guatemala	EU185112	EU220350
	SP12606	Izabal, Guatemala		EU220351
	SP12615	Izabal, Guatemala	EU185105	EU220352
	SP12650	Izabal, Guatemala		EU220353
	SP12771	Zacapa, Guatemala	EU185129	EU220380 EU220381
	SP12772	Zacapa, Guatemala	EU185133	EU220382 EU220383
	AK 25022	El Progreso, Guatemala		EU220384 EU220385
	AK 25023	El Progreso, Guatemala		EU220386
	AK 25024	El Progreso, Guatemala		EU220387 EU220388
	AK 25065	Izabal, Guatemala		EU220354
	AK 25093	Izabal, Guatemala		EU220355
Rhogeessa velilla	TK134692	Guayas, Ecuador	EU185120	EU220329
	TK134792	Guayas, Ecuador	EU185121	EU220331
	TK134868	Guayas, Ecuador		EU220333
	TK134869	Guayas, Ecuador		EU220332
	TK134870	Guayas, Ecuador	EU185122	EU220330
	TK134872	Guayas, Ecuador	EU185123	EU220334
Bauerus dubiaquercus	SP12598		EU185102	EU220299 EU220300
Myotis tricolor	SP13200		EU185100	

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