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KARYOTYPIC DIVERSITY AND SPECIATION IN *AGRODIAETUS* BUTTERFLIES

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That chromosomal rearrangements may play an important role in maintaining postzygotic isolation between well-established species is part of the standard theory of speciation. However, little evidence exists on the role of karyotypic change in speciation itself—in the establishment of reproductive barriers between previously interbreeding populations. The large genus *Agrodiaetus* (Lepidoptera: Lycaenidae) provides a model system to study this question. *Agrodiaetus* butterflies exhibit unusual interspecific diversity in chromosome number, from $n = 10$ to $n = 134$; in contrast, the majority of lycaenid butterflies have $n = 23/24$. We analyzed the evolution of karyotypic diversity by mapping chromosome numbers on a thoroughly sampled mitochondrial phylogeny of the genus. Karyotypic differences accumulate gradually between allopatric sister taxa, but more rapidly between sympatric sister taxa. Overall, sympatric sister taxa have a higher average karyotypic diversity than allopatric sister taxa. Differential fusion of diverged populations may account for this pattern because the degree of karyotypic difference acquired between allopatric populations may determine whether they will persist as nascent biological species in secondary sympatry. This study therefore finds evidence of a direct role for chromosomal rearrangements in the final stages of animal speciation. Rapid karyotypic diversification is likely to have contributed to the explosive speciation rate observed in *Agrodiaetus*, 1.6 species per million years.

KEY WORDS: ***Agrodiaetus*, chromosomal speciation, comparative phylogenetic analysis, differential fusion, postzygotic isolation.**

The origin of new species (i.e., speciation) involves the establishment of reproductive isolation (e.g., Dobzhansky 1937, 1940). A species' chromosomal complement (i.e., its karyotype) is one of the few morphological characters that can contribute to the formation of postzygotic isolation between biological species (for review, see Dobzhansky 1937; White 1973). Mating between species with different karyotypes produces hybrids that are heterozygous for chromosomal rearrangements fixed between parental species. These hybrids typically have reduced fertility due to missegregation of homologous chromosomes during the first meiotic division (e.g., Lorkovíc 1974; John et al. 1983; Forejt 1996). Although different kinds of chromosomal rearrangements have various effects on the fertility of heterozygous hybrids (for review, see King 1993), hybrid fertility is generally negatively correlated with the extent of karyotypic divergence between parental taxa (e.g., White 1973; Gropp et al. 1982).

Chromosomal rearrangements can also indirectly promote genetic divergence and/or preserve co-adapted gene complexes by blocking recombination within rearranged chromosomal regions (Noor et al. 2001; Rieseberg 2001; Navarro and Barton 2003). Theoretical models have suggested that recombination can oppose species formation by breaking the allelic association between

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adaptation and mate choice, thus, preventing divergence of hybridizing taxa (Felsenstein 1981; Barton and Bengtsson 1986). By purging recombinant products, chromosomal rearrangements (e.g., paracentric inversions in *Drosophila*) effectively prevent genetic introgression between hybridizing taxa, whereas extensive introgression can occur in colinear chromosomal regions (e.g., Noor et al. 2001). Therefore, chromosomal rearrangements can alleviate the selection–recombination antagonism for loci within rearranged genome regions and promote speciation (for review, see Ortíz-Barrientos et al. 2002; Butlin 2005).

Most taxa do not undergo extensive karyotypic changes during speciation and tend to be karyotypically conserved at the genus or family level. New chromosomal rearrangements usually appear as heterozygotes and are often (but not always) associated with heterozygote disadvantage (i.e., underdominance). Therefore, their spread to fixation within a population and/or species is considered unlikely (e.g., Spirito 1998). For example, cat species from the family Felidae (Mammalia: Carnivora) acquired only a few chromosomal rearrangements during the 11 million years of their evolution (Johnson et al. 2006), and every felid has the same chromosome number $2n = 39$. Most butterflies in the family Lycaenidae (Insecta: Lepidoptera) have a haploid chromosome number of either 23 or 24 (Lesse 1960; Lorkovíc 1990).

Nevertheless, species in a few animal genera exhibit extreme interspecific karyotypic diversity. In mammals, for example, the genus *Muntiacus* (Mammalia: Cervidae) includes species with different karyotypes, a range of $n = 3$ to $n = 23$ (Yang et al. 1997), and the genus *Sigmodon* (Mammalia: Cricetidae) has an interspecific karyotypic diversity from $n = 11$ to $n = 26$ (Zimmerman 1970). The greatest ranges in chromosome numbers at the genus level are found in insects. The genera *Apiomorpha* (Hemiptera: Eriococcidae) and *Agrodiaetus* (Lepidoptera: Lycaenidae) have interspecific karyotypic diversities from $n = 2$ to $n = 96$ (Cook 2000) and from $n = 10$ to $n = 134$ (Lukhtanov et al. 2005), respectively. Because these karyotypically diverse genera tend to have many morphologically similar species, these groups present ideal study systems to examine the potential role of chromosomal rearrangements in animal speciation.

Chromosomal rearrangements are known to contribute to postzygotic isolation between well-established species (e.g., Piálek et al. 2001; Delneri et al. 2003), but whether such rearrangements can play a direct role in speciation remains an open question (but see Noor et al. 2001). Chromosomal rearrangements may have been fixed after actual speciation and may not have contributed directly to the origin or strengthening of reproductive isolation prior to its completion (e.g., Charlesworth et al. 1982). Aside from special cases such as polyploidy and monobrachial centric fusion, chromosomal speciation has remained a controversial mechanism, especially in animals other than mammals (e.g., Coyne and Orr 2004). Part of the controversy stems from a lack of information: "It is remarkable that no one has systematically studied the number of [chromosomal] rearrangement differences between taxa as a function of divergence time (measured molecularly)" (Coyne and Orr 2004, pp. 260–261).

Recently, we have shown that initial differentiation in the genus *Agrodiaetus* most likely accumulates between geographically isolated populations, and that older phylogenetic groups in the genus tend to have higher levels of karyotypic diversity (Lukhtanov et al. 2005). Here we use a comparative phylogenetic approach to test whether chromosomal rearrangements may have directly contributed to the formation of new species in this genus. If rearrangements have contributed to the establishment and/or maintenance of reproductive isolation between nascent *Agrodiaetus* species, (1) karyotypic diversity should accumulate gradually among taxa with allopatric distributions (see Kandul et al. 2004; Lukhtanov et al. 2005), and (2) young sister taxa that have secondary sympatric distributions should be more karyotypically differentiated than the corresponding sister taxa with allopatric distributions. This higher karyotypic diversity between sympatric sister taxa is expected from differential fusion of karyotypically divergent allopatric taxa when they meet again in secondary sympatry. We also test for an association between karyotypic diversity and diversification rate in the genus using sister-group comparisons.

AGRODIAETUS BUTTERFLIES

Agrodiaetus Hübner [1822] is a large and karyotypically diverse genus of blue butterflies, Lycaenidae (Lepidoptera). This genus contains at least 120 species distributed throughout the Western Palearctic and Central Asian regions (for the latest revision, see Häuser and Eckweiler 1997). The highest species diversity in the genus is found in the Caucasus region, Iran, and Turkey. Adults of *Agrodiaetus* species are small (i.e., wing span of 1.8–4.0 cm). The genus was estimated to have originated only about three million years ago (Kandul et al. 2004) and, thus, *Agrodiaetus* species may have not had sufficient time to acquire extensive morphological differences. However, many species of the genus have developed distinctive karyotypes. *Agrodiaetus* shows one of the highest interspecific karyotypic diversities known in the animal kingdom. Its haploid chromosome numbers range from $n = 10$ in *A. caeruleus* and *A. birunii* to $n = 134$ in *A. shahrami* and *A. achaemenes* (Lukhtanov and Dantchenko 2002a; Lukhtanov et al. 2005). Therefore, karyotypic characters provide additional identification characters for many described species that are virtually indistinguishable by wing patterns and/or aspects of the male and female genitalia.

Multiple chromosomal rearrangements fixed between *Agrodiaetus* taxa presumably affect the fertility of hybrids and, thus, likely promote postzygotic isolation between these taxa. Potential segregational problems, like multivalent formations

and misalignments, have been repeatedly observed during the first meiotic division in hybrids between karyotypically distinct *Agrodiaetus* species collected in nature (Lukhtanov, unpubl. data). However, the fertility of chromosomal hybrids has not been experimentally studied in the genus. *Agrodiaetus* taxa occur at high elevation, feed on localized plant species of *Onobrychis* and *Hedysarum* (Fabaceae), and are sufficiently difficult to culture that hybridization experiments in the laboratory are not feasible at this stage. Nevertheless, hybridization experiments done by Schurian and Hotman (1980) and Schurian (1989) on *Lysandra,* a closely related genus to *Agrodiaetus,* indicated that hybrids produced between karyotypically distinct *Lysandra* species have reduced fertility. Thus, major chromosomal rearrangements, like fusion and fragmentation, most likely induce or enhance postzygotic isolation between *Agrodiaetus* taxa.

The modal chromosome number of lycaenids ($n = 23/24$) is the likely ancestral number for *Agrodiaetus.* This number is present in most closely related genera to *Agrodiaetus* (e.g., Kandul et al. 2004). This ancestral karyotype has diversified toward both lower and higher chromosome numbers in the genus. The evolution of the *Agrodiaetus* karyotype has not been caused by polyploidy and/or accompanied by a significant increase in genome size. The areas occupied by native metaphase I plates of *Agrodiaetus* species with diverse chromosome numbers are almost identical (for examples, see Lesse 1960; Lukhtanov and Dantchenko 2002a). In addition, an inverse relationship exists between the chromosome number of a species' karyotype and the relative sizes of its bivalents (Lorkovíc 1990). Therefore, the karyotypic diversity in *Agrodiaetus* is likely to have arisen through multiple fusions and fragmentations of its chromosomes (Lorkovíc 1990). The holocentric organization of Lepidoptera chromosomes makes the butterfly genome especially amenable to chromosomal fusion and fragmentation. The kinetochore activity of a holocentric chromosome is not localized to a single site, the centromere, but is spread along the full length of the chromosome (see Wolf et al. 1997; Lukhtanov and Dantchenko 2002a). The fusion of holocentric chromosomes does not dramatically change the kinetics of meiotic segregation, as in the case of a monocentric chromosome that can become dicentric after fusion. The fragments from chromosomal fission can therefore attach to mitotic and meiotic spindles and be protected from loss at cell division.

Material and Methods

AGRODIAETUS SPECIMENS

To estimate the accumulation of karyotypic diversity at both inter- and intraspecific levels, we analyzed different *Agrodiaetus* species, subspecies, and chromosomal races (i.e., populations with stable chromosome numbers that differ from the modal number of the species) collected over five years. All traditionally recognized *Agrodiaetus* species groups (Hesselbarth et al. 1995; Häuser and Eckweiler 1997) and phylogenetic clades (Kandul et al. 2004) are represented in this study. Our sample includes a total of 147 specimens representing at least 100 recognized *Agrodiaetus* species (see online Appendix S1).

KARYOTYPE EXAMINATION

Testes from males were fixed for karyotype examination before the body was stored in 100% ethanol for DNA preservation. We used the squash method of karyotypic analysis specially modified for studing species with high chromosome numbers (Lukhtanov et al. 2006). Karyotypes of at least 10 individuals were examined for each population. In most cases, the individual specimens sampled for karyotypic analyses were also used for phylogenetic analyses. We could not examine karyotypes from collected specimens in 26 cases, and in these instances, we used karyotypic data previously obtained for the same population (if available) or from a different population of the same species. The collection data, haploid chromosome numbers (when available), and their references are presented for all taxa in Appendix S1. All specimens are deposited in the DNA and Tissues Collection of the Museum of Comparative Zoology (Harvard University, Cambridge, MA).

DNA EXTRACTION AND SEQUENCING

Two mitochondrial genes, *Cytochrome Oxidase subunit I* (*COI*) and *Cytochrome Oxidase subunit II* (*COII*), were used to infer the phylogeny of *Agrodiaetus*. DNA extraction techniques, primers used for PCR amplification, and sequencing methods are described in Appendix S2 and elsewhere (Monteiro and Pierce 2001; Kandul et al. 2004).

PHYLOGENETIC ANALYSIS

All 148 continuous sequences of *COI*, tRNA-*leu,* and *COII* genes were unambiguously aligned in a dataset using Sequencher 3.1 (Genecodes Corporation, Ann Arbor, MI), then the dataset was partitioned into the respective genes in PAUP* 4.0b10 (Swofford 2000). Only protein-coding sequences were used for a phylogeny inference. We applied three different methods (i.e., maximum parsimony, Bayesian Inference, and maximum likelihood) to infer the phylogeny. Maximum parsimony (hereafter, MP) analysis was performed in PAUP* 4.0b10 (see Appendix S2). Hierarchical Likelihood Ratio Tests, hLRTs, (Huelsenbeck and Crandall 1997) as implemented in Model test 3.06 (Posada and Crandall 1998) were used to determine the substitution model for model-based phylogeny inferences. Bayesian inference (hereafter, BI) was performed as implemented in MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). PHYML 2.4 (Guindon and Gascuel 2003) was used for maximum likelihood (hereafter, ML) analysis.

The ML tree inferred under the Hasegawa–Kishino–Yano model (Hasegawa et al. 1985) with invariable sites and gamma distribution (hereafter, HKY + I + γ) was used for comparative phylogenetic analysis. According to the Shimodaira–Hasegawa test (see Appendix S2, Shimodaira and Hasegawa 1999) the ML tree was not significantly different from BI and MP majority consensus trees. The ML tree permitted calculation of phylogenetic independent contrasts (Felsenstein 1985), because it was a strictly bifurcating tree with assigned branch lengths. Differences in substitution rates across the ML tree were smoothed using the penalized likelihood method as implemented in r8s (Sanderson 2002) to obtain ultrametric branch lengths in the ML tree. Six taxa with unknown chromosome number were pruned from the tree. Finally, the hard polytomies assigned by r8s were resolved manually as soft polytomies (i.e., branch lengths close to 0), which were consistent with the original topology of the ML tree.

A thorough description of the phylogenetic analysis and obtained results is presented in Appendices S2 and S3 published online (see Supplementary Material). (http://lifesciences.asu.edu/evolution/).

MODELING EVOLUTION OF CHROMOSOME NUMBER IN *AGRODIAETUS*

A karyotype is the stochastic product of different chromosomal rearrangements (e.g., Imai et al. 1986, 2001). Bickham and Baker (1979) argued that species karyotype contributes to the fitness of an individual (i.e., the canalization model of karyotype evolution), but available empirical data are inconsistent with the canalization model (e.g., Coyne 1984; King 1985; Imai et al. 1986). The variable probabilities of appearance and fixation of distinct chromosomal rearrangements, which themselves are contingent on population structure (e.g., Sites and Moritz 1987), genomic sequences (e.g., Lönnig and Saedler 2002), adaptive selection (e.g., Perez-Ortin et al. 2002), and their underdominance effects (e.g., King 1993), shape karyotypic evolution. Only major chromosomal rearrangements such as fusions or fragmentations will change chromosome numbers. Because the relative frequencies of appearance and fixation of chromosomal fusions and fragmentations are not known in Lepidoptera, we used the most parsimonious conceivable model, a Brownian motion model, that assumes equal frequencies of fusions and fragmentations to analyze the evolution of haploid chromosome numbers in *Agrodiaetus.*

PHYLOGENETIC COMPARATIVE ANALYSIS OF CHROMOSOME NUMBERS

Sister taxa at internal nodes on the ML tree had more similar chromosome numbers than those for a random pair of taxa (test for serial independence: $P < 0.001$, Abouheif 1999). To account for the effect of common ancestry on the distribution of haploid chromosome numbers among sample specimens, we used Felsenstein's (1985) method of independent contrasts to study the evolution of chromosome number. This method is based on the assumption that a character evolved according to a Brownian motion model.

Independent contrasts were calculated using the PDTree program from the Phenotypic Diversity Analysis Program's (PDAP) module (Midford et al. 2002) in Mesquite (Maddison and Maddison 2004). Each independent contrast was scaled (i.e., standardized) by its expected standard deviation (Felsenstein 1985), here the square root of the sum of the sister clades' branch lengths in the ultrametric ML tree. To check that contrasts had been adequately standardized (i.e., whether phylogenetic autocorrelation was effectively removed), we used a Pearson product–moment correlation between the standardized independent contrasts and their standard deviations (Garland et al. 1992) as implemented in PDTree (see Garland et al. 2005).

The average genetic distance, relative age, raw contrasts of chromosome numbers, standardized independent contrast of chromosome numbers, and range overlap were estimated for every pair of sister clades in the ultrametric ML tree. The average genetic distance was calculated directly from the ML tree by successively averaging genetic distances at internal nodes moving from the tips through to the root of the tree. The relative age of each internal node was estimated in r8s. To calculate raw contrasts of chromosome numbers, we first averaged haploid chromosome numbers between every pair of sister clades in the ML tree, starting from the tips and working toward the root of the tree, and then took the absolute difference between averaged chromosome numbers at every node in the tree. In other words, we calculated raw contrasts assuming equal branch lengths on the ML tree. The distribution of sister clades at every internal node was classified as either sympatric or allopatric. Two sister clades were considered to have sympatric distributions if they shared at least one pair of basal taxa with sympatric distributions (for more, see Appendix S4). This method of ancestral range reconstruction is not as strongly biased as Lynch's method (1989), which infers an ancestral geographic range as the sum of the ranges of its descendents (thus, older pairs, of sister taxa tend to have sympatric distributions). However, the key assumption of both methods is the same: geographic ranges of both extant and ancestral species have not changed significantly since the time of speciation, or these ranges can at least be inferred from current distributions (for more, see Barraclough and Vogler 2000; Losos and Glor 2003; Hunt et al. 2005). To estimate the ranges of *Agrodiaetus* species, we used comprehensive dot distribution maps of butterflies published in four guides to butterflies from the Palearctic, including Western Russia, Ukraine and the Caucasus (Kudrna 2002), Turkey (Hesselbarth et al. 1995), Iran (Nazari 2003), and Central Asia (Lukhtanov and Lukhtanov 1994). In addition, data from recent publications (e.g., Lukhtanov

and Dantchenko 2002b; Carbonell 2003; Dantchenko and Churkin 2003) supplemented the distributional information for species in Turkey and Central Asia.

RANGES OF KARYOTYPIC DIVERSITIES AMONG ALLOPATRIC AND SYMPATRIC SISTER CLADES

Both raw and standardized contrasts of chromosome numbers were used to estimate the range of karyotypic diversity found separately among allopatric and sympatric sister clades. The basal node connecting *Agrodiaetus* taxa with *Polyommmatus icarus* was not considered.

ACCUMULATION OF KARYOTYPIC DIVERSITY

We used every node on the ML tree to estimate the accumulation of karyotypic diversity, measured by haploid chromosome number in *Agrodiaetus*, except the seven most basal nodes in the tree (Appendix S5). However, their exclusion would not affect any of the results presented here. These basal sister clades are likely to show the greatest amount of noise; most were not resolved on the MP and BI majority consensus trees, and were inferred to have sympatric distributions. Two different approaches were used to study the accumulation of karyotypic diversity. Both approaches assume that ancestral distributions are successfully reconstructed for every node on the ML tree, and that all available data are used.

First, because a Pearson product–moment correlation between the relative age of sister clades and their raw contrasts of chromosome numbers was significantly positive ($r = 0.524$, $n = 134$, $P < 0.000$), we used absolute values of residuals (hereafter, residuals) from the linear regression of the raw contrast (i.e., response) over the relative age (i.e., regressor) to study whether the distribution of sister clades (allopatric/sympatric) affected the fit. This analysis does not assume that the chromosome number evolves under a Brownian motion model. Second, we tested whether the distribution of sister clades affected the relationship between the relative age and the standardized contrast of chromosome numbers that were calculated in the ultrametric ML tree under the assumption of a Brownian motion model. We performed separate analyses of covariance (ANCOVA) for both datasets. The residuals and the standardized contrasts were each treated as a response variable, the sister clades' age was the predictor variable (i.e., covariate), and allopatry/sympatry were two levels of a single factor.

Finally, we used standardized contrasts of chromosome numbers to compare the average karyotypic diversity among sympatric sister clades to the average diversity found in allopatric sister clades. First, all sister clades were considered; then, only young sister clades (i.e., genetic distance less or equal to 0.05 per bp, see Lukhtanov et al. 2005) were examined. Pairs of closely related (i.e., young) sister clades are more informative for this analysis. Younger species have had a shorter time to acquire secondary characters after speciation was complete (e.g., Coyne and Orr 2004), and the geographical ranges of extant species at the time of speciation can be more accurately estimated for younger species (e.g., Losos and Glor 2003). However, because a few young allopatric taxa have "uncertain" taxonomic status and in the long run may not be valid species, their inclusion in the analysis could lead to the underestimation of karyotypic diversity among allopatric sister clades. This is particularly noteworthy because out of a total of 13 pairs of young sister taxa with identical chromosome numbers, 12 pairs were formed by allopatric sister taxa. In contrast, chromosomal races can be objectively defined for both allopatric or sympatric sister clades. Thus, to account for this bias, only young sister clades with different chromosome numbers were considered in our analysis.

KARYOTYPIC DIVERSITY AND SPECIES NUMBER

To study the association between karyotypic diversity and species richness, we used sister-group comparisons (see Barraclough et al. 1998) in the interspecific phylogeny of *Agrodiaetus* as implemented in MacroCAIC 1.0.1 (Agapow and Isaac 2002). Only sequences representing different species of *Agrodiaetus* with known karyotypes were considered, and so an additional 41 taxa (i.e., subspecies and chromosomal races) were pruned from the ultrametric ML tree. A total of 100 different *Agrodiaetus* species and one outgroup species, *Polyommatus icarus*, remained in the interspecific tree. The nexus format of the tree was converted into a CAIC format using TreeEdit 1.0a10 (Rambaut and Charleston 2002). Both equal branch lengths and ultrametric branch lengths were used to calculate standardized independent contrasts of chromosome numbers in MacroCAIC. All pairs of sister clades that had different intra-cladal standardized contrasts of chromosome numbers and conformed to the criteria described below were analyzed. We measured the difference in species richness between sister clades as the difference in relative rates (RRD, $\ln(n_i/n_j)$, where n_i and n_j are the number of species in the clade with larger and smaller values, respectively, of the standardized contrasts). Pairs of sister clades that included at least either three (i.e., default settings) or five species were considered. First, we analyzed all pairs of sister clades chosen with MacroCAIC. Of these, only nonnested sister clades at the tips of the tree were analyzed. Because the relative rate difference did not have constant variance at all clade sizes, we could not use regression through the origin to estimate the significance of the association between the standardized contrast and the species richness (see Isaac et al. 2005). Instead, we performed a Wilcoxon signed-rank test on the direction of the relative rate difference.

STATISTICAL ANALYSIS

All statistical analyses were performed in JMP 6.0 (SAS Institute, Cary, NC).

Results

INTERSPECIFIC KARYOTYPIC DIVERSITY

We examined karyotypes from 100 different *Agrodiaetus* species. These species represent approximately 83% of species diversity in the genus. The sampled species karyotypes span the entire range of interspecific karyotypic diversity known in the genus, a range of $n = 10$ to $n = 134$ (Appendix S1). The most frequent chromosome numbers found among the sampled *Agrodiaetus* species were in the range from $n = 20$ to $n = 29$ (27 out of 100 examined species; Fig. 1). The majority of sampled *Agrodiaetus* species have chromosome numbers higher than $n = 23/24$.

PHYLOGENY RECONSTRUCTION

Detailed results of phylogenetic analyses and comparisons of inferred tree are presented in Appendix S2. Briefly, phylogenies inferred using MP, BI, and ML methods were not significantly different (i.e., the Shimodaira–Hasegawa test: the worst fit for the MP majority consensus tree, $P < 0.08$; see Appendix S2). The ML tree used in the comparative phylogentic analysis is shown in Appendix S3. This tree supports earlier *Agrodiaetus* trees inferred from smaller datasets (Kandul et al. 2004; Lukhtanov et al. 2005).

STANDARDIZED INDEPENDENT CONTRASTS OF CHROMOSOME NUMBERS

A Pearson product–moment correlation between the standardized independent contrasts and their standard deviations (Appendix S5) was not significant ($r = 0.029$, $n = 141$, $P = 0.730$). Thus, these independent contrasts were adequately standardized and could be weighted equally in our comparative phylogenetic analyses (Garland et al. 1992, 2005).

ACCUMULATION OF KARYOTYPIC DIVERSITY

Overall, karyotypic diversity accumulated gradually in *Agrodiaetus* (Lukhtanov et al. 2005) and, thus, left a phylogenetic signal (i.e., closely related taxa have similar chromosome numbers). According to raw contrasts of chromosome numbers, the range of karyotypic diversity present among allopatric sister clades exceeded the karyotypic diversity found among sympatric sister clades (Fig. 2A). This general pattern did not change when the standardized contrasts were considered (Fig. 2B). Only one pair of sympatric sister clades had a standardized contrast that fell outside of the karyotypic diversity found among allopatric sister clades (node #81, *A. karindus* and *A. peilei*, Appendices S3, S5).

Sister clades with sympatric distributions had significantly higher residuals than allopatric sister clades (*t*-test with unequal variance: $P < 0.030$). The residuals for allopatric sister clades increased with their relative ages (linear regression: $b = 0.300 \pm 0.058$, $t = 5.22$, $P < 0.000$; Fig. 3A). No trend was found

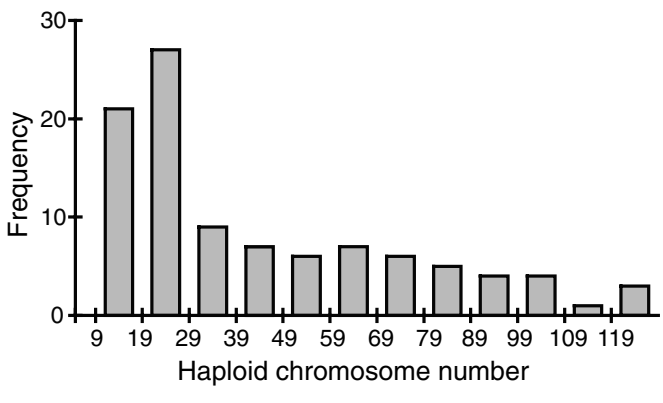

Figure 1. Frequency distribution of haploid chromosome numbers among sampled *Agrodiaetus* species. Karyotypes of 100 different *Agrodiaetus* species (i.e., 83% of known species in the genus) were examined in this study.

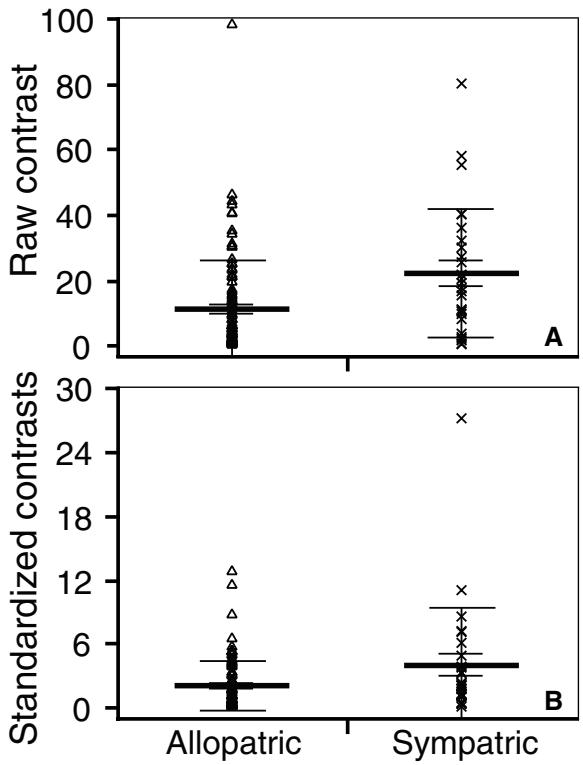

Figure 2. Ranges of karyotypic diversities found among sister clades with allopatric and sympatric distributions. Two measures of karyotypic diversity between sister clades were used: (A) absolute values of residuals from a linear regression of the raw contrast of chromosome numbers (i.e., response) over the sister clades' age (i.e., regressor); and (B) standardized independent contrasts of chromosome number inferred from the ultrametric ML tree. Sister clades have either allopatric (triangles) or sympatric (crosses) geographic distributions. A mean (bold line), its standard error (thin short line), and standard deviation (thin long line) are indicated for each distribution. Taken together, the ranges of karyotypic diversity found among sympatric and allopatric sister clades overlap, and likely came from the same overall distribution.

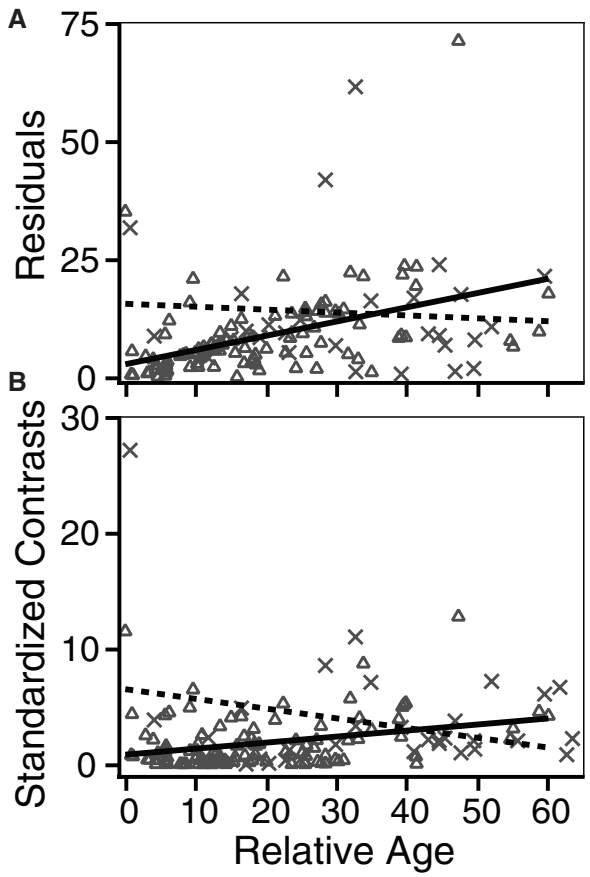

Figure 3. Plot of the relative age of sister clades on the ultrametric ML tree versus their karyotypic diversity. Two measures of karyotypic diversity between sister clades were used: (A) the absolute values of residuals from a linear regression of the raw contrast of chromosome numbers (i.e., response) over the sister clades' age (i.e., regressor); and (B) standardized independent contrasts of chromosome numbers inferred from the ultrametric ML tree. Pairs of sister clades have either allopatric (triangles) or sympatric (crosses) geographic distributions. According to analyses of covariance (ANCOVA), the distribution ($P < 0.035$*) and the distribution × relative age ($P < 0.008$*, see Table 1) have a significant effect on both absolute residuals and standardized contrast.

between the residuals for sympatric sister clades and their relative ages ($b = -0.061 \pm 0.166$, $t = -0.37$, $P < 0.716$). The difference in the slopes was significant (ANCOVA: age × distribution, $F = 7.326$, $P < 0.008$; Table 1 and Fig. 3A).

We found that although the effect of relative age (hereafter, age) on the standardized contrast was not significant (ANCOVA: age, $F = 0.518$, $P < 0.473$; Table 1 and Fig. 3B), the distribution of sister clades had a significant effect on the standardized contrast (ANCOVA: distribution, $F = 12.588$, $P < 0.000$; Table 1 and Fig. 3B). Furthermore, the distribution affected the relationship between the age of sister clades and their standardized contrasts (ANCOVA: age × distribution, $n = 134$, $F = 9.483$, $P < 0.003$, Table 1). Thus, the slopes of the linear regressions for the standardized contrast over the age were different for allopatric and sympatric sister clades (Fig. 3B). A significant positive correlation was found between the standardized contrast and the age for allopatric sister clades (linear regression: $b = 0.052 \pm 0.016$, $t = 3.29$, $P < 0.001$), whereas a nonsignificant negative slope was found for sympatric sister clades ($b = -0.084 \pm 0.066$, $t = -1.27$, $P < 0.214$). The removal of sister clades (both sympatric and allopatric) with identical chromosome numbers (i.e., their standardized contrasts are equal to 0) did not affect the outcome of the ANCOVA and individual regression analyses (Table 1).

The sister clades with sympatric distributions had significantly higher standardized contrasts of chromosome numbers than allopatric sister clades (*t*-test with unequal variance, $P < 0.032$). Furthermore, young sister clades with sympatric distributions had significantly higher standardized contrasts than the corresponding allopatric sister clades (*t*-test with unequal variance, $P < 0.043$; Fig. 4A). When sister clades formed by taxa with identical chromosome numbers were removed, the difference was still significant (all sister clades, $P < 0.043$; young sister clades, $P < 0.047$; Fig. 4B).

We could not find a correlation between the standardized contrast of chromosome numbers and the difference in relative rates using any of the settings in MacroCAIC (Table 2).

Discussion

SPECIATION RATE IN *AGRODIAETUS*

There are at least 120 species in the genus *Agrodiaetus* (see Lesse 1960; Forster 1961; Häuser and Eckweiler 1997; Lukhtanov and Dantchenko 2002b; Nazari 2003). Using the average substitution rate of *COI* measured in diverse groups of Arthropoda (Quek et al. 2004), the age of the genus *Agrodiaetus* was estimated to be three million years (Kandul et al. 2004; Kandul 2005; but for a systematic bias in the estimation of recent divergence times see also Ho et al. 2005). The net diversification interval in *Agrodiaetus* (i.e., the average time between the appearance of a new species in the same lineage) is approximately 0.6 million years. Thus, the diversification rate is 1.6 species per million years, which is among the fastest rates yet estimated (see Coyne and Orr 2004). To our knowledge, it is exceeded only by African lake cichlids, the classic example of explosive animal speciation (e.g., Salzburger et al. 2005), and *Laupala* crickets in Hawaii (Mendelson and Shaw 2005). Karyotypic diversification may well have contributed to the extreme diversification rate in the genus.

KARYOTYPIC DIVERSIFICATION

The chromosome numbers of *Agrodiaetus* species diversified toward both lower and higher numbers from the modal number for lycaenids, $n = 23/24$. The haploid chromosome numbers for *Agrodiaetus* species sampled in this study range from $n = 10$ in *A. birunii, A. caeruleus,* and *A. masulensis* to $n = 134$ in *A.*

Table 1. Analysis of covariance table for testing effects on karyotypic diversity. Two measures were used to study the accumulation of karyotypic diversity: (1) Residuals obtained from fitting the raw contrast of chromosome numbers over the relative age; and (2) standardized contrasts of chromosome numbers calculated from the ultrametric ML tree. Seven pairs of sister clades were not considered in the ANCOVA (see Materials and Methods). $^*P < 0.05$.

Source	Degrees of freedom (df)	Sum of squares	*F* ratio	*P*-value
Absolute residuals				
All sister clades considered ($N = 134$)				
Distribution of sister clades (Dist.)	1	391.641	4.526	0.035*
Sister clades' age (Age)	1	277.147	3.203	0.758
Dist. × Age	1	633.873	7.326	0.008*
Standardized contrasts of chromosome numbers				
All sister clades considered ($N = 134$)				
Distribution of sister clades (Dist.)	1	118.193	12.588	0.000*
Sister clades' age (Age)	1	0.397	0.065	0.473
Dist. × Age	1	35.271	5.786	0.003*
Standardized contrasts of chromosome numbers				
Only sister clades with nonzero standardized contrast considered ($N = 121$)				
Distribution of sister clades (Dist.)	1	115.725	11.6403	0.000*
Sister clades' age (Age)	1	11.736	1.1805	0.279
Dist. × Age	1	93.451	9.399	0.003*

achaemenes and *A. shahrami* (Appendix S1). The most frequent range of haploid chromosome numbers found among 100 species karyotypes sampled here, $n = 20$–29 (Fig. 1), includes the lycaenid modal number. Three species, *Agrodiaetus stempffferi*, *A. poseidonides,* and *A. turcicus*, have karyotypes with 23 or 24 equally sized pairs of chromosomes (Appendix S1). Because all these species are clustered close to the base in the ML tree (Appendix S3), they are likely to have conserved the ancestral karyotype of *Agrodiaetus.* We could not reconstruct the ancestral chromosome number of *Agrodiaetus* because the basal nodes (i.e., nodes #1–4 and 64, Appendix S3) were poorly supported in the ML tree or collapsed in the MP and BI consensus trees.

The majority of sampled *Agrodiaetus* species have chromosome numbers higher than $n = 23/24$. Inclusion of unsampled *Agrodiaetus* species with known chromosome numbers would not change this pattern; in general, a greater number of species have evolved higher numbers than lower ones. A formal analysis of forces generating this pattern is beyond the scope of this paper. Mechanisms of chromosomal fusions and fragmentations are not fully understood for monocentric chromosomes (e.g., Shaffer and Lupski 2000; Lönnig and Saedler 2002), let alone holocentric chromosomes. However, one provisional explanation can be offered (see also "karyotypic orthoselection" in White 1973, pp. 450–454). A single fusion event involves active participation of two nonhomologous chromosomes, whereas a fragmentation involves only one chromosome. Thus, fragmentations might occur more readily than fusions. However, this reasoning also assumes that the daughter chromosomes can easily develop telomeric sequences at the breakpoints, which may not be the case. Before

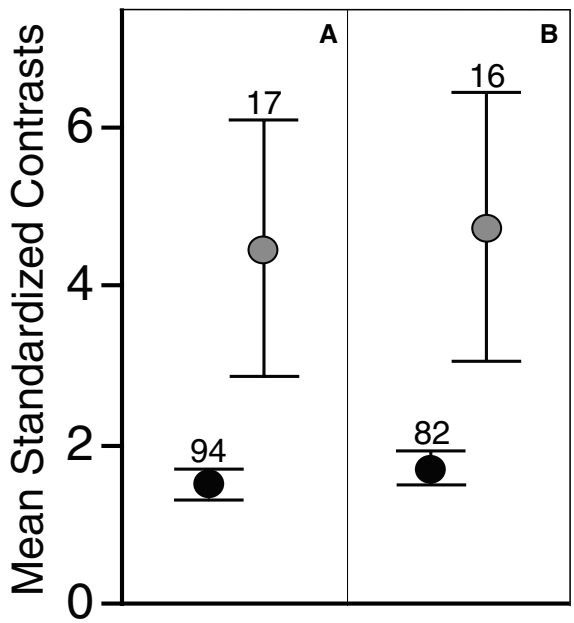

Figure 4. Difference in mean standardized contrasts of chromosome numbers between young sympatric sister clades and allopatric sister clades. Pairs of sister clades with genetic distances less than 0.05 substitutions per bp were considered young pairs (for details, see Lukhtanov et al. 2005). All young pairs were used in comparison (A). Only young sister clades formed by taxa with different chromosome numbers were considered in comparison (B). Numbers of sister clades for each set are shown above the error bar. In both comparisons, young sister clades with sympatric distributions (gray circle) have a significantly higher mean contrast of chromosome numbers than the corresponding allopatric sister clades (black circle; $P < 0.043^*$ and $P < 0.047^*$, respectively).

Table 2. Significance of association between karyotypic diversity and species richness.

Minimal node size	Sister clades	*N*	*P*-value
Standardized contrasts calculated using equal branch lengths			
Three species	all sister clades considered	70	0.199
	nested clades removed	17	0.592
Five species	all sister clades considered	47	0.249
	nested clades removed	11	0.185
Standardized contrasts calculated using ultrametric branch lengths			
Three species	all sister clades considered	70	0.102
	nested clades removed	17	0.059
Five species	all sister clades considered	47	0.135
	nested clades removed	11	0.648

Standardized contrasts of chromosome numbers were used to estimate karyotypic diversity (independent variable), and differences in relative rates (i.e., $\ln(n_i/n_j)$) were used to measure the difference in species richness (response variable) between two sister clades. Pairs of sister clades that have at least three or five species were analyzed. First, we analyzed all designated pairs of sister clades; then, we analyzed only nonnested pairs of sister clades at the tips of the tree. The number of sister clades is shown for each analysis in column *N*. The significance of the association was estimated using a Wilcoxon signed-rank test on the direction of the difference in relative rates (see Materials and Methods).

possible differences in appearance frequencies of fusions and fragmentations are incorporated into models of karyotype evolution, the mechanism(s) underlying these chromosomal rearrangements must be elucidated.

REARRANGEMENTS AND SPECIES CHROMOSOME NUMBER

We assume that differences in species chromosome numbers correlate with the number of chromosomal rearrangements differentiating their karyotypes. The examination of butterfly karyotypes is a notoriously difficult procedure due to the small size of the chromosomes, the absence of visible morphological markers, and the generally high number of chromosomes. Only two characters of the species karyotype are commonly used in butterfly cytogenetics: haploid chromosome number and relative sizes of bivalents. We focused on the chromosome number because it can be easily analyzed in a comparative study. Sister species with different chromosome numbers acquired them through the fixation of a number of chromosomal rearrangements. However, we do not know the true number of chromosomal rearrangements separating their karyotypes. Species with conserved chromosome numbers can still have chromosomal rearrangements fixed between them that will be invisible to our analysis (cf. paracentric inversions among *Drosophila* species). Therefore, the true number of chromosomal rearrangements among *Agrodiaetus* taxa is probably underestimated in our study.

ESTIMATION OF GENETIC DISTANCE

Genetic distance estimates between sympatric species may be systematically deflated by gene flow, leading to the false impression that sympatric species diverged more recently than comparable allopatric species (e.g., Kulathinal and Singh 2000). However, we used mitochondrial DNA sequences to estimate genetic distance, and mtDNA is unlikely to introgress between *Agrodiaetus* species because lepidopteran females are the heterogametic sex and more prone to sterility (e.g., Haldane 1922; Presgraves 2002). As a result, the potential gene flow between nascent species with sympatric distributions is unlikely to result in systematic underestimation of genetic distances.

SPECIATION AND KARYOTYPIC DIVERSIFICATION

We examined the relationship between karyotypic diversity and age using a sister-clade analysis. If chromosomal rearrangements have contributed to the establishment or maintenance of reproductive isolation among nascent species, we expect to find that (1) karyotypic diversity has accumulated gradually among allopatric taxa and (2) young sympatric sister taxa have more marked karyotypic differences than the corresponding (i.e., young) allopatric sister taxa. The observation of this pattern of karyotype diversity does not prove that chromosomal rearrangements directly caused speciation. Nevertheless, such a pattern would be consistent with a direct role for chromosomal rearrangements in the persistence of nascent sympatric species and, thus, in the speciation process.

Sister-clade analysis of karyotypic diversity in the genus *Agrodiaetus* yields an expected pattern. First, the range of karyotypic diversity among allopatric sister clades is generally larger than the range found among sympatric sister clades, regardless of whether raw or standardized contrasts were used (Fig. 2). One pair of sympatric sister species, *A. karindus* $n = 70$ and *A. peilei* $n = 38$, has a remarkably high standardized contrast for its age (i.e., 3 substitutions from 2145 bp, Appendix S3), but this single outlier comes from the subsample that is expected to be biased toward higher karyotypic divergence (see below). In

addition, because our scoring of karyotype is limited to chromosome number and size, we have failed to sample all existing forms of karyotypic variation among allopatric sister clades. Therefore, we cannot reject the null hypothesis (e.g., Templeton 1981; Coyne and Orr 2004) that the karyotypic diversity found among sister clades with sympatric distributions represents a nonrandom sample of the entire karyotypic diversity that originated in allopatry.

Second, we found that karyotypic diversity accumulated gradually between allopatric sister clades. (1) We calculated the residuals from the linear regression of the raw contrast of chromosome number over the age of the clade. The residuals for allopatric sister clades increased gradually with age ($P < 0.000$, Fig. 3A), whereas sympatric clades showed no significant association between residuals and age. Furthermore, sympatric sister clades deviated more from the regression line than allopatric sister clades ($P < 0.03$). The analysis of the residuals did not rely directly on the assumption that karyotype evolved according to a Brownian motion model. A positive linear correlation between the square root of the relative age and the raw contrast in chromosome numbers corroborates this assumption ($r = 0.462$, $n = 134$, $P < 0.001$). (2) The same pattern was inferred from the analysis of the standardized contrasts of chromosome numbers. The distribution of sister clades significantly affected the relationship between standardized contrasts and age; standardized contrasts increased gradually with age among allopatric sister clades, whereas no significant association was found for sympatric sister clades (Fig. 3B).

Third, sympatric sister clades have significantly higher average karyotypic diversity than allopatric sister clades ($P < 0.032$). The removal of old sister clades did not affect the difference between average karyotypic diversities in sympatric and allopatric sister clades ($P < 0.047$; Fig. 4B).

Our findings indicate that differential fusion (Templeton 1981) of karyotypically divergent allopatric populations in secondary sympatry likely enhances karyotypic diversity among sympatric sister clades and is, thus, responsible for the observed pattern. The fundamental difference between allopatric and sympatric pairs of sister clades is that although an allopatric pair could conceivably be derived from two specimens of the same species from geographically different populations, a sympatric pair is always formed by at least two distinct biological species. Therefore, pairs of young sympatric clades (i.e., tips on a phylogeny) are distinct biological species that managed to develop sufficient reproductive isolation to be able to persist in sympatry. The initiation of significant karyotypic diversity in *Agrodiaetus* was likely acquired in allopatry (Lukhtanov et al. 2005), because chromosomal rearrangements can become fixed through drift within small, geographically isolated populations (White 1973; King 1993). Differential fusion (Templeton 1981) will operate when allopatric populations come back into contact (i.e., secondary sympatry). The more chromosomal rearrangements accumulated between diverging allopatric populations, the higher the level of postzygotic isolation between them (see King 1993: p. 165). Populations that have accumulated multiple chromosomal rearrangements can persist in secondary sympatry and become true biological species, whereas populations whose karyotypes are insufficiently diverged are likely to fuse and lose their distinctiveness. In other words, differential fusion purges low karyotypic diversity among nascent *Agrodiaetus* species in sympatry, meaning that only those species pairs with relatively high levels of karyotypic divergence persist as distinct taxa.

Differential fusion of karyotypically divergent populations would be expected to operate in this system because chromosomal rearrangements play a role in the formation of postzygotic isolation (e.g., White 1973; Gropp et al. 1982; Delneri et al. 2003). Any additional characters that were serendipitously independently acquired in allopatry and could indirectly contribute to pre- and postzygotic isolation would be tested in secondary sympatry as well. A thorough analysis of morphological and ecological characters in the genus has thus far yielded only one further isolating character that could contribute to the persistence of divergent populations in secondary sympatry, male wing coloration. The data from sister-clade analysis of changes in male wing color argued against differential fusion as the mechanism that strengthened the color differences between sympatric *Agrodiaetus* species. Color differences are likely to have evolved in situ in sympatry of nascent species through the process of reinforcement of prezygotic isolation (Lukhtanov et al. 2005). Lukhtanov et al. (2005) argued that the persistent sympatric co-occurrence of karyotypically divergent but nascent species sets the stage for the reinforcement of male wing coloration. The evidence of the direct role of chromosomal rearrangements in the persistence of nascent species in *Agrodiaetus* further supports this hypothesis.

We did not find an association between karyotypic diversity and species richness (Table 2) using sister-group comparisons as implemented in MacroCAIC. All pairs of sister clades in the interspecific tree that conformed to the set criteria were objectively considered. Both measures of species richness, the difference in relative rates (see methods) and the proportional dominance index (i.e., $n_i/(n_j + n_i)$; data not shown), were used in the analysis.

A positive association between karyotypic diversity and species richness has been found earlier in comparative studies (Wilson et al. 1975; Bush et al. 1977; Petitpierre et al. 1993; Olmo et al. 2002; Olmo 2005). However, phylogenetic relationships were not taken into account in these studies.

Using sister-clade analysis to identify correlates of net species diversification (see Barraclough et al. 1998) insures that each analyzed pair of sister clades shared a relatively recent common ancestor (i.e., in the genus *Agrodiaetus*, less than three million years ago). Thus, each sister lineage recently inherited the same

ecological propensities, and observed differences among them must have been acquired during their independent evolution. Nevertheless, speciation is a process contingent on many factors, including habitat partitions, population structure, and effective population size. The relative effects of these factors on net diversification rate may be stronger than that of karyotypic diversity. In addition, these factors themselves can shape karyotypic diversity. For example, differences in effective population sizes (e.g., Bush et al. 1977; Coyne 1984) and population structure (e.g., Sites and Moritz 1987) affect the fixation rate of chromosomal rearrangements. Therefore, although a comparative phylogenetic approach is a useful method for inferring factors promoting speciation (Barraclough et al. 1998; Barraclough et al. 2000), it has its limits in identifying individual effects of these inter-dependent factors.

CONCLUSION

Distinct chromosomal rearrangements fixed in different species play a role in the maintenance of postzygotic isolation between them (e.g., Noor et al. 2001; Delneri et al. 2003); however, conclusive data supporting their direct and general role in speciation have been lacking (for a review see Coghlan et al. 2005). A few studies have systematically examined the accumulation of chromosomal rearrangements (but see Wang and Lan 2000) among young species using phylogenetic methods (Coyne and Orr 2004). The genus *Agrodiaetus* has acquired extreme interspecific karyotypic diversity during a relatively short time period. Using sister-clade analysis, we show that (1) karyotypic diversity among sympatric taxa is likely to be a nonrandom subsample of the karyotypic diversity found among allopatric taxa, (2) karyotypic diversity accumulates gradually among allopatric *Agrodiaetus* taxa, and (3) chromosome numbers between young pairs of sympatric taxa are more different than those between corresponding pairs of allopatric taxa. The differential fusion of allopatric populations in secondary sympatry probably generated this pattern: the degree of karyotypic divergence acquired between allopatric populations of *Agrodiaetus* will determine whether they persist as nascent species in secondary sympatry or fuse and lose their distinctiveness. The results of this comparative phylogenetic study provide evidence for a direct role of chromosomal rearrangements in the final stages of animal speciation.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Appendices s1–s5.

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