Reinforcement of pre-zygotic isolation and karyotype evolution in *Agrodiaetus* butterflies

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The reinforcement model of evolution argues that natural selection enhances pre-zygotic isolation between divergent populations or species by selecting against unfit hybrids[1,2] or costly interspecific matings[3]. Reinforcement is distinguished from other models that consider the formation of reproductive isolation to be a by-product of divergent evolution[4,5]. Although theory has shown that reinforcement is a possible mechanism that can lead to speciation[6–8], empirical evidence has been sufficiently scarce to raise doubts about the importance of reinforcement in nature[6,9,10]. *Agrodiaetus* butterflies (Lepidoptera: Lycaenidae) exhibit unusual variability in chromosome number. Whereas their genitalia and other morphological characteristics are largely uniform, different species vary considerably in male wing colour, and provide a model system to study the role of reinforcement in speciation. Using comparative phylogenetic methods, we show that the sympatric distribution of 15 relatively young sister taxa of *Agrodiaetus* strongly correlates with differences in male wing colour, and that this pattern is most likely the result of reinforcement. We find little evidence supporting sympatric speciation: rather, in *Agrodiaetus*, karyotypic changes accumulate gradually in allopatry, prompting reinforcement when karyotypically divergent races come into contact.

Speciation is the process whereby previously interbreeding populations develop reproductive isolation. Geographic barriers can arise and prevent gene flow, enabling populations to diverge genetically in allopatry[4,11]. Occasionally, incipient allopatric species come into secondary contact through the expansion of their ranges before they have developed pre-zygotic isolating mechanisms. In such cases, natural selection acting against maladaptive hybrids[1,2,6] or against costly interspecific mating[6–8] can produce an indirect selection pressure favouring trait divergence and assortative mating. This process, called reinforcement, is of particular significance because it provides a role for natural selection in the formation of pre-zygotic isolation and eventually in speciation, processes that are otherwise incidental. Despite its plausibility[7] and increasing attention from evolutionary biologists[8], only a few well-documented cases of reinforcement have been published[6,11–15].

In the broad sense, reinforcement of pre-zygotic isolation can take place at both intraspecific and interspecific levels (see page 354 of ref. 3). Reinforcement between divergent populations that exchange genes[9] can lead to speciation (termed "true reinforcement" by ref. 3), whereas reinforcement without gene flow is an adaptive genetic change that can occur after speciation has been completed. At both levels, reinforcement can give rise to a particular pattern of reproductive character displacement (RCD) involving greater interspecific mate discrimination between sympatric species than between allo-

patric species. Such patterns have long been considered evidence for reinforcement.

However, RCD is a common phenomenon[5] and the same pattern of RCD may be generated by at least three other mechanisms: differential fusion[9,16,17], ecological character displacement[6,15] and runaway sexual selection[18]. Under differential fusion, RCD arises as a by-product of evolution in allopatry. Those populations that have serendipitously evolved strong mating discrimination can persist in secondary sympatry, whereas those populations lacking such discrimination fuse and lose their distinctiveness. Species that persist in sympatry will demonstrate a high level of mating discrimination even though reinforcement has not operated[11,16]. Similarly, ecological divergence may cause concomitant changes in mate recognition signals that make sympatric populations of two nascent species less likely to mate with one another[6,15]. Runaway sexual selection can also generate RCD if selection has favoured dramatic differences in mate recognition characters directly within a single population arrayed along an ecological gradient[18].

We have studied RCD in the species-rich genus *Agrodiaetus*. This genus is estimated to have arisen 2.5–3.8 million years ago[19], and exhibits one of the widest diversities of chromosomal complements (that is, karyotypes) found in the animal kingdom, with haploid chromosome numbers of different species ranging from $n = 10$ to $n = 134$ (refs 20, 21). This range in karyotype is not caused by polyploidy: the similarity in genome sizes among *Agrodiaetus* species suggests that karyotype diversity arose through fusion and fragmentation of chromosomes[19–21]. Hybrids between heterokaryotypic *Agrodiaetus* species have been observed in nature[22,23], but segregational problems during meiosis would result in their having reduced fertility. Karyotypic differences thus form a partial post-zygotic reproductive barrier[20,24]. Although females are uniformly brown, *Agrodiaetus* species show considerable variability in male wing colour. Wing coloration, both in visible and ultraviolet wavelength ranges, is an important mate recognition characteristic in butterflies[14,25] involved in the formation of pre-zygotic reproductive barriers[14,15,26]. In lycaenids, both sexes typically exhibit mate choice[27], and females accept only those males with appropriate conspecific coloration[27]. Females of polyommatine species such as *Agrodiaetus* mate only once[28], and thus heterospecific matings that fail to give rise to viable offspring are strongly selected against. The combination of rapid karyotypic evolution, the role of karyotypic differences in reducing hybrid fitness, the reproductive biology of lycaenids, and a simple wing-colour-based criterion in mate choice makes *Agrodiaetus* a promising candidate for studies of reinforcement.

We reconstructed a phylogeny using 1,938 base pairs from two mitochondrial genes, *Cytochrome oxidase I* and *II* (*COI* and *COII*; see Supplementary Information), for 89 species and subspecies of

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Agrodiaetus. We determined the genetic distance on the phylogeny for each pair of sister taxa, and noted each taxon's wing colour and whether the taxa were sympatric or allopatric in their distribution. Traits involved in mate differentiation showed greater differences between sympatric pairs of species as opposed to allopatric pairs, when comparing taxa separated by relatively small genetic distances (ML distances ranging from 0–0.050 changes per nucleotide; see Supplementary Information).

By mapping taxon wing colour on the inferred phylogeny, we observed 19 changes in wing colour among sampled taxa (Fig. 1). *A. cyaneus* and *A. gorbunovi* (Fig. 1) are the closest related pair of species found in sympatry (0.008 changes per nucleotide, under the HKY + I + Γ model of DNA substitution). Despite their genetic similarity, *A. cyaneus* has already acquired a new wing colour. Sampled sister taxa separated by genetic distances smaller than this value occur only in allopatry (Fig. 1). At genetic distances between 0.008 and 0.050, sympatric pairs of species begin to appear, and they generally exhibit different wing colours.
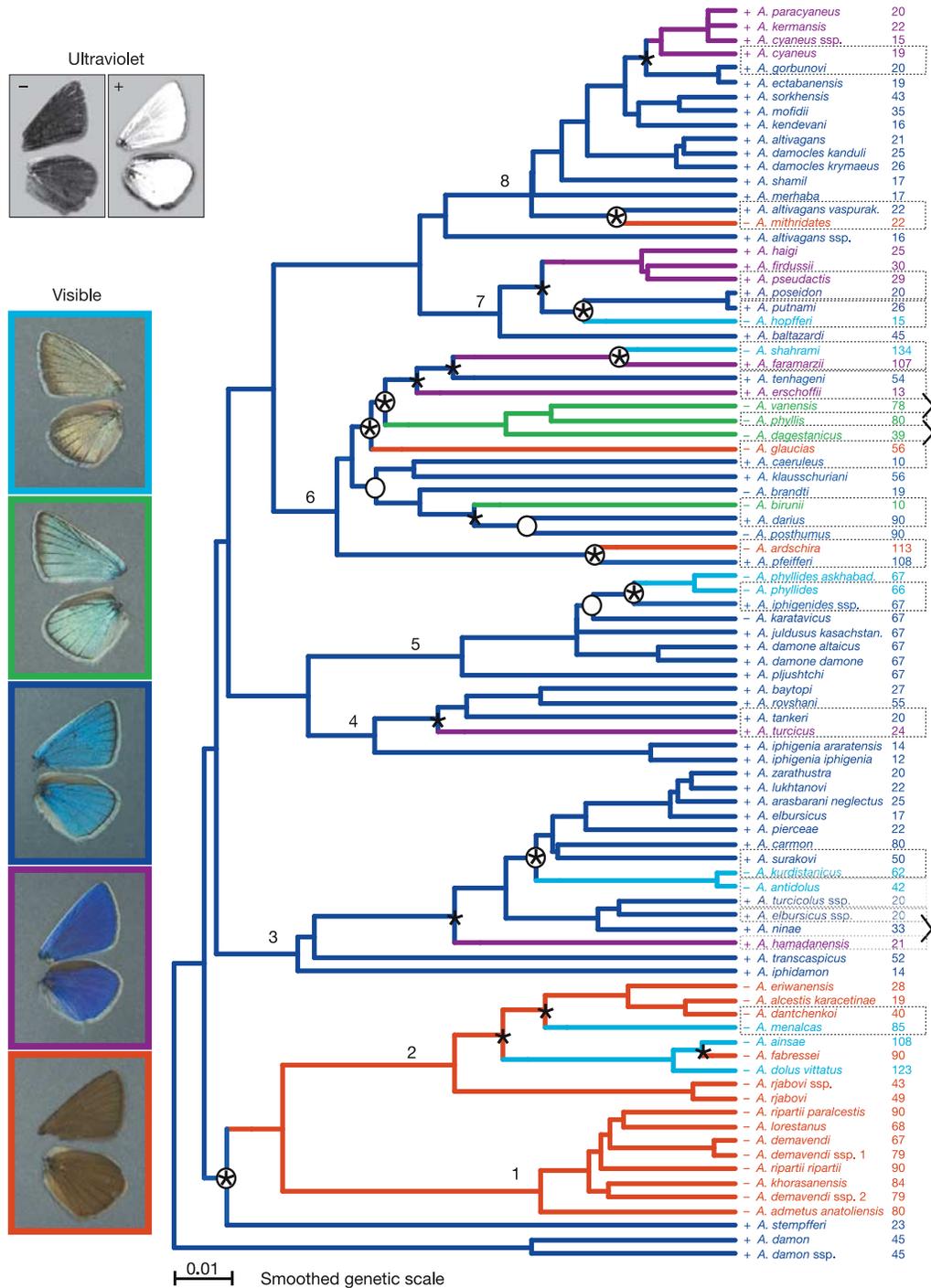
Figure 1 | Changes in male wing coloration along the phylogeny of *Agrodiaetus*. Five unordered states of male wing colour and presence (+) or absence (−) of wing ultraviolet reflectance were mapped on the ML tree. The inferred changes in wing visible coloration and ultraviolet reflectance are labelled on the tree with an asterisk and a circle, respectively. Relatively young sympatric pairs of taxa with markedly different visible colours are boxed. The column to the right of the taxon names shows haploid chromosome numbers. Eight clades (1–8) were used to examine karyotypic divergence in *Agrodiaetus* (Fig. 4).

To account for dependencies arising from common ancestry, we analysed 88 independent pairs, or nodes, of sister clades[29]. Of these nodes, 28 were classified as sympatric pairs, and the remaining 60 were allopatric. Out of 19 cases of colour changes (Fig. 1), 15 were observed between sympatric sister clades, and 4 between allopatric sister clades. In other words, colour changes occurred preferentially between sympatric, as opposed to allopatric, sister clades (Fisher's exact test, $P < 0.000002$). Considering only the sympatric pairs, most of the young sister clades (ML distances of 0–0.05 changes per nucleotide; Fig. 2a) exhibit colour differences, whereas old sister clades (0.50–0.12 changes per nucleotide; Fig. 2a) tend to have the same wing colour. Conversely, allopatric pairs of sister clades exhibit only a small number of colour differences, and these differences are distributed independently of genetic distance (Fig. 2b). A phylogenetic analysis of changes in wing ultraviolet reflectance produced a similar pattern of RCD (Figs 1 and 2; see also Supplementary Information).

Comparative phylogenetic methods applied to the geographic distributions of extant species enable us to discriminate between reinforcement and the three other mechanisms that could generate similar patterns of RCD. Reinforcement predicts that primarily young phylogenetic lineages will demonstrate RCD because older lineages are less likely to hybridize, having already acquired full reproductive isolation in allopatry. In contrast, differential fusion and ecological character displacement do not predict a strong correlation between RCD and lineage ages (see Supplementary Information). Moreover, according to differential fusion, RCD should be equally rare among old and young sympatric species, and changes in wing colour found among sympatric pairs of species should comprise a subset of the changes in colour seen among

allopatric pairs of species[11,12]. The distribution of colour changes found in *Agrodiaetus* is therefore unlikely to be generated by differential fusion or ecological character displacement (Fig. 2), and is more consistent with the predictions of the reinforcement model.

The specific pattern of RCD found in this study (Fig. 2) could be caused by differential fusion if wing colour evolves rapidly, for example by sexual selection between allopatric populations, whereas other forms of pre-zygotic isolating characters are more stable in the genus *Agrodiaetus*. Our data argue against this possibility. Wing colour remained unchanged in 69 nodes among sampled *Agrodiaetus* taxa, and once evolved, new wing colours passed unchanged through multiple subsequent allopatric speciation events (Fig. 1). At the same time, other potentially pre-zygotic isolating characters such as host plant use and ecological preferences vary even between purely allopatric populations of *Agrodiaetus* (see Supplementary Information). The third alternative mechanism, runaway sexual selection, generates RCD within "a single population distributed across an ecological cline" (ref. 18; in sympatry), whereas primary divergence in allopatry is necessary for the appearance of RCD under reinforcement. In our data, the smallest genetic divergences occurred between sister taxa with allopatric distributions. An additional age–range correlation test[30] did not reveal a pattern consistent with frequent sympatric speciation in *Agrodiaetus* (Fig. 3). Thus, the relationships between genetic distance, male wing colour variability and geographic distribution exhibited by *Agrodiaetus* are consistent with reinforcement as a mechanism generating RCD, and appear to reject three alternative mechanisms: differential fusion, ecological character displacement and runaway sexual selection.
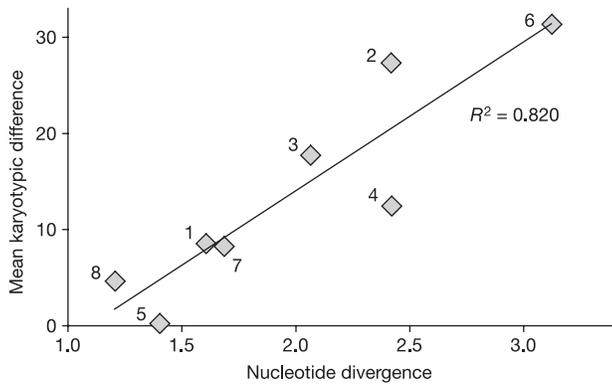
Eight independent clades (1–8; Fig. 1) were chosen to examine the accumulation of karyotypic diversity in the genus. These clades
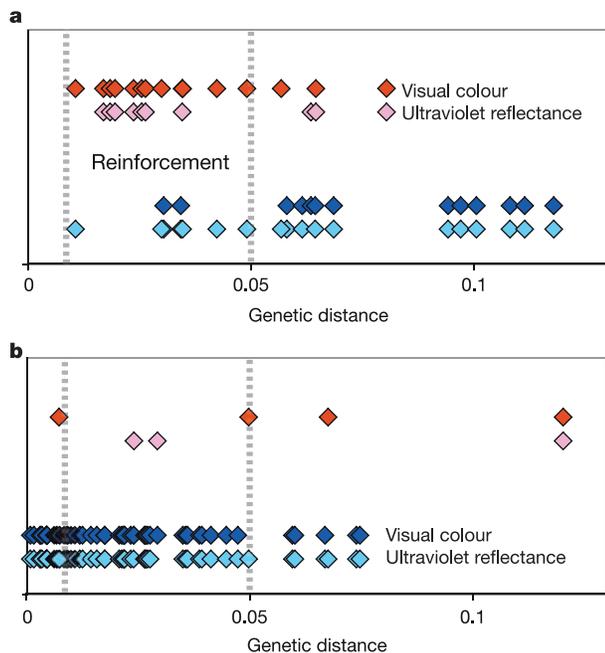
Figure 2 | **Changes in male wing coloration between *Agrodiaetus* sister clades as a function of their genetic distance.** According to our reconstruction, pairs of sister clades exhibit a change in visible colour at the node that separates them (red) or remain the same colour (blue). Ten changes in ultraviolet reflectance (pink) coincided with changes in visible coloration. Six changes in visible coloration between young sympatric sister clades did not affect wing ultraviolet reflectance (turquoise). **a**, Among sympatric sister clades, changes in visible colour occur primarily between recently divergent clades. **b**, Conversely, among allopatric sister clades, recently divergent clades retain the same coloration (visible and ultraviolet), and colour changes are otherwise rare and happen at random throughout the entire range of genetic distances.
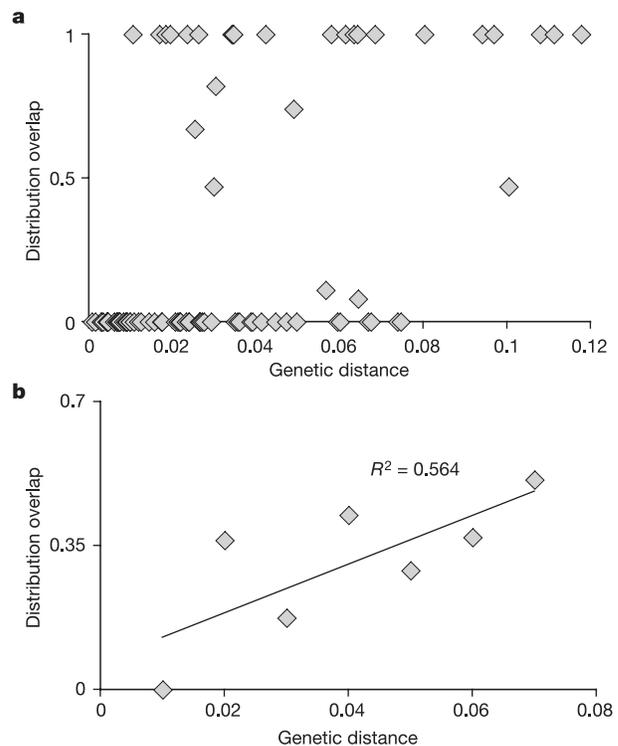
Figure 3 | **Age–range correlation plot. a**, The distribution overlap for every pair of sister clades was plotted against the genetic distance between them. **b**, The cumulative age–range correlation plot shows distribution overlap averaged over genetic distance. Because the number of pairs of relatively old sister clades was too small to calculate a mean distribution overlap, its values are not shown for genetic distances greater than 0.08 changes per nucleotide (under HKY + I + Γ).

Figure 4 | *Agrodiaetus* **karyotypic diversity strongly correlates with nucleotide divergence ($R^2 = 0.820$; $P < 0.002$).** Mean intraclade karyotypic differences in eight independent clades (1–8, Fig. 2) are plotted against nucleotide divergences in the same clades.

exhibited a significant correlation between nucleotide divergence and mean karyotypic difference (Fig. 4; $R^2 = 0.820$; $P < 0.002$). Because genetic diversity among lineages is also proportional to the time since their divergence, it seems that chromosome numbers diverge with time. This conclusion is consistent with allopatric speciation as the main mode of cladogenesis in the genus, because karyotypic differences can accumulate within geographically isolated, small populations. Given the frequent chromosomal rearrangements in *Agrodiaetus*, karyotypic characters could act synergistically with geographic isolation to enhance reproductive barriers between nascent species of *Agrodiaetus*, despite their high vagility as adult butterflies. This synergistic action could impede gene flow between populations, facilitating the evolution of pre-zygotic isolating characters.

Although interspecific hybridization can occur within *Agrodiaetus*[22,23], we do not know whether nascent *Agrodiaetus* species exchange genes in sympatry before they acquire full reproductive isolation. Biological species can evolve pre-zygotic isolating barriers without gene flow, for example when their hybrids are sterile[3,9]. At the same time, the absence of gene flow between extant sympatric species does not necessarily imply that these species did not exchange genes when they first came into contact. Therefore, although we cannot distinguish at what level (intraspecific or interspecific) reinforcement has operated, our comparative study demonstrates that natural selection against maladaptive matings is likely to have caused widespread divergence in pre-zygotic isolating characters between sympatric species of *Agrodiaetus*, and could have led to speciation.

METHODS

Methods are described in greater detail in the Supplementary Information.

Sampled species. *Agrodiaetus* butterflies belong to the section *Polyommatus* of the family Lycaenidae (Insecta: Lepidoptera). Females are brown, whereas males have a variety of background colours ranging from silver and blue to brown on the upper side of their wings (Fig. 1). With the exception of male wing coloration, which is a relatively labile character[19] in the genus, *Agrodiaetus* species have remarkably similar genitalia and other external morphological characteristics. The taxa sampled for this study represent the entire range of known karyotypic diversity in *Agrodiaetus*, from $n = 10$ in *A. caeruleus* and *A. birunii* to $n = 134$ in *A. shahrami*. Identification of a number of *Agrodiaetus* species is based on karyotype; therefore the karyotypes of most specimens were examined before their DNA was extracted for gene sequencing, with the exception of 19 cases where individuals from populations with well characterized karyotypes were used[20,21]. Eighty-nine well-differentiated taxa (76 species and 13 subspecies) of *Agrodiaetus* were used in this study (Supplementary Appendix 1), and of these, 52 karyotyped specimens of *Agrodiaetus* were analysed for the first time[19].

Phylogenetic analysis. Two mitochondrial genes, *Cytochrome oxidase subunit I* (*COI*) and *Cytochrome oxidase subunit II* (*COII*), were amplified by polymerase chain reaction (PCR). PCR products were of equal length and directly

sequenced. Eighty-nine continuous sequences of *COI*, tRNA-*leu* and *COII* genes were aligned in a data set that was partitioned into the respective genes using PAUP* 4.0b10. For phylogeny reconstruction, we used three main methods: maximum parsimony (PAUP* 4.0b10), bayesian inference (MrBayes 3.01) and maximum likelihood (PHYML). Hierarchical likelihood ratio tests (hLRTs) were used to identify the model of DNA substitution that best fit the data for maximum likelihood and bayesian inference analyses. To ensure that the bayesian inference analysis was not trapped in local optima, we ran three independent rounds of the procedure. Average log-likelihood values at stationarity were calculated during each round and compared for convergence. The support of tree branches recovered by maximum parsimony and maximum likelihood methods was estimated with nonparametric bootstrap values. To align the tips of the recovered maximum likelihood tree (Fig. 1), we homogenized substitution rates across lineages using Sanderson's nonparametric rate-smoothing algorithm as implemented in TreeEdit.

Reconstruction of ancestral colour. Wing colour was treated as a multi-state unordered character with a total of five distinct states (Supplementary Appendix 1). Wing ultraviolet reflectance was coded as present or absent. A test of serial independence rejected the null hypothesis that the wing colour was not correlated with phylogeny ($P = 0.0003$). We used a maximum likelihood method of ancestral character reconstruction because this method accounts for branch lengths on the tree and estimates probabilities of reconstructing different states. We reconstructed ancestral wing coloration on the maximum likelihood tree (see Supplementary Information for branch support) inferred under the HKY + I + Γ model of DNA substitution (Fig. 1) in Mesquite 1.0. Maximum likelihood optimizations were done using the Markov *k*-state one-parameter model.

Sister-clade analysis. We compared reconstructed and extant states of species colours for every pair of sister clades on the maximum likelihood tree inferred under the HKY + I + Γ model of DNA substitution (Fig. 1; see Supplementary Information for branch support). Average genetic distances between sister clades were estimated from the maximum likelihood tree. We classified a pair of sister clades as sympatric if they shared at least one pair of basal taxa with a sympatric distribution (Supplementary Appendix 2). We have considered all nodes independent of their age. For simplicity, we have assumed that sister clades separated by relatively older nodes gained sympatry only recently. The observed pattern of RCD is even stronger if we exclude sister taxa separated by older nodes from our analysis.

Karyotype evolution. A test of serial independence ($P = 0.0003$) showed that the distribution of chromosome number is correlated with phylogenetic placement on the maximum likelihood tree. Although karyotype (including chromosome number and relative size of bivalents) is a labile character in *Agrodiaetus*[19,21], we must control for changes attributable to common ancestry. We chose eight independent clades (that is, lineages), recovered on the maximum likelihood tree (Fig. 1), to examine the accumulation of karyotypic divergence in *Agrodiaetus*. Nucleotide divergence in a clade was estimated in Arlequin. To calculate mean intraclade karyotypic differences, we first averaged haploid chromosome numbers between every pair of sister clades from the maximum likelihood tree, starting from the tips and working towards the root of the tree, took the absolute difference between averaged chromosome numbers at every node on the tree, and then averaged these differences at the internal nodes included in the eight well-defined clades (Fig. 1).

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