LETTERS

Reinforcement drives rapid allopatric speciation

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Allopatric speciation results from geographic isolation between populations. In the absence of gene flow, reproductive isolation arises gradually and incidentally as a result of mutation, genetic drift and the indirect effects of natural selection driving local adaptation[1–3]. In contrast, speciation by reinforcement is driven directly by natural selection against maladaptive hybridization[1,4]. This gives individuals that choose the traits of their own lineage greater fitness, potentially leading to rapid speciation between the lineages[1,4]. Reinforcing natural selection on a population of one of the lineages in a mosaic contact zone could also result in divergence of the population from the allopatric range of its own lineage outside the zone[4–6]. Here we test this with molecular data, experimental crosses, field measurements and mate choice experiments in a mosaic contact zone between two lineages of a rainforest frog. We show that reinforcing natural selection has resulted in significant premating isolation of a population in the contact zone not only from the other lineage but also, incidentally, from the closely related main range of its own lineage. Thus we show the potential for reinforcement to drive rapid allopatric speciation.

Although the role of reinforcing natural selection in driving allopatric speciation within a lineage has received little attention[4,6], its role in the final stages of speciation between divergent sister lineages has had a long and contentious history—some consider that there are compelling examples[7], whereas others argue that the empirical evidence is generally inconclusive[8,9]. Uncertainty remains because strict criteria must be satisfied to demonstrate the process of reinforcement unambiguously[4]: first, heterospecific matings occur in the field; second, there is selection against hybridization; third, displacement of a trait is perceived by the other sex; fourth, variation in the trait is heritable and responds to selection; and last, displacement has not occurred for other reasons, such as ecological divergence. Demonstrating that this process can complete speciation requires showing that it has resulted in significant reproductive isolation between the lineages. Further, evidence for reinforcement is most compelling within a well-corroborated historical biogeographic framework[10].

The green-eyed tree-frog *Litoria genimaculata* is a stream-breeding hylid frog common in rainforest throughout the 'Wet Tropics' region of northeast Queensland, Australia. Like many other Wet Tropics

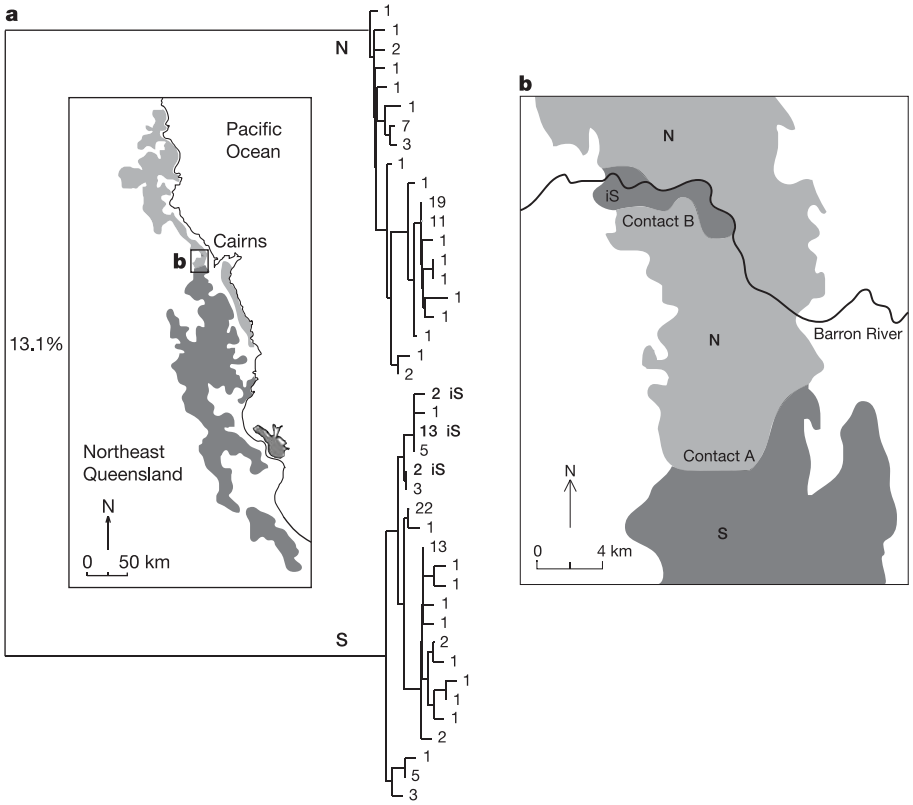

Figure 1 | Distribution of the N (pale shading) and S (dark shading) lineages of *L. genimaculata*. **a**, The distribution of N and S and a tree showing genetic divergence (percentage) and the number of individuals with each haplotype. **b**, An enlargement of the region within the rectangle in **a**, showing the mosaic contact between the N and S lineages. The iS population shares haplotypes (with 0.1% net divergence overall) with nearby populations from the main range of the S lineage (**a**) but is currently isolated from them (population subdivision statistic $F_{st} = 0.20$, $P < 0.001$). By contrast, the N populations are continuous and genetically connected (north versus south of Barron River; $F_{st} = -0.01$, $P = 0.60$) through the mosaic contact.

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species, *L. genimaculata* consists of two highly divergent lineages, northern (N) and southern (S) (Fig. 1a), reflecting long-term isolation to northern and southern rainforest refugia during the cooler, drier periods of the Pliocene and Pleistocene epochs[11]. The rainforests of the northern and southern refugia reconnected about 6,500 years ago, bringing the *L. genimaculata* lineages into secondary contact in a suture zone[12]. The secondary contact zone in this species is a mosaic that consists of the main 'contact A', as well as a second contact (B) involving a recent geographical isolate of the S lineage (iS hereafter) within the range of the N lineage (Fig. 1b). Here we test whether speciation by reinforcement has occurred between the two lineages at either contact A or contact B, and, if so, whether this has incidentally driven allopatric speciation between the iS population and the genetically similar main range of the S lineage.

Genetic analysis revealed that hybridization between the lineages occurs in the field, although it is geographically broader (6.0 km versus 0.6 km), and significantly more frequent (3.1–6.8% hybrids versus 0–1.4%; likelihood ratio test, LI = 8.46, $P = 0.015$), at contact A than at contact B. For the process of reinforcement to operate, there must be selection against hybridization[1,4]. We tested this by performing experimental crosses between the N and S/iS lineages. Viability of hybrid crosses was highly asymmetric (likelihood ratio test, LI = 20.73, $P < 0.001$): all crosses involving an S or iS female with an N male failed at the early tadpole stage, whereas all the reciprocal crosses succeeded to termination of the experiment at late tadpole stage or metamorphosis, albeit with significantly slower development than the control crosses within the N lineage (univariate analysis of variance (ANOVA), $F_{1,10} = 9.526$, $P = 0.012$). The asymmetry in viability is supported by the genetic analysis of the contact zones, which revealed that none of the hybrids carried S mitochondrial DNA (mtDNA). There is therefore strong selection against hybridization involving S or iS females and potentially mild selection against hybrids from N female crosses.

We assessed character displacement in morphology and mating call in the contact zone. A pattern of character displacement is a potential signature of reinforcement, although it could also be caused by other factors[4,13]. Morphology is not the direct determinant of mate choice in these frogs; however, it was assessed to test whether any divergence in this trait is linked to the mate choice trait (male call)[14,15] or to ecological divergence. As found for other species from the Wet Tropics with divergent phylogeographic lineages[16], there was no difference between N and S body size or shape away from the contact for either sex (Supplementary Information). Further, across the contact zone, there was no difference between N, S and iS for female size and male shape (Supplementary Information). The only morphological difference detected was a 20% reduction in mean body size of iS males in contact B (Fig. 2). Like most frogs[15], mate choice in *L. genimaculata* is mediated primarily by female choice of a single trait—male call. This makes frogs one of the best groups for studying reinforcement[14]. An analysis of field recordings across the contact region revealed that the N, S and iS male calls all differ significantly from one another (Fig. 3). The iS males have diverged in size and call from all other S males, whereas the S males at contact A do not differ from those outside the contact region (Supplementary Information and Supplementary Figs 3, 4).

If reinforcement is operating in this system, we can make two predictions about the impact of call divergence on mate choice. First, southern females should choose correctly more often than northern females because the cost of hybridization is greater for them, and second, the greater divergence in call of iS males should result in stronger assortative mating at contact B than at contact A. Consistent with these predictions, female two-choice trials revealed the following: first, females of the southern lineage (iS and S) were significantly better at choosing their own lineage than northern females (Fig. 4), and second, there was significantly stronger premating isolation at contact B than at contact A, to the point of highly significant positive assortative mating between the lineages at contact B (Fig. 4). The genetic results support these patterns in that there are significantly fewer hybrids at contact B (0–1.4%) than at contact A (3.1–6.8%), and none with S mtDNA. The almost complete reproductive isolation at contact B leads us to conclude that the two lineages have speciated at contact B but not at contact A.

Above, we have satisfied four of Howard's[4] five criteria for demonstrating reinforcement, and furthermore we have shown that this process has resulted in speciation: hybridization occurs in the field; there is strong postzygotic selection against hybrids; mate choice in this system is determined primarily by a single genetically determined character, frog call[15]; and divergence in this character at contact B has enhanced premating isolation between the two lineages to the point of significant reproductive isolation. The final requirement is to disentangle the effects of ecology on trait divergence[4,13]. Divergence in body size and call of the iS males does not seem to relate to ecological factors for the following reasons: first, females do not show size divergence across the contact region, and there is no divergence in male shape, as might be expected for ecologically driven divergence; and second, the distributions of N, S and iS do not show any relationship to habitat characteristics (for vegetation,

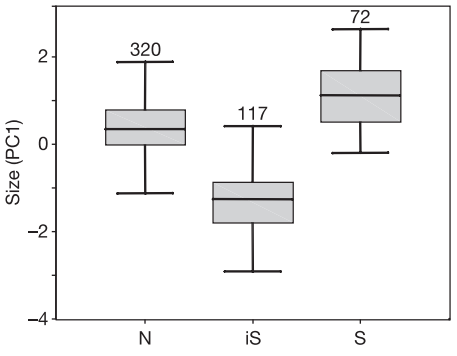

Figure 2 | Divergence in male body size across the mosaic contact zone. The box plots show the median, 25th and 75th quartiles, and minimum and maximum data of the first principal component (PC1). Numbers shown above the box plots indicate the number of individuals. PC1 represents body size and explains 90% of the variation between N, iS and S. Male size differs significantly (ANOVA on PC1, $F_{2,24} = 35.42$, $P < 0.001$) because iS males are significantly smaller than both N males (contrast, $F_{1,16} = 20.63$, $P < 0.001$) and S males (contrast, $F_{1,16} = 18.58$, $P < 0.001$).

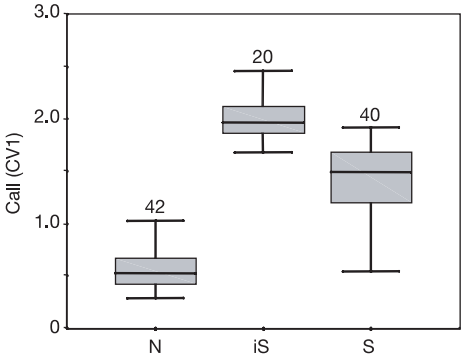

Figure 3 | Divergence in call across the mosaic contact zone. The box plots show the median, 25th and 75th quartiles, and minimum and maximum data of the first canonical variate (CV1). Numbers shown above the box plots indicate the number of individuals. CV1 explains 93.8% of call variation between N, iS and S. Call differs significantly between the groups (MANOVA on three call characters, $F_{6,12} = 11.28$, $P < 0.001$), with the S call differing significantly from the N call (contrast, $F_{3,6} = 12.80$, $P = 0.005$), and the iS call differing significantly from both the N (contrast, $F_{3,6} = 34.67$, $P < 0.001$) and the S calls (contrast, $F_{3,6} = 9.29$, $P = 0.011$).

LI = 7.77, $P = 0.133$; for stream substrate, LI = 1.49, $P = 0.837$; for stream flow, LI = 0.41, $P = 0.888$).

The call divergence of iS, resulting in speciation from N at contact B, has incidentally led to significant divergence of the iS call from that of the remainder of the southern lineage (Fig. 3 and Supplementary Fig. 4). To test whether this call divergence has resulted in premating isolation, mate choice trials were conducted in which iS and S females could choose between an iS and an S male call. There was complete premating isolation—in all experiments both iS and S females chose the male call of their own group (likelihood ratio test, LI = 19.12, $P < 0.001$). This allopatric speciation within the southern lineage seems to have occurred rapidly because there is very low sequence divergence between iS and S and they share haplotypes (Fig. 1). This demonstrates a twofold role of natural selection in speciation: natural selection acting directly against hybridization in a contact zone can also indirectly drive rapid allopatric speciation. This significantly extends the role of reinforcement from previous work[5,6] by showing, unconfounded by ecology and intervening populations, speciation of a contact zone population, both from another lineage and from its own lineage, as a result of reinforcement of a mate choice trait. Reinforcement has been viewed to have a role only in the final stages of speciation between already differentiated lineages[1,17], whereas the present results show the potential for reinforcement to be the sole cause of speciation.

Questions arising from this research include the following. First, why are iS males so small, given the lack of morphological divergence in the iS females? We propose that the reduction in male size might have been driven by sexual selection on iS male call. Because of a strong relationship between call divergence and body size in iS (Supplementary Information, and Supplementary Fig. 5), the more divergent the iS male call chosen by the females is from the other groups, the smaller the selected male is. Second, given that the postmating penalty for hybridization by the southern lineage is the same at both contacts, why has reinforcement been effective at contact B but not at contact A? The primary difference between contacts A and B is the range size of the southern lineage parental population—at contact A it is many times greater than the contact zone, whereas at contact B it is of smaller width than the contact zone. Our data are consistent with the suggestion[4,18,19] that if the parental population area is sufficiently small relative to both the area of overlap and the dispersal distance (not measured in this study), the diluting effects of gene flow from the parental population would be limited[20–22], and enough of the population may be exposed to maladaptive hybridization that a reinforced trait could spread throughout[1,2,23], resulting in speciation. The prevalence of such speciation in nature may be limited by the likelihood of extinction of the small population on initial contact[17].

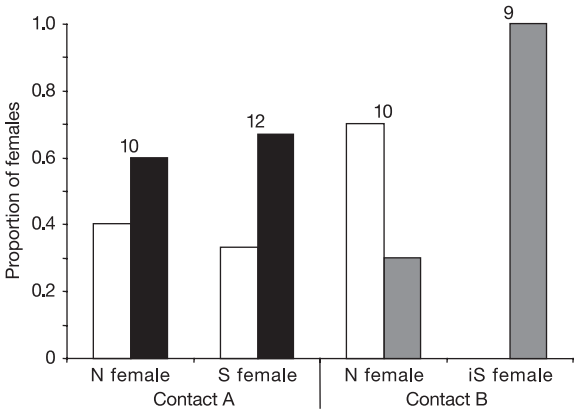

Figure 4 | Female choice of male calls at contacts A and B. Females of the southern lineage (iS and S) displayed significantly better choice, corrected for the effect of contact, than N females (generalized linear model, change in deviance $\Delta_{dev} = 4.33$, degrees of freedom, d.f. = 1, $P = 0.037$). Further, premating isolation, corrected for lineage, was significantly stronger at contact B than at contact A (generalized linear model, $\Delta_{dev} = 5.42$, d.f. = 1, $P = 0.020$). There is significant premating isolation between the northern and southern lineages at contact B (likelihood ratio test, LI = 12.79, $P = 0.003$) but not at contact A (likelihood ratio test, LI = 0.105, $P = 1.000$). White columns, N male; black columns, S male; grey columns, iS male. Numbers shown indicate the number of females.

METHODS

For full details of all methods see Supplementary Information.

Sampling and genetic analyses. Sampling sites are presented in Supplementary Figs 1 and 2. Sampling at contacts A and B consisted of stream transects and scattered sites covering the breadth of the zone. Genetic material was taken from toe-pads. The tree was constructed from 527 base pairs of *CO1* mtDNA (primers Cox and Coy[11]) from 141 individuals (58 N, 66 S and 17 iS) from across the range, by using neighbour-joining with Kimura two-parameter distance estimates. *CO1* (sequencing and restriction-fragment-length polymorphism test) was used to screen 787 individuals from 68 sites across 40 drainages. The contact zone was characterized by using *CO1* and two nuclear markers.

Two near-diagnostic nuclear loci, LITGEN02 and CRYBA[24], were screened across 619 individuals: 63 N and 80 S (6 drainages in each) from outside of the contact region, and 248 (13 sites, 4 drainages) and 228 (10 sites, 4 drainages) from contacts A and B, respectively. The status of individuals was inferred by using a combination of mtDNA and bayesian inference (using 'NewHybrid'[25]) of nuclear genotypes. We present percentage hybrids from both a stringent criterion, P (mismatch of nuclear genotype to mitochondrial genotype) >0.9, and a more relaxed criterion, $0.7 > P > 0.5$, for identifying potential hybrids. Both criteria gave similar results.

Statistical analyses: general comments. All analyses of morphology and call were nested by drainage. The planned, non-orthogonal contrasts—N versus S, N versus iS, and S versus iS—were used for the analyses of morphology and calls. Significance values were compared with sequential Bonferroni values[26]. All two-way contingency tables were analysed as tests of independence[26]. Given the relatively small expected frequencies in some cells, exact tests (likelihood ratio tests) were performed[26,27].

Experimental crosses. Males and gravid females were collected from the field and paired in the lab until eggs were laid. Once the hatchlings of a clutch reached stage 23 (ref. 28), two replicates of ten tadpoles each were placed in random positions beneath ultraviolet lamps and raised on lettuce. The difference in viability of inter-lineage crosses involving N ($n = 8$) and S/iS ($n = 6/1$) females was analysed with a likelihood ratio test. An ANOVA was used to test the difference in development rate (time until hindlimb buds, stage 26 (ref. 28)) between the N female hybrid clutches ($n = 8$) and the N lineage control clutches ($n = 4$).

Morphological analyses. Measurements taken were snout-to-vent length (SVL), tarsus length, head width, and weight. A total of 924 mature males (34 drainages) and 153 mature females (23 drainages) were measured. To remove confounding effects of altitude, analyses of covariance (ANCOVAs) were performed on each of the morphological traits, followed by linear regressions from which the unstandardized residuals were taken. Principal-component analyses (PCAs) were conducted because the traits were all highly correlated. The first principal component (PC1) was used as the measure of body size in nested ANOVAs. The remaining PCs were used in nested multivariate analyses of variance (MANOVAs) to assess differences in body shape. To maximize drainage coverage for the analyses of size divergence in females across the contact region and males across the S lineage, only SVL was used in nested ANOVAs; shape was not assessed.

Call analyses. Call duration, dominant frequency and note rate were measured in four calls in each of 132 males (19 drainages). To remove confounding effects of air temperature, ANCOVAs were performed on the three call characters, followed by linear regressions from which the unstandardized residuals were taken. Nested MANOVAs were run on the three call characters, followed by multivariate contrasts. Nested canonical variates (CVs) were calculated for presentation and analysis of call divergence against body size.

Mate choice. Gravid females were presented with two field-recorded calls in a laboratory mate-choice chamber. Females from contact A ($n = 22$) were presented with an N and an S call, and those from contact B ($n = 19$) were presented with an N and an iS call. After a 5-min listening period, a 10-min trial was conducted. The trials to assess premating isolation between S ($n = 8$ females) and iS ($n = 6$ females) were performed in the same manner except that some of the females and male calls came from outside the contact region.

The difference in mate choice between the southern (S and iS) and northern (N) lineages, and the difference in premating isolation at contacts A and B, were compared by using a generalized linear model with a binomial error specified. The level of premating isolation at each contact, and premating isolation between S and iS were analysed with likelihood ratio tests.

Habitat association. Three broad habitat characteristics (stream-side vegetation, stream substrate and stream flow) were classified into categories at each site ($n = 46$) in the contact region. The association between N, S and iS and each of the three habitat characteristics was assessed by using a likelihood ratio test.

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