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Faster evolution of a premating reproductive barrier is not associated with faster speciation rates in New World passerine birds

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Why are speciation rates so variable across the tree of life? One hypothesis is that this variation is explained by how rapidly reproductive barriers evolve. We tested this hypothesis by conducting a comparative study of the evolution of bird song, a premating barrier to reproduction. Speciation in birds is typically initiated when geographically isolated (allopatric) populations evolve reproductive barriers. We measured the strength of song as a premating barrier between closely related allopatric populations by conducting 2339 field experiments to measure song discrimination for 175 taxon pairs of allopatric or parapatric New World passerine birds, and estimated recent speciation rates from molecular phylogenies. We found evidence that song discrimination is indeed an important reproductive barrier: taxon pairs with high song discrimination in allopatry did not regularly interbreed in parapatry. However, evolutionary rates of song discrimination were not associated with recent speciation rates. Evolutionary rates of song discrimination were also unrelated to latitude or elevation, but species with innate song (suboscines) evolved song discrimination much faster than species with learned song (oscines). We conclude that song is a key premating reproductive barrier in birds, but faster evolution of this reproductive barrier between populations does not consistently result in faster diversification between species.

1. Introduction

How fast new species form varies tremendously within larger taxonomic groups [1]. For example, speciation rates, defined as the splitting rate on a phylogenetic tree, vary over 30-fold within New World birds [2]. One intuitive expectation is that speciation rates are faster when populations more rapidly evolve reproductive barriers. After all, speciation is often defined as the evolution of reproductive isolation between related populations [3,4]. However, the sole empirical study testing this 'reproductive isolation' hypothesis found no association between speciation rates and evolutionary rates of reproductive barriers in two taxonomic groups: fruit flies, where premating and postmating barriers were estimated using laboratory experiments, and birds, where postmating barriers were estimated using data on crosses, predominately from aviaries and zoos [5]. For these taxa, the rate of evolution of the measured reproductive isolation between populations over shorter timescales apparently fails to leave an imprint on speciation rates measured over longer timescales. Alternatives to the reproductive isolation hypothesis are that speciation rates are largely idiosyncratic, or set by demographic and ecological factors that govern rates of population formation, population persistence and geographic range expansion [6–8]. For example,

speciation rates are positively correlated with rates of population differentiation in New World birds [9], and speciation in Himalayan birds appears to be limited by rates of population persistence and range expansion [10].

Here, we test these contrasting hypotheses by measuring the association between evolutionary rates of song divergence and recent speciation rates in New World passerine birds. Song is traditionally considered to be a potent premating reproductive barrier in passerine birds. Birds use song to attract and choose mates, and a common observation is that when closely related species are sympatric, they differ in song (and sometimes little else) [11–15]. However, this perspective has been challenged by recent work suggesting premating barriers such as song have relatively little impact on speciation [16]. We focus on how song diverges between geographically isolated (allopatric) populations because speciation in birds is typically initiated in allopatry [17,18], and divergence in song between allopatric populations is thought to constitute a barrier to reproduction that promotes speciation [19–21]. The reproductive isolation hypothesis predicts that evolutionary rates of song divergence between allopatric populations are positively associated with speciation rates; other hypotheses predict no such association.

We tested these predictions by conducting 2339 field playback experiments to measure song divergence for 175 taxon pairs. Each taxon pair consisted of a pair of related allopatric or parapatric populations; playback experiments always occurred in the allopatric portion of population's ranges. We quantified song divergence as song discrimination, measured as the degree to which birds behaviourally discriminated against allopatric song relative to local song. In some taxon pairs, birds responded just as strongly to allopatric song as to local song. In others, birds ignored allopatric song while responding strongly to local song. We assumed that song constitutes a strong reproductive barrier for taxon pairs where territorial individuals completely ignored allopatric song. To test this assumption, we focused on 26 parapatric taxon pairs for which we have information on their propensity to interbreed in parapatry. We used these cases to test whether response to simulated secondary contact—behavioural response to playback experiments, which took place in allopatry—was correlated with observations of mate choice decisions in real secondary contact. Our study thus uses empirical data to test the hypothesis that the faster evolution of a premating reproductive barrier is associated with faster recent speciation rates, while simultaneously examining the often-held but seldom-tested assumption that song divergence in geographic isolation constitutes a reproductive barrier in birds.

2. Methods

(a) Taxon pair selection

We studied 175 taxon pairs of New World passerines. Most taxon pairs were entirely allopatric ($N = 131$), with a smaller number that were mostly allopatric but parapatric in a portion of their range ($N = 44$). All experiments were conducted in allopatric regions of species' ranges. We studied pairs of populations without regard to their taxonomic status. Taxon pairs included allopatric populations ranked as subspecies ($N = 89$) and as species ($N = 86$; typically sister species), and came from both major clades of passerines: oscines, which learn their song at young ages from listening and copying adult birds ($N = 108$), and suboscines, which have innate, genetically controlled song ($N = 67$) [22].

(b) Field experiments

We conducted 2339 experiments (mean of 13.4 experiments per taxon pair, range = 4 to 35) to measure bird behaviour in response to two treatments: (i) song from the local population (sympatric treatment) and (ii) song from the related, allopatric population (allopatric treatment). We conducted fieldwork at multiple sites within four regions: (i) the Pacific Northwest (British Columbia and Washington state: 430 experiments on 32 taxon pairs); (ii) southwestern United States (southeastern Arizona: 93 experiments on 11 taxon pairs; southern Texas: 22 experiments on four taxon pairs: and southern California: 17 experiments on three taxon pairs); (iii) southern Central America (Costa Rica and western Panama: 785 experiments on 59 taxon pairs); and (iv) the tropical Andes (Ecuador and northern Peru: 1002 experiments on 83 taxon pairs). Some taxon pairs were studied in multiple regions. Consequently, the number of taxon pairs described above for different regions sums to more than the total number of taxon pairs in this study.

Prior to fieldwork, we downloaded multiple high-quality natural vocalizations for each population for each taxon pair from the online archives of xeno-canto.org and the Macaulay Library of Natural Sounds (mean unique recordings used per population = 6.8; interquartile range = 5–8). In the field, we searched for individuals or pairs of one of our taxon pairs, then initiated an experiment when we detected one or more individuals. We synced a wireless speaker (UE Roll or JBL Charge 2+) to a smartphone with Bluetooth, placed the speaker at least 15 m from the nearest bird, retreated to approximately 10 m from the speaker and began the first treatment by broadcasting a song at a natural volume (approx. 80 dB) for 2 min. We alternated the order of the first treatment in an experiment (sympatric or allopatric) between experiments. We then observed behavioural responses during these 2 min and over a subsequent 5 min observation periods. The key behavioural response we measured was the distance of the bird's closest approach to the speaker, quantified as the minimum distance in the horizontal plane from the bird, whether perched or in flight, to the speaker. We stopped playback if the bird(s) approached within 5 m of the speaker during the initial 2 min of song playback, as we considered this to represent an obvious strong approach response. At the end of the observation period, birds had typically left the area (greater than 15 m from the speaker) and ceased vocalizing at an elevated rate. If not, we waited to start the second treatment until birds were greater than 15 m from the speaker and had returned to pre-playback vocal activity. In this experimental design, the sympatric treatment is a positive control, and we therefore included only experiments in which birds approached to within 15 m of the speaker in the sympatric treatment, as we assume a bird defending a territory *should* approach the speaker in response to playback of local conspecific song. Indeed, the mean closest approach in response to sympatric song was 4 m.

For each taxon pair, we quantified song discrimination as the proportion of tested territories where birds ignored allopatric song. We defined 'ignoring' allopatric song as failing to approach within 15 m of the speaker in response to allopatric song. Responses to allopatric song were symmetric within a taxon pair (see electronic supplementary material, figure S1; Pearson's $r = 0.90$; d.f. = 34, $t = 11.93$, $p < 0.0001$ for 36 taxon pairs with five or more experiments for each population), so we included all experiments performed on both populations when calculating the taxon pair's song discrimination score. Results were unchanged when using an alternative method that uses continuous data (closest approach measured in metres) to calculate song discrimination [23]. Both approaches have potential weaknesses: the categorical metric we employ may lead to false positives when birds approach the speaker out of curiosity, while the continuous metric can lead to exaggerated estimates of song discrimination (e.g. a bird that approaches local song

to 1 m and foreign song to 2 m is scored as showing substantial song discrimination).

Our experiments measured the response of territorial birds, typically mated pairs or males alone, to allopatric song. We assume that a species that consistently ignores a signal in a territorial context will also ignore that signal in a mate choice context, as selection should be stronger on mate choice decisions than on territorial defense decisions. Empirical evidence across the bird tree of life supports this assumption, showing that birds are indeed 'pickier' about the signals they respond to in a mate choice context. That is, a broad range of signals can elicit a territorial defense response but a narrow range of signals are accepted by individuals choosing mates (reviewed by [24]).

(c) Genetic divergence

We calculated genetic divergence for all taxon pairs using homologous sequences of mitochondrial genes downloaded from GenBank (typically cytochrome b or ND2) and quantified mtDNA divergence as uncorrected p-distances. Mitochondrial DNA divergence evolves in a roughly clock-like fashion in birds, with approximately 2% divergence per million years in isolation [25]. Here, we follow many previous comparative studies by using mitochondrial divergence as a proxy for the amount of time since the two populations in a taxon pair last shared a common ancestor.

(d) Statistical analysis

We followed previous studies and modelled the evolution of song discrimination by fitting Michaelis–Menten models [23,26]. We fit the Michaelis–Menten model used by Weir & Price [23] where song discrimination = genetic distance/(β + genetic distance). In this formulation, β is the value of genetic distance at which song discrimination reaches 0.5, meaning that half of territorial birds ignore song from their allopatric relative. Larger values of β indicate slower evolution of song discrimination; we thus term β the 'song discrimination waiting time'. We tested our hypotheses by fitting a model that included the variable of interest (e.g. recent speciation rate) as a modifier to β, and comparing the fit of this full model with a reduced model that did not include the variable of interest using F tests (the 'anova' function in R). All statistics were conducted in R [27].

First, we tested the assumption that song discrimination in allopatry indicates song is a reproductive barrier. If so, taxon pairs with strong song discrimination in allopatry should fail to regularly interbreed in parapatry. We tested this prediction using the parapatric taxon pairs; we inspected the primary literature and reference volumes [28], finding field data on patterns of interbreeding for 26 of the parapatric taxon pairs. We compared the fit of a full model that included regular interbreeding as a binary predictor variable to a reduced model that did not include this covariate.

Second, we tested whether evolutionary rates of song discrimination between populations in passerine birds are associated with macroevolutionary rates. Oscines and suboscines are sister clades. But despite their equivalent evolutionary age, oscines have nearly four times more species than suboscines. We tested whether evolutionary rates of song discrimination were associated with this diversity disparity by comparing model fit between a full model with clade (oscine versus suboscine) as a predictor variable with a reduced model that did not include clade.

Third, we tested whether evolutionary rates of song discrimination were associated with recent speciation rates. We estimated recent speciation rates using the diversification rate statistic, which infers speciation rates for each tip of the phylogeny based on the distribution of nodes and branch lengths leading to it [29,30], and is more reflective of recent speciation than long-term diversification (speciation minus extinction). We computed recent speciation rates for taxon pairs using species-level molecular phylogenies. For suboscines, we used the recently published suboscine tree [31], for oscines, we used the Emberizoidea tree [32], and, for the non-emberizoid passerines, we used a consensus tree built using TreeAnnotator [33] from a pseudo-posterior distribution of 10 000 phylogenies downloaded from birdtree.org [29]. For taxon pairs classified as two distinct species, we used the average diversification rate statistic of the two species as our estimate of recent speciation rate for the taxon pair. We tested the importance of recent speciation rate by comparing model fit between a full model with clade (oscine versus suboscine) and logged recent speciation rate as predictor variables with a reduced model that did not include recent speciation rate. Related taxon pairs in our dataset may have similar recent speciation rates because they share many branches in the tree. We therefore also analysed our data with models that incorporated phylogenetic branch length information (see electronic supplementary material).

Last, we tested whether latitude and elevational zone were linked to song evolution and recent speciation rates, as both song evolution [23,34] and species formation [35] have been reported to occur at faster rates in high latitudes in New World birds than in the tropics. We coded latitudinal zone as temperate ($N = 47$) or tropical ($N = 128$) based on the locations where playback experiments were conducted. For the elevational zone, we restricted our analysis to 110 tropical taxon pairs that reside in humid forest and categorized each of these taxon pairs as lowland (midpoint elevation 0–1000 m), mid-mountain (midpoint elevation 1000–2000 m) or high elevation (midpoint elevation greater than 2000 m) using a reference volume [36]. We tested the relationship between geography and the evolution of song discrimination by fitting full models that included clade and the geographic variable (latitudinal zone or elevational zone) as predictor variables to reduced models that lacked the geographic variable. In addition, we tested whether recent speciation rates differed between latitudinal zones using a t-test, and between elevational zones by fitting a linear model with elevational zone (lowland, mid-mountain or high mountain) as a predictor variable.

3. Results

(a) Song discrimination is a metric of reproductive isolation

Taxon pairs that show strong song discrimination in allopatry tend not to interbreed routinely with one another in parapatry (t-test; d.f. = 7.85, $t = 3.89$, $p = 0.0050$, figure 1a). This result holds when taking evolutionary age into account (F test; d.f. = 1, $F = 10.53$, $p = 0.0034$, figure 1b).

(b) Song discrimination evolution is faster in suboscines

Suboscines have faster evolutionary rates of song discrimination than do oscine taxon pairs (figure 2). A full model that included clade (oscine/suboscine) as a predictor variable was a better fit than a reduced model that lacked clade (F-test; d.f. = 1, $F = 34.41$, $p < 0.0000001$). We estimate that suboscine taxon pairs evolve song discrimination three times faster than oscines (song discrimination waiting times of 2.7 ± 0.40 and 8.4 ± 1.1, respectively).

(c) Song discrimination evolution is not associated with speciation rate

Evolutionary rates of song discrimination are not statistically associated with higher recent speciation rates (figure 3).
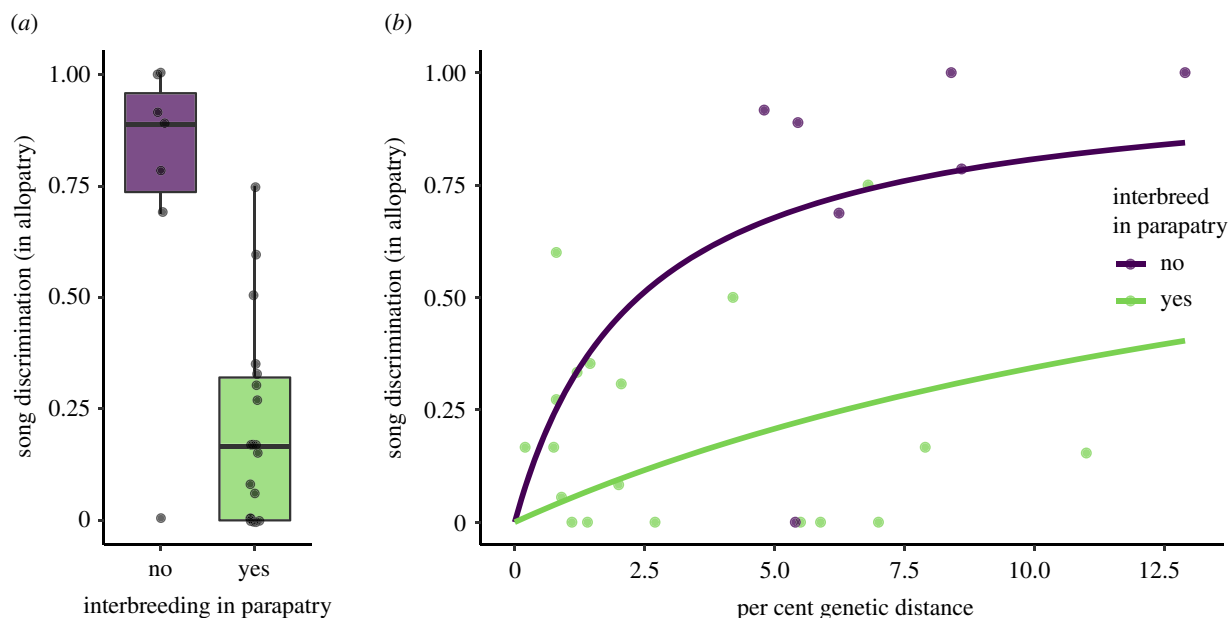
Figure 1. Greater song discrimination in allopatry is associated with reduced interbreeding in parapatry. This analysis is based on 26 taxon pairs that have narrow zones of parapatry; all playback experiments were conducted in the allopatric portion of the range. Song discrimination in allopatry is lower in taxon pairs that routinely interbreed with one another in parapatry ((a); d.f. = 7.85, t = 3.89, p = 0.0050). This result holds when accounting for the fact that taxon pairs with strong song discrimination are older and have larger genetic distances (b). Predictions from the full Michaelis–Menten that included regular interbreeding in parapatry are plotted as trendlines in (b); raw data are shown as points. This full model was a much better fit compared to a reduced model that did not include regular interbreeding in parapatry as a covariate (F test; d.f. = 1, F = 10.48, p = 0.0036). (Online version in colour.)
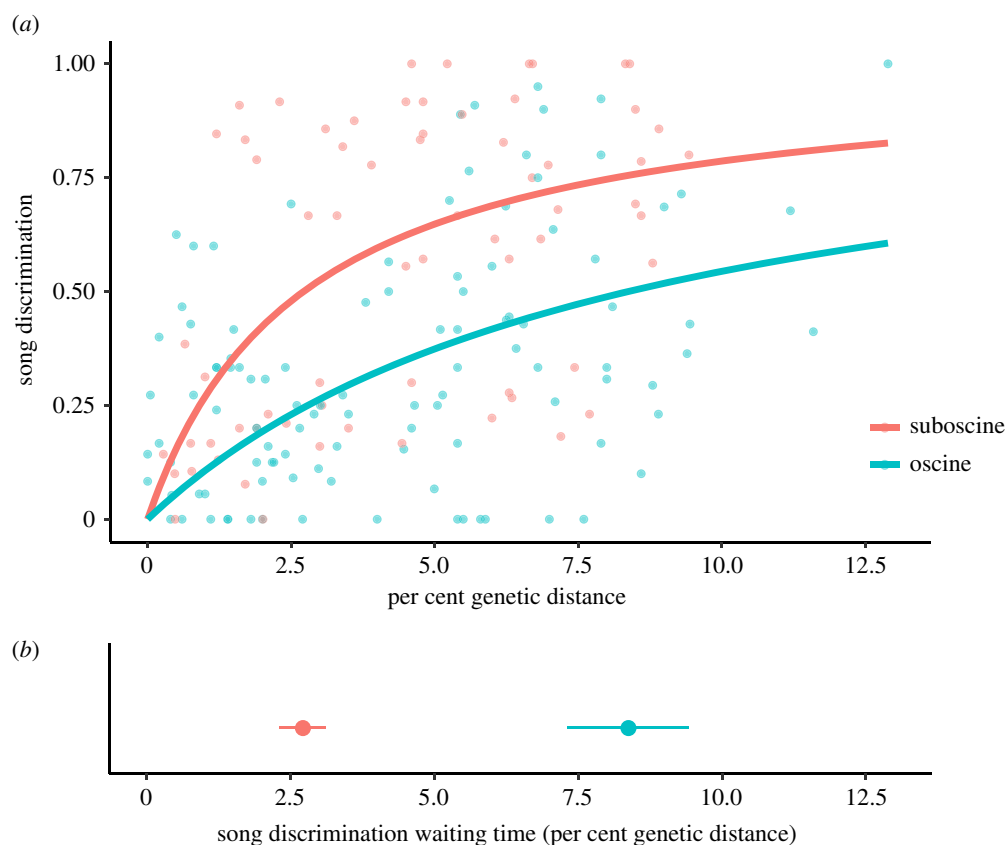

Figure 2. The evolution of song discrimination is faster in taxon pairs with innate song (suboscines) than taxon pairs with learned songs (oscines). Predictions from the full Michaelis–Menten that included clade (oscine versus suboscine) are plotted as trendlines in (a); raw data are shown as points. This full model was a much better fit compared to a reduced model that did not include clade information (F test; d.f. = 1, F = 34.41, p < 0.0000001). The song discrimination waiting time is 3.1 times smaller for suboscines (2.7 ± 0.40) than for oscines (8.4 ± 1.1; (b)); this measures the genetic distance at which half of territories ignore allopatric song. (Online version in colour.)

A full model that included recent speciation rate and clade as a predictor variable was not a better fit than a reduced model that lacked recent speciation rate (F-test; d.f. = 1, F = 0.57, p = 0.45). We recovered this same result in phylogenetic models (see electronic supplementary material). We visualize this lack-of-association by fitting a model in which we coded
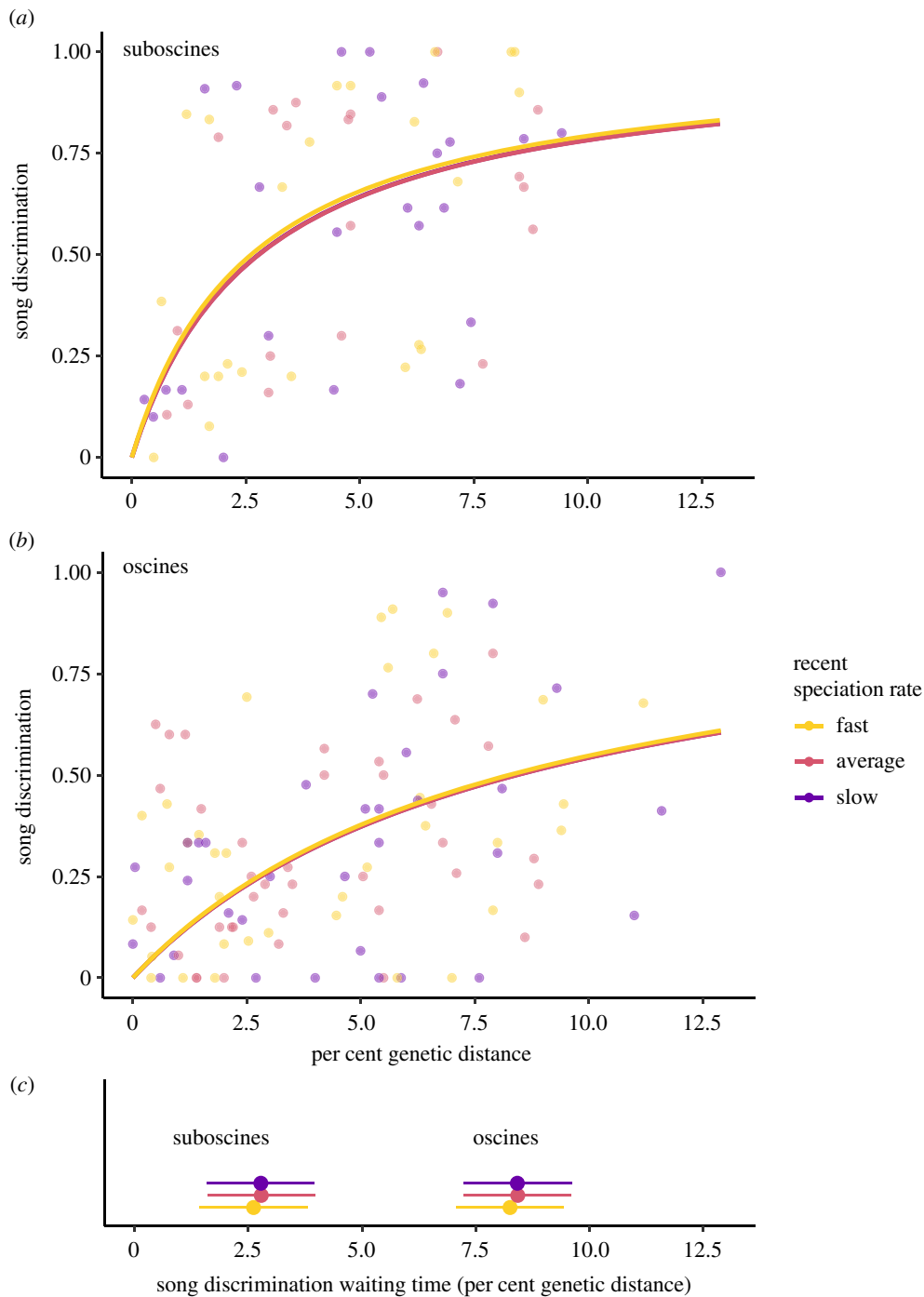
Figure 3. Evolutionary rates of song discrimination are not correlated with recent speciation rates. A full model that included log-transformed recent speciation rate as a continuous variable was not a better fit compared to a reduced model that did not include recent speciation rate (F-test; d.f. = 1, F = 0.57, p = 0.45). Relationships are visualized using a model where recent speciation rate was coded as a categorical variable with three levels (slow, average and fast; each level contained one-third of data), with predictions plotted as trendlines and raw data are shown as points. The song discrimination waiting time is similar between taxon pairs with slow and fast recent speciation rates (c); this measures the genetic distance at which half of territories ignore allopatric song. (Online version in colour.)

recent speciation rate as a categorical variable with three levels (slow, average and fast; each level contained one-third of the data; figure 3). Oscines and suboscines in this dataset do not consistently differ in their recent speciation rates (mean values = 0.28 and 0.27 species produced per million years, respectively; t-test; d.f. = 166.4, t = −0.27, p = 0.79).

(d) Song discrimination evolution is not explained by geography

Song discrimination evolves at similar rates in the tropics and the temperate zone (figure 4; F test; d.f. = 1, F = 0.015, p = 0.70) and at similar rates across different elevational zones within

the tropics (electronic supplementary material, figure S2). In our dataset, recent speciation rates were unrelated to latitudinal zone (d.f. = 146.4, t = 0.37, p = 0.71) or elevation (estimate and standard error for elevational zone in a univariate linear model = −0.0081 ± 0.022; t = −0.37, p = 0.71).

4. Discussion

A recent review declared 'The connection between metrics of diversification and reproductive isolation remains in its infancy, but it is one of the most urgent questions in speciation biology' [37]. Here, we addressed this urgent question by conducting thousands of field experiments to measure the strength of a
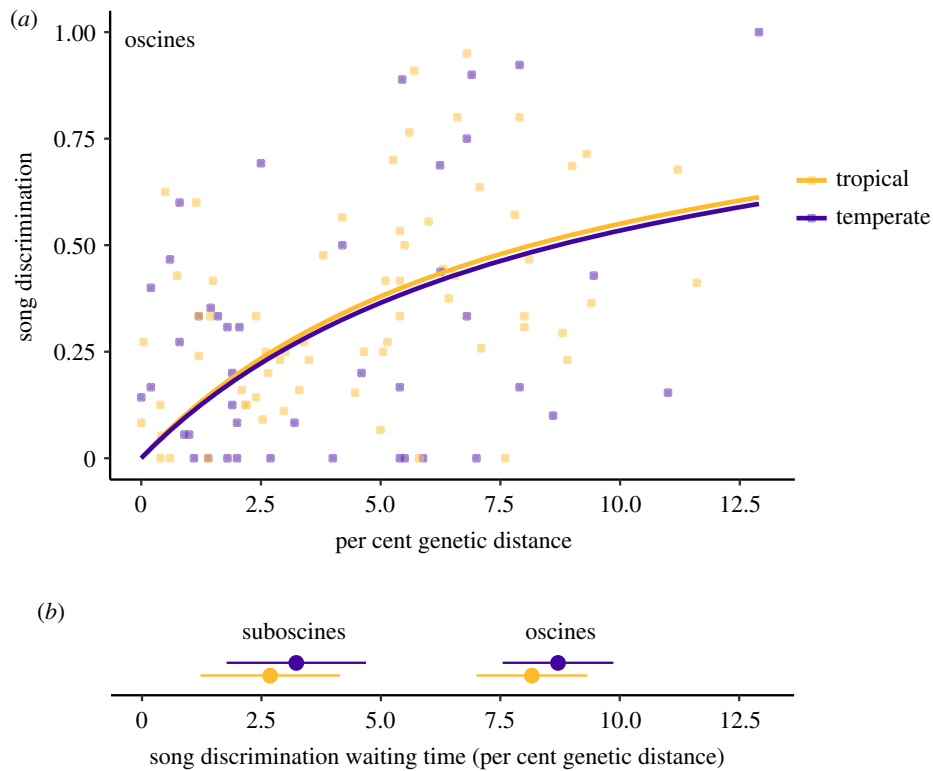
Figure 4. Evolutionary rates of song discrimination are similar between the tropics and temperate zone; data are shown only for oscines (*a*) because nearly all suboscines are tropical. A full model that included latitudinal zone was not a better fit compared to a reduced model that did not include latitudinal zone (*F* test; d.f. = 1, *F* = 0.015, *p* = 0.70). Predictions from the full Michaelis–Menten model are plotted as trendlines; raw data are shown as points. Song discrimination waiting times were similar for tropical and temperate taxon pairs for both suboscines and oscines (*b*). (Online version in colour.)

premating barrier—bird song—in hundreds of comparisons of passerine birds. We provide evidence that the song discrimination we experimentally measured in the field does indeed measure the strength of a reproductive barrier. However, faster evolutionary rates of song discrimination were not associated with faster recent speciation rates. That is, rates of evolution of a barrier to gene flow between populations are not consistently linked to rates of speciation.

(a) Song is a reproductive barrier in passerine birds
We find that the evolution of strong song discrimination between allopatric populations constitutes a barrier to reproduction. Taxon pairs that ignore allopatric song do not regularly interbreed in parapatry, while those that respond to allopatric song do regularly interbreed in parapatry. That is, behavioural responses to simulated secondary contact in our playback experiments align with observed mating patterns in real-world secondary contact. This constitutes comparative evidence that song is a potent reproductive barrier in birds, and supports our assumption that the song discrimination we measured quantifies the strength of a reproductive barrier. These conclusions can be tested independently as follows. We measured song discrimination in allopatry for 16 taxon pairs that occur in parapatry, but it is unknown whether they interbreed in parapatry. We use the results of our playback experiments to generate testable predictions for each of these 16 cases (electronic supplementary material, table S1).

(b) Faster evolution of song discrimination is not associated with faster macroevolutionary rates
We found no association between evolutionary rates of song discrimination and recent speciation rates. Why might faster

evolution of a reproductive barrier *not* lead to faster recent speciation rates? There are at least three possible explanations. First, evolutionary rates of reproductive isolation may indeed be associated with speciation rates, but this association is only detectable when all barriers that contribute to reproductive isolation are measured, for example, postmating barriers that are important in avian speciation [16,38]. However, we found evidence that the single barrier we studied—birdsong—constitutes a strong reproductive barrier, suggesting that our failure to find an association between song discrimination and speciation rates will not be explained away by other measures. Second, rates of reproductive isolation may be evolutionarily labile. If rates of reproductive isolation are highly variable within clades, they may not predict much about local clade diversity. Third, speciation rates may truly be unrelated to the rate of evolution of reproductive isolation; speciation rates may be better explained by demographic and ecological factors that set rates of population formation, population persistence or range expansion [6–8]. If so, speciation rates reflect the large variety of processes that determine when new populations form, and if those populations persist long enough to evolve reproductive isolation. This accumulation of events may be idiosyncratic, related to factors including geography, population dynamics, traits of species and levels of genetic variation.

(c) Birds with innate song (not song learners) have faster evolutionary rates of song discrimination
A longstanding hypothesis in birds is that song learning accelerates rates of song evolution, promoting more rapid speciation. This is a special case of the more general hypothesis that phenotypic plasticity leads to faster rates of evolution and

speciation [39]. Faster evolution of reproductive isolation in song learners is a classic explanation for why, in passerine birds, song learners (oscines) have nearly four times as many species as their suboscines (approx. 4700 versus approximately 1300) [40–42]. However, we reject this hypothesis for passerine birds in the Americas. We find that allopatric taxon pairs of suboscines (innate song) evolve song discrimination much faster than do allopatric taxon pairs of oscines (song learners). We previously reported the same result when analysing an earlier version of our song playback dataset that contained 69 taxon pairs, versus the current dataset of 175 taxon pairs. In our previous analysis, we found that song learners and species with innate song evolve divergence in acoustic traits at roughly similar rates. However, within-population variation in acoustic traits of songs was much greater for song learners than for species with innate song, as expected if plasticity increases trait variation [26]. We suggested that greater within-population variation in song leads to lower evolutionary rates of song discrimination because song learners can only discriminate against a foreign signal when it falls outside the relatively wide range of variation present within their population, an example where learning may impede rather than accelerate speciation.

(d) Geography does not drive song discrimination

Accumulating evidence suggests that evolutionary rates tend to be fastest in the temperate zone [43]. However, we do not recover this result in our dataset. Instead, we find similar evolutionary rates of song discrimination in the tropics and temperate zone, and also across different elevational zones in the tropics. This contrasts with a recent report that song discrimination evolves faster in temperate North America than in the Amazon basin [23]. There are at least two reasons that could explain these different results. First, our tropical field experiments did not include sites in the lowland Amazon basin, but instead took place in Central America and the tropical Andes; song discrimination may evolve particularly slowly in the Amazon. Second, differences in experimental design of field playbacks may impact estimates of song discrimination.

(e) Concluding remarks

Speciation is often defined as the evolution of reproductive isolation. Fast evolution of reproductive isolation underlies rapid speciation in adaptive radiations in taxa including three-spined sticklebacks [4], Lake Victoria cichlids [44] and Hawaiian crickets [45]. Here, we provide evidence that evolutionary rates of a premating barrier, song, fail to have cascading effects on speciation rates in New World passerine birds. It is perhaps noteworthy that the case examples cited above occur on recently formed lakes or oceanic islands, environments that may feature unusually high levels of ecological opportunity. In continental systems, it appears that faster rates of evolution of reproductive isolation do not leave a clear imprint on longer term macroevolutionary processes, though studies remain few [5,8]. Testing the reasons why metrics of diversification are only loosely connected with reproductive isolation remains an urgent question in speciation research.

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