

# Rapid evolution of sexual signals in sympatric *Calopteryx* damselflies: reinforcement or 'noisy-neighbour' ecological character displacement?

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## Abstract

Enhanced prezygotic isolation in sympatry is one of the most intriguing patterns in evolutionary biology and has frequently been interpreted as evidence for reinforcement. However, the frequency with which reinforcement actually completes speciation remains unclear. The Jewelwing damselflies (*Calopteryx aequabilis* and *C. maculata*) have served as one of the few classic examples of speciation via reinforcement outside of *Drosophila*. Although evidence for wing pattern displacement and increased mate discrimination in this system have been demonstrated, the degree of hybridization and gene flow in nature are unknown. Here, we show that sympatric populations of these two species are the result of recent secondary contact, as predicted under a model of speciation via reinforcement. However, we found no phenotypic evidence of hybridization in natural populations and a complete association between species-specific haplotypes at two different loci (mitochondrial CO I and nuclear EF1- $\alpha$ ), suggesting little or no contemporary gene flow. Moreover, genealogical and coalescent-based estimates of divergence times and migration rates indicate that, speciation occurred in the distant past. The rapid evolution of wing colour in sympatry is recent, therefore, relative to speciation and seems to be better explained by selection against wasting mating effort and/or interspecific aggression resulting from a 'noisy neighbour' signalling environment.

## Introduction

Reinforcement, as originally proposed by Dobzhansky (1937), is the increase in prezygotic reproductive isolation between recently diverged, hybridizing species via natural selection against the production of unfit offspring. This has been one of the most controversial ideas in evolutionary biology because of the lack of good examples from nature and prior views that it was theoretically improbable. Coyne and Orr's (1989, 1997)

striking finding, however, that prezygotic isolation is stronger among sympatric than allopatric taxa, in contrast to patterns of post-zygotic isolation, was compelling enough to rejuvenate interest in the process of reinforcement as a mechanism of speciation and more recent theoretical work has established that this process may operate under a much broader range of conditions than traditionally recognized (e.g. Liou & Price, 1994; Kelly & Noor, 1996; Servedio & Kirkpatrick, 1997; Kirkpatrick & Ravigné, 2002). In fact, there are now several convincing examples of reinforcement in nature (e.g. Noor, 1995; Saetre *et al.*, 1997; Nosil *et al.*, 2003; Pfenning, 2003; Hoskin *et al.*, 2005).

Although the renewed enthusiasm for reinforcement as a significant force in the origin of new species is clearly warranted, it remains important to distinguish between

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reinforcement as a mechanism of speciation and enhanced isolation between species that have already evolved complete barriers to gene exchange (Butlin, 1987a, b, 1989). In the former case, hybridization occurs between partially isolated populations and hybrids have nonzero fitness. Therefore, natural selection against hybrids (narrow sense reinforcement) or against interspecific matings (broad sense reinforcement) acts to reduce gene flow and increase mate discrimination (Servedio & Noor, 2003; Servedio, 2004). In the latter case, completely unfit hybrids might still be produced via the same process but the displacement of sexual signals has little to do with speciation as full post-zygotic reproductive isolation has already been achieved (Coyne & Orr, 2004).

Distinguishing between true reinforcement (*sensu* Coyne & Orr, 2004), which is a microevolutionary process that completes speciation, and the equivalent process operating on fully isolated taxa may initially appear arbitrary. However, this distinction is crucial if we are to answer the most important unresolved question about reinforcement; namely, has the process been a significant force in the origins of biological diversity throughout history (Servedio, 2004). Unfortunately, demonstrating that reinforcement has indeed led to the completion of reproductive isolation between species that currently show displaced characters is not trivial. First, reproductive character displacement is not necessarily an outcome of reinforcement (Lemmon *et al.*, 2004), and several other mechanisms can also lead to displaced sexual signals in sympatry (e.g. differential fusion or ecological character displacement, Templeton, 1981). Second, as mentioned above, enhancement of sexual isolation can occur when taxa that are fully isolated via intrinsic post-zygotic barriers subsequently come into secondary contact. Thus, although reproductive character displacement is common (Howard, 1993; Noor, 1999), it is impossible to directly ascribe this pattern to the process of reinforcement.

Consequently, without in-depth, systematic studies of putative examples of reinforcement it is not possible to determine what larger role this process has played in speciation in general. Although there is now substantial evidence that selection against unfit hybrids has played an important role during speciation events in *Drosophila* (Noor *et al.*, 2001), flycatchers (Saetre *et al.*, 1997) and sticklebacks (Rundle & Schuller, 1998; Albert & Schluter, 2004), many other putative examples of reinforcement have yet to be carefully investigated. In this study, we critically review one of the most classically cited examples of reinforcement outside of *Drosophila*; enhanced isolation among sympatric populations of Jewelwing damselflies (*Calopteryx*) (Waage, 1975, 1979; Howard, 1993; Futuyma, 1998; Noor, 1999).

Previous behavioural work on this system conclusively demonstrated that wing colouration is strongly displaced among sympatric female River (*Calopteryx aequabilis*) and

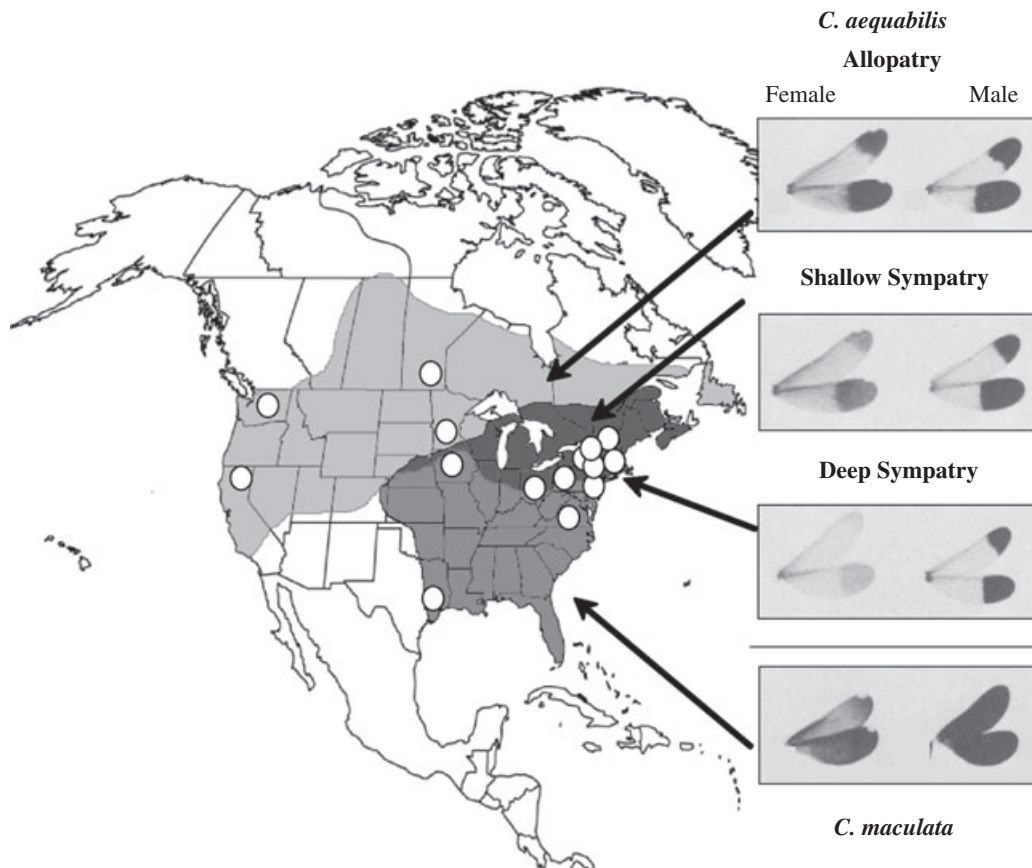
Ebony (*Calopteryx maculata*) Jewelwing damselflies. *Calopteryx maculata* has dark wings and shows little geographic variation in the wing pattern, whereas *C. aequabilis* displays paler wings and smaller spots in sympatry; a pattern that increases with the geographic depth of sympatry and the relative frequency of heterospecifics (Waage, 1975, 1979). Moreover, sympatric male Jewelwings are better able to discriminate between conspecific and heterospecific females (Waage, 1979), demonstrating that there is, indeed, enhanced isolation in sympatry. These results have led authors to portray this system in evolutionary biology textbooks as one of the few convincing examples of speciation via reinforcement of prezygotic isolation (e.g. Futuyma, 1998). However, without rigorous genetic studies alternative hypotheses are equally plausible.

Here, we show that sympatric populations of River and Ebony Jewelwings are the result of recent secondary contact; a scenario compatible with the speciation by reinforcement. However, if enhanced isolation and displaced sexual signals in this system are the result of narrow sense reinforcement, then we expect to find evidence of gene flow in the form of introgression. In contrast, we found no phenotypic evidence of hybridization in natural populations and a complete association between species-specific haplotypes at two different loci. Furthermore, our coalescent-based estimates of migration are consistent with little or no contemporary gene flow between these two species, and both genealogical and coalescent-based estimates of divergence times indicate that these damselflies are deeply divergent and not sister taxa. Taken together, these results suggest that speciation in this system occurred in the distant past and that the wing pattern displacement is recent relative to species divergence.

## Material and methods

### Taxon sampling

Specimens of all but one North American *Calopteryx* species and two European species, *Calopteryx virgo* and *Calopteryx splendens*, were collected to investigate the evolutionary relationships of our focal species with the Nearctic representatives of this damselfly genus. We sampled adult *C. aequabilis* and *C. maculata* from both allopatric and sympatric populations, spanning their respective geographic ranges (Fig. 1, Table 1) and generated mitochondrial sequence data for 131 individuals including; 27 *C. aequabilis* from sympatric populations, 24 *C. aequabilis* from allopatric populations, 50 *C. maculata* from sympatry, and 30 *C. maculata* from allopatry. An additional 43 sympatric *C. aequabilis* and 27 sympatric *C. maculata* were scored for species-specific mitochondrial haplotypes using a polymerase chain reaction (PCR)-based diagnostic protocol developed during the course of the project. In total, we determined the mitochondrial



**Fig. 1** Map of geographic sampling for ranges of *Calopteryx aequabilis* and *Calopteryx maculata* showing area of sympatry in dark grey (images of wing pattern phenotypes adapted from Waage, 1979).

haplotype of 94 *C. aequabilis* and 97 *C. maculata* for a final sample size of 191 individuals (Table 1). For a subsample of 13 individuals (three *C. aequabilis* and five *C. maculata*), we also generated EF1- $\alpha$  sequence data to aid in phylogeny reconstruction.

### DNA amplification and sequencing

DNA was isolated from  $\sim 25 \mu\text{g}$  of flight muscle using a Qiagen (Valencia, CA, USA) extraction kit. A 1-kb fragment of the mitochondrial CO I was initially amplified using universal, degenerate insect primers: Ron and Pat (Willett *et al.*, 1999). Subsequent amplifications were done using newly designed internal primers (Int-1: 5'-GGATCGATGAAGAACG-3'; Int-2: 5'-GAATCGTG GGCTGCAAT-3') to optimize the PCR and sequencing conditions for all taxa. We sequenced this  $\sim 850$ -bp region of CO I and a  $\sim 1.2$ -kb fragment of the nuclear gene EF1- $\alpha$  from the same 13 individuals for our phylogenetic analysis, including sympatric and allopatric representatives of both focal taxa. Elongation factor was amplified and sequenced using the universal primers Ef44 and EfcM4 described by Monteiro & Pierce (2001).

In addition, we sequenced CO I from 123 individuals for our broader geographic survey of mitochondrial haplotype sharing.

All PCR (10- $\mu\text{l}$  volume) contained 3 mM  $\text{MgCl}_2$ , 0.2 mM dNTPs, 50 mM KCl, 20 mM Tris (pH 8.4), 2.5 ng of each primer and 1 U of *Taq* DNA polymerase (Invitrogen Platinum® *Taq*, Invitrogen, Carlsbad, CA, USA) and 1  $\mu\text{L}$  of genomic DNA. PCR amplifications were performed using a OmniGene (Hybaid) thermal cycler. The mitochondrial gene CO I was amplified using a touchdown protocol (95, 56–46 and 72 °C for 1 min), although cycle conditions for EF1- $\alpha$  were similar but the annealing temperature was fixed at 52 °C. Successfully amplified products were cleaned for sequencing by incubating with 1  $\mu\text{l}$  of Exonuclease I (20 units/ $\mu\text{l}$ ) and Shrimp Alkaline Phosphatase (5 units/ $\mu\text{l}$ ) (0.5  $\mu\text{l}$  of each/sample) at 37 °C for at least 45 min. Cleaned PCR fragments for both loci were sequenced directly on an ABI PRISM 377 automated sequencer using BigDye terminator labelling (Applied Biosystems, Foster City, CA, USA). Sequences were aligned using a delayed CLUSTAL W algorithm (Higgins *et al.* 1996). Trimming during the editing and alignment

**Table 1** Collection information for *Calopteryx* specimens that were sequenced.

Species	Collecting locality	No. of sequenced	Allopatry vs. sympatry	Voucher no.	Accession
<i>Calopteryx aequabilis</i>	Susan River, Susanville, CA	2	Allopatry	CUEM 1–2	DQ411575-76
<i>C. aequabilis</i>	Stossel Creek, 6 mi E Duvall, WA	10	Allopatry	CUEM 3–12	DQ411616-25
<i>C. aequabilis</i>	Winnipeg, Manitoba	4	Allopatry	CUEM 13–16	DQ411577-80
<i>C. aequabilis</i>	Iron Creek, Morrison Co., MN	8	Allopatry	CUEM 17–24	DQ411581-88
<i>C. aequabilis</i>	Mississippi river, Old Ferry, MN	5	Sympatry	CUEM 25–29	DQ411589-93
<i>C. aequabilis</i>	Raritan River, New Brunswick, NJ	7	Sympatry	CUEM 30–36	DQ411594-00
<i>C. aequabilis</i>	Fall Creek, Ithaca, NY	6	Sympatry	CUEM 37–42	DQ411601-06
<i>C. aequabilis</i>	Ellis Hollow Creek, Ithaca, NY	5	Sympatry	CUEM 43–47	DQ411607-11
<i>C. aequabilis</i>	Lewis Creek, VT	4	Sympatry	CUEM 48–51	DQ411612-15
<i>Calopteryx maculata</i>	Lafayette and Taylor Counties, FL	6	Allopatry	CUEM 52–57	DQ411629-34
<i>C. maculata</i>	Little Volga River, Maynard, IA	8	Sympatry	CUEM 58–65	DQ411635-42
<i>C. maculata</i>	Concord & Shawsheen Rivers, MA	6	Sympatry	CUEM 66–71	DQ411643-48
<i>C. maculata</i>	Big Flat Brook, Sussex, NJ	5	Allopatric	CUEM 72–76	DQ411649-53
<i>C. maculata</i>	Ellis Hollow Creek, NY	12	Sympatry	CUEM 77–88	DQ411654-65
<i>C. maculata</i>	Cayuta lake outlet, NY	3	Sympatry	CUEM 89–91	DQ411666-68
<i>C. maculata</i>	Jenksville State Forest, NY	5	Sympatry	CUEM 92–96	DQ411669-73
<i>C. maculata</i>	Buttermilk Falls, Ithaca, NY	8	Sympatry	CUEM 97–104	DQ411674-81
<i>C. maculata</i>	Mississippi river, Old Ferry, MN	2	Sympatry	CUEM 105–06	DQ411626-27
<i>C. maculata</i>	Cincinnati, OH	2	Sympatry	CUEM 107–08	DQ411682-83
<i>C. maculata</i>	Angelina Nat. For., TX	8	Allopatry	CUEM 109–16	DQ411684-91
<i>C. maculata</i>	G.W. Nat. For. Front Royal, VA	11	Allopatry	CUEM 117–27	DQ411692-02
<i>C. maculata</i>	Lewis Creek, VT	4	Sympatry	CUEM 128–31	DQ411703-10
<i>Calopteryx dimidata</i>	Web's Mill Branch, Ocean Co., NJ	1	Source – M. May	CUEM 132	DQ411710
<i>Calopteryx amata</i>	Big Flat Brook, Sussex, NJ	1	Source – M. May	CUEM 133	DQ411628
<i>Calopteryx splendens</i>	East Jutland, Denmark	1	Source – T. Simonsen	CUEM 134	DQ411707
<i>Calopteryx virgo</i>	East Jutland, Denmark	2	Source – T. Simonsen	CUEM 135–6	DQ411708-9

**Table 2** Additional individuals sampled from sympatric populations and scored for mitochondrial haplotype by species-specific restriction digest.

Population	<i>Calopteryx aequabilis</i> Male	<i>C. aequabilis</i> Female	<i>Calopteryx maculata</i> Male	<i>C. maculata</i> Female
Fall Creek, Ithaca, NY	21	14	11	6
Ellis Hollow Creek, Ithaca, NY	5	3	4	6

process resulted in final aligned data sets of 804 bp for CO I and 1193 bp for EF1- $\alpha$ .

Based on initial sequencing results, we designed species-specific primers and scored the mitochondrial haplotypes of an additional 70 individuals from two sympatric localities (see Table 2). PCR were carried out using a touchdown protocol to increase amplification specificity. Annealing temperature was reduced by 2 °C (from an initial annealing temperature of 60 °C) every two cycles for the first 10 cycles. The remaining 25 cycles were performed at 95, 50 and 72 °C for a minute. Two PCR were performed for each individual using either primer specific to *C. maculata* (Mac-F: 5'-ATGAAGGC CCCAGGAATAAAA-3' and Mac-R: 5'-AAAATGTTGGGG GAAGAATGT-3') or *C. aequabilis* (Aequa-F: 5'-TAACAT AAAGGCCCCAGGGATAAAAT-3' and Aequa-R: 5'-AAG AAGTGTGAGGGAAAAATGTC-3'). These two primer

pairs are species specific and produce PCR products that differ slightly in size, allowing rapid haplotype assignment.

### Phylogenetic analysis

Phylogenetic analyses were done using PAUP\* 4.0b10 (Swofford, 2003) and MrBayes 3.1.1 (Ronquist & Huelsenbeck, 2003). For each locus, the most appropriate DNA substitution model was determined with Modeltest 3.7 (Posada & Crandall 1998). Hierarchical likelihood ratio tests (hLRTs) implemented in Modeltest selected a GTR + I +  $\Gamma$  model for CO I (Nst = 6, Rates = gamma, Shape = 1.0182 and Pinvar = 0.5259) and an HKY +  $\Gamma$  for EF1- $\alpha$  (Nst = 2, TRatio = 1.0252, Rates = gamma, Shaper = 0.1644 and Pinvar = 0). Support for the resulting maximum-likelihood trees was determined using 100 maximum-likelihood bootstrap replicates. In

addition, we further tested the nonparametric support for nodes recovered in the ML tree using 10 000 parsimony bootstrap replicates.

A Bayesian phylogenetic approach was used to investigate the topological support of the larger mitochondrial data set because of computational limitations of traditional maximum-likelihood methods. The appropriate model of DNA substitution was specified in MrBayes 3.1.1 using the general lset values (nst = 6, rates = invgamma) and allowing the programme to converge on the best estimates of these model parameters. Four chains, one cold and three heated incrementally (0.2 temp), were employed for a series of 1 million generation (Monte Carlo Markov chain, MCMC) searches sampled every 100 generations. Average standard deviation of the split frequencies was 0.00361 at the end of the run and the average PSRF was 1.001 for the 12 parameters estimated. Very consistent clade credibility values were observed among runs suggesting that convergence had been reached. Posterior probabilities of the consensus tree were obtained after discarding as burn-in the first 1000 sampled trees.

### Historical origins of sympatry

If character displacement in wing colouration is the result of a reinforcement process in this system, then we expect sympatric populations to be the result of secondary contact between *C. aequabilis* and *C. maculata*. If so, then we expect the topology of our mtDNA phylogenetic tree to reflect that sympatric populations are derived, given sufficient time for lineage sorting. To test this prediction, we compared the likelihood scores of the best trees with those in which sympatric populations were constrained to be basal to allopatric populations using parametric bootstrapping (Huelsenbeck *et al.*, 1995, 1996; Swofford *et al.*, 1996; Goldman *et al.*, 2000). Using Mesquite (Maddison & Maddison, 2004), we simulated 100 data sets using the topology, branch lengths and DNA substitution model parameters of the basal constraint. Differences in the log-likelihood scores of the best trees recovered under the constrained and unconstrained heuristic searches were calculated and used to generate a null distribution of expected values. The observed difference in the log-likelihoods for the real data set was then compared with this simulated distribution to determine the *P*-value.

### Estimates of inter-specific gene flow

Demonstrating that displaced populations are the result of secondary contact is necessary but not sufficient to invoke speciation via reinforcement. The critical assumption is the existence of limited gene flow upon secondary contact between the interacting species. To assess the degree of recent and/or current gene flow, we used two different, complementary strategies. First, we assessed the degree of association between wing colour phenotype

and specific haplotypes of the mtDNA marker CO I. Second, we used a coalescent approach to simultaneously estimate population divergence time and levels of gene flow between *C. aequabilis* and *C. maculata* as well as among sympatric and allopatric populations within each species. Coalescent-based analyses were carried out using the distinguishing migration from isolation (MDIV) software developed by Nielsen & Wakeley (2001); whereas more sophisticated models of isolation and migration, including population growth, can currently be implemented in IM (Hey and Nielsen 2004); this programme is better suited for multilocus data sets because of the difficulty in estimating a large number of demographic parameters from a single gene.

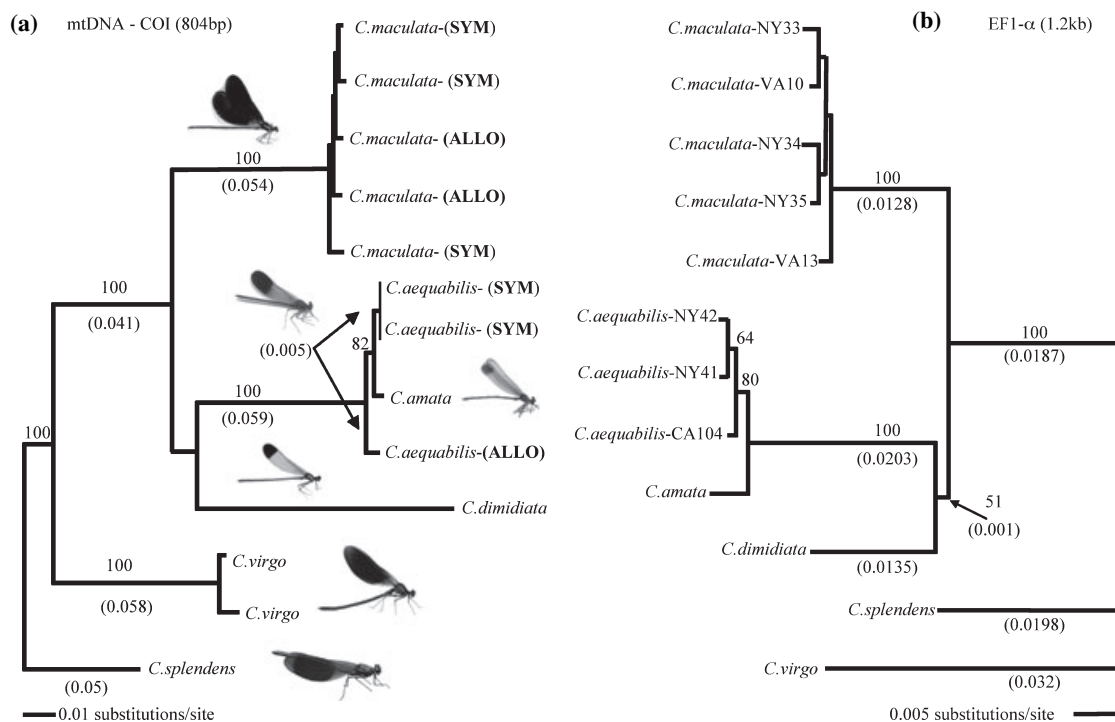
MDIV calculates the posterior distribution of several coalescent parameters using a Metropolis-Hastings MCMC algorithm and has been shown to generate reliable joint estimates of migration rates and divergence times based on single-locus DNA sequence data (Nielsen & Wakeley, 2001). The programme estimates  $\theta$  ( $2N\mu$ ), gene flow ( $M = 2Nm$ ) and divergence time ( $T = t/N$ ); where  $N$  is the effective female population size (for mitochondrial DNA),  $\mu$  is the gene-specific mutation rate per generation,  $m$  is the proportion of individuals from one population that are replaced by individuals from the second population each generation, and  $t$  is the divergence time in generations.

For each pairwise comparison, we ran the Markov chain three times with different random seeds for 5 million cycles under a finite-site model (burnin = 500 000; max migration = 10; max divergence time = 20, max theta = auto initialize). Parameter estimates were highly consistent among runs suggesting that chain length was sufficient for parameter stabilization. Pairwise estimates of  $T$  were then converted to time since population divergence (in years) as follows:  $T_{\text{div}} = T\theta / (2\mu)$ . We used Brower's (1994) rough molecular clock approximation of 2.3% mitochondrial sequence divergence/million years, or 1.15% per lineage per million years, as our neutral mutation rate (i.e.  $\mu = 9.25 \times 10^{-6}$  for 804 bp). Credibility intervals (95%) were calculated by assuming that  $2\Delta \ln L$  is  $\chi^2$  distributed (Rice, 1995).

## Results

### Phylogenetic relationships and wing pattern evolution among Nearctic *Calopteryx*

The results of our mitochondrial and nuclear gene genealogies are highly concordant and well resolved. Both loci indicate that *C. maculata* is a monophyletic and deeply divergent lineage that is basal to the remaining North American species (Fig. 2). In addition, we found that the sparkling Jewelwing (*C. dimidiata*) is the sister lineage of both *C. amata* and *C. aequabilis* and is deeply divergent from both of these species. Previous work on the evolutionary relationships of the genus *Calopteryx* and



**Fig. 2** Maximum-likelihood gene genealogies of unique haplotypes for (a) CO I mtDNA and (b) EF1- $\alpha$ . Numbers above branches indicate nonparametric parsimony bootstrap values. Values below branches indicate Bayesian posterior probabilities. GTR + I +  $\Gamma$  Model: Rmat = (2.3484 15.3956 1.0612 0.8663 15.3956) Shape = 1.0182, Pinvar = 0.5259.

broader subfamily (Calopterygidae) suggested little differentiation between *C. aequabilis* and *C. amata* (Misof *et al.*, 2000; Dumont *et al.*, 2005). Our results support the close relationship between these two wing pattern forms and also indicate that, at least for mitochondrial DNA, *C. aequabilis* is not a monophyletic grouping. Finally, although sample size is small, both gene genealogies indicate that sequences from western (allopatric) *C. aequabilis* are basal to those generated from individuals collected in eastern (sympatric) populations. No clear geographic relationships are evident among allopatric and sympatric populations of *C. maculata*.

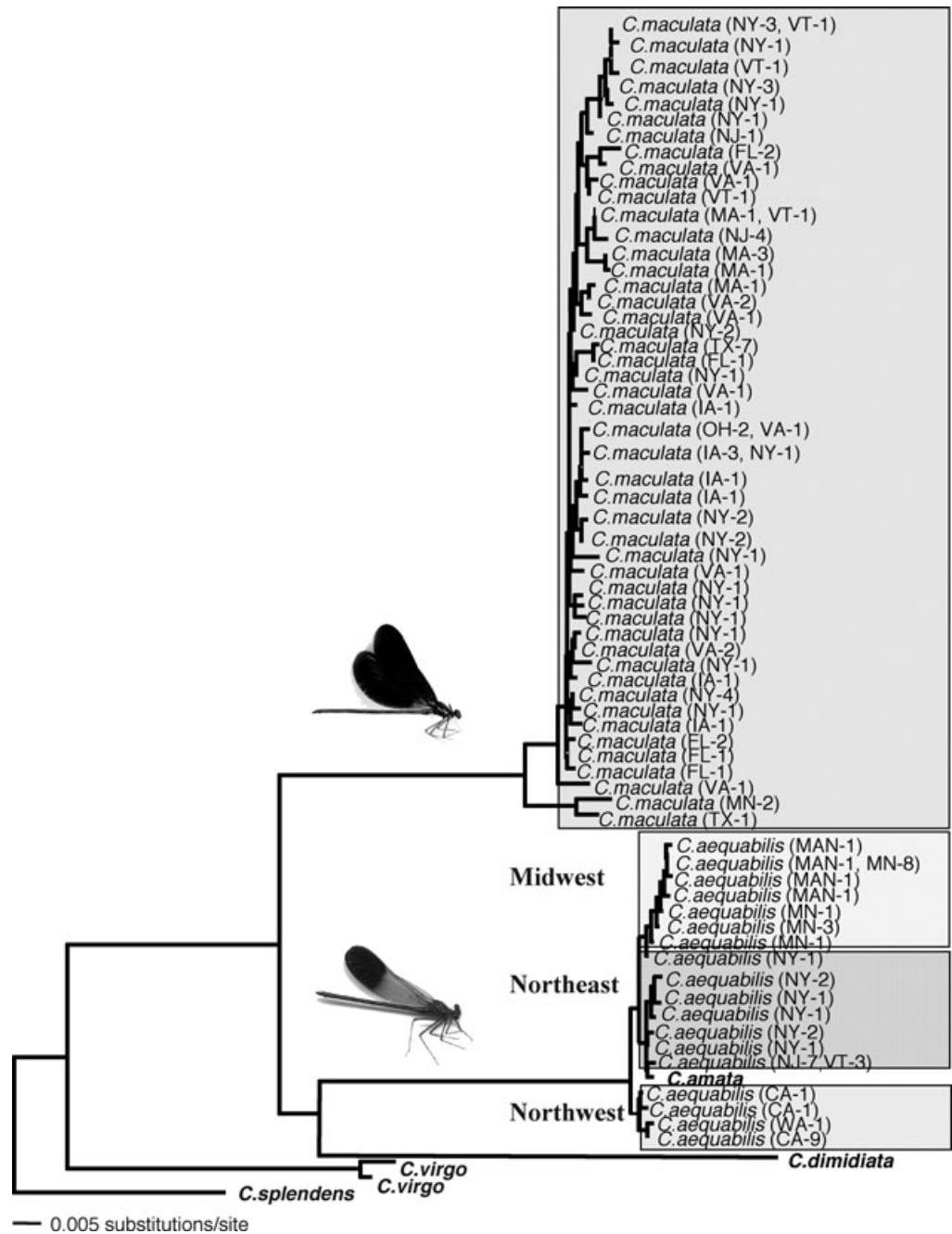
### Secondary origins of sympatry

The recovered topology of the best-unconstrained maximum-likelihood mitochondrial gene tree is consistent with a scenario of secondary contact between *C. aequabilis* and *C. maculata*. Sympatric (displaced) populations of *C. aequabilis* are derived from conspecific allopatric (nondisplaced) populations in the maximum-likelihood tree (Fig. 3). Moreover, the intraspecific genetic divergence for CO I between sympatric and allopatric populations of *C. aequabilis* is 10-fold less ( $\sim 1.5\%$ ) than the observed levels of interspecific genetic divergence ( $\sim 15\%$ ). To test if this topology is significantly better than trees with ancestral sympatric populations, we carried out an LRT against a model constrained to force

sympatric populations of *C. aequabilis* to be basal. The observed difference assessed by parametric bootstrap yielded an exact value of  $P = 0.07$ .

### Interspecific gene flow

If sympatric populations of *C. maculata* and *C. aequabilis* are currently hybridizing or have experienced gene flow in the recent past, then we predict that mitochondrial haplotypes will be shared to some degree. In contrast, our phylogenetic analyses did not show any evidence of haplotype sharing between these two lineages (Figs 3 and 4). The results of our maximum-likelihood analyses show that for both the mitochondrial (CO I) and nuclear (EF1- $\alpha$ ) genealogies, sequences obtained from *C. maculata* and *C. aequabilis* each form monophyletic clades with high nodal support (see Fig. 2). In addition, our relatively extensive survey ( $n = 70$ ) of two different sympatric populations using a PCR-based assay found full concordance between wing pattern phenotype and mitochondrial lineage (Fig. 5). Furthermore, the MDIV coalescent estimate of migration between Ebony (*C. maculata*) and River Jewelwings (*C. aequabilis*) was extremely low ( $M = 2Nm = 0.08$ , 95% CI 0.0–0.54) and consistent with divergence with no gene flow between these two species. In addition, MDIV estimated a time since divergence between these two Jewelwing damselfly species ( $T_{div} = 6.38$  Ma, 95% CI 3.64–10.26 Ma) consistent with Miocene speciation.

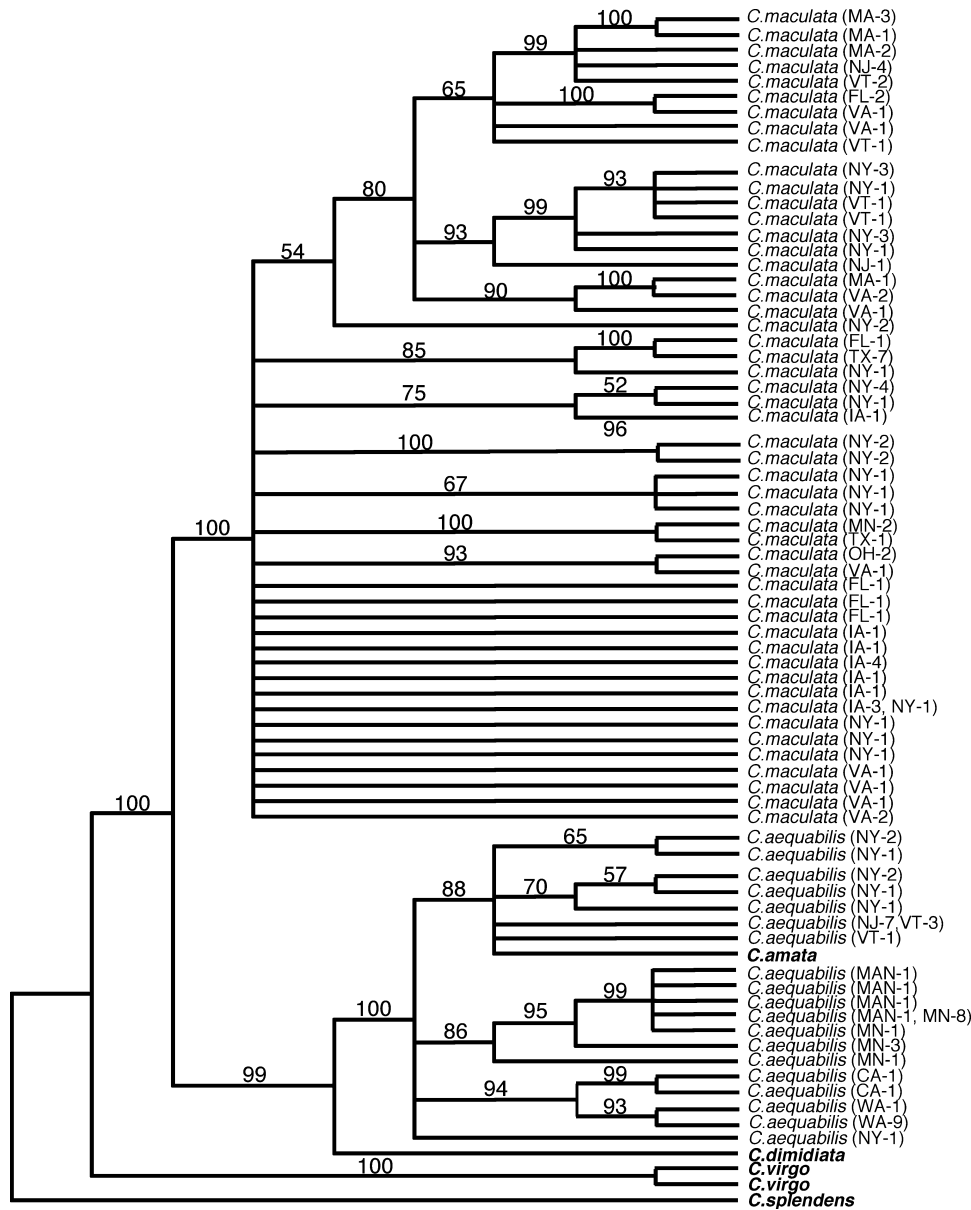


**Fig. 3** Reconstructed maximum-likelihood topology showing relationships among mtDNA haplotypes and wing pattern phenotypes. Sampling locality and the number of individuals possessing each mitochondrial haplotype are in parentheses.

**Intraspecific levels of gene flow**

Migration estimates between sympatric and allopatric populations of *C. aequabilis* ( $M = 2Nm = 0.1$ , 95% CI 0.0–0.52) indicate little or no mitochondrial gene flow between the populations sampled for this analysis. In contrast, sympatric and allopatric populations of *C. maculata* appear to exchange migrants at a much

higher rate ( $M = 2Nm = 1.82$ , 95% CI 0.72–4.96). Conversion of MDIV estimates of  $T$  into time since divergence resulted in very similar estimates of divergence for both sympatric and allopatric populations of *C. aequabilis* and *C. maculata* ( $T_{div} = \sim 199\ 000$  vs.  $198\ 000$  years before present (ybp), 95% CI 65 000–333 000 vs. 119 000–439 000) despite very different estimates of  $\theta$  for each species ( $\theta$  *aequabilis* = 2.79;  $\theta$  *maculata* = 18.39).



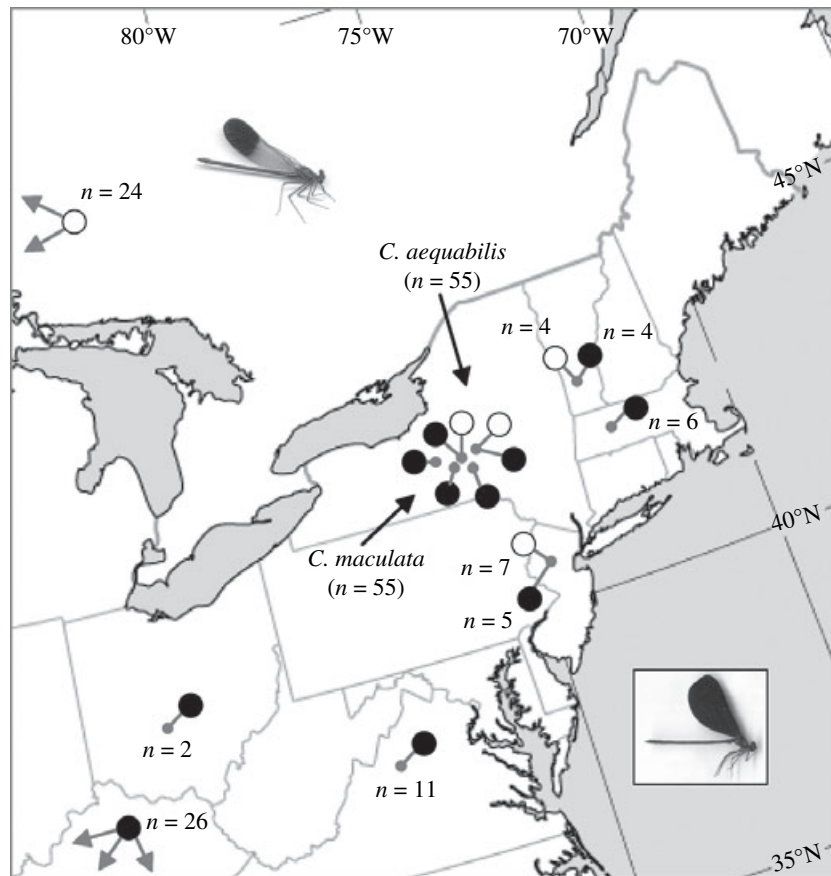
**Fig. 4** Bayesian reconstruction of mtDNA genealogy (numbers about branches are clade credibility values). Sampling locality and the number of individuals possessing each mitochondrial haplotype are in parentheses.

**Discussion**

Historically, the damselfly genus *Calopteryx* has provided several examples of reproductive character displacement and enhanced isolation that have been widely interpreted as evidence for the importance of the reinforcement process during speciation events within this group. The wing pattern displacement among sympatric populations of Ebony and River Jewelwings (Waage, 1975) and the associated increase in the ability of male Ebony Jewelwings to correctly discriminate between con- and heterospecific females (Waage, 1979) have

served as one of the classic examples for reinforcement outside of *Drosophila* (Howard, 1993; Futuyma, 1998; Coyne & Orr, 2004). Although this pattern of wing character displacement (and its associated preference) is consistent with speciation via reinforcement, this hypothesis critically relies upon the assumption that selection against hybridization has acted historically to reduce gene flow and promote speciation in this system. However, several lines of evidence arising from our study strongly suggest that this is not the case.

First, although our data support that sympatric populations of *C. aequabilis* and *C. maculata* are the result



**Fig. 5** Distribution of sympatric populations of *Calopteryx maculata* and *Calopteryx aequabilis* sampled. Small grey circles indicate populations (i.e. streams). Large circles indicate the proportion of individuals with a wing pattern phenotype characteristic of *C. maculata* (black) or *C. aequabilis* (white) that also possessed an mtDNA haplotype indicative of that species in allopatry.

of secondary contact, both of the recovered gene genealogies indicate that these species are not sister taxa and represent deeply divergent lineages (Figs 2–4). Second, our coalescent-based estimates of divergence and migration between *C. maculata* and *C. aequabilis* strongly support a historical scenario of allopatric speciation with no gene flow. Third, our broad-scale survey of mitochondrial haplotypes failed to find any evidence for the production of hybrids, suggesting that reproductive isolation between these two damselflies is complete.

In contrast, levels of mitochondrial sequence polymorphism within *C. maculata* and *C. aequabilis* are low (< 1.5%), and intraspecific divergence time estimates between allopatric and sympatric populations (~200 000 ybp) indicate that both species have only recently (Pleistocene) experienced population differentiation. Moreover, Waage (1979) presented clear evidence that both wing pattern displacement and mate discrimination increase with the degree of sympatry between these two damselfly species. Taken together with our results, this strongly suggests that wing pattern displacement and, hence, the associated increase in

prezygotic isolation between these two damselflies must have arisen recently, long after the initial divergence between these two species.

A potential criticism to this argument arises from the fact that low levels of hybridization might be difficult to detect (Noor *et al.*, 2000) even if contact is recent relative to divergence. However, our sampling should be able to detect relatively low levels of gene flow because even if there is significant hybrid unfitness because of nuclear incompatibilities, introgression of mitochondrial DNA is expected to be substantial (Takahata & Slatkin, 1984). In addition, we found no phenotypic evidence for hybridization among the collected specimens. Therefore, although it is impossible to conclusively rule out very low levels of gene flow, our current data suggest that no contemporary introgression is occurring.

A more serious concern is that selective differences may exist in the relative fitness of each mitochondrial genome depending upon nuclear background of each species. If this were the case, then selection would retard mitochondrial introgression and obscure evidence of historical hybridization. To fully address this hypothesis,

that the current lack of evidence for mitochondrial haplotype sharing is because of historical gene flow and reciprocal selection against mtDNA genomes, will require additional genetic markers. However, our coalescent estimates of divergence and migration strongly suggest that speciation in this system is ancient and vastly predated the origins of enhanced isolation.

A further potential caveat to our conclusions is that evolutionary inference can be confounded by the effects of inherited symbionts (Hurst & Jiggins, 2005). Although vertically transmitted endosymbionts can affect patterns of mtDNA diversity in a variety of ways, of concern here is the possibility that *C. maculata* and *C. aequabilis* harbour different strains of passenger symbionts, such as *Wolbachia*, and that cytoplasmic incompatibility maintains the deeply divergent haplotype structure in this system via linkage disequilibrium in spite of nuclear gene flow. If so, then we expect to find discordant genealogical patterns at additional nuclear markers.

In contrast, although our sampling of the nuclear gene *E1- $\alpha$*  is limited, the genealogical pattern at this locus is completely concordant with the mtDNA results. In addition, two other phylogenetic studies using both mitochondrial (Misof *et al.*, 2000) and nuclear rDNA (Dumont *et al.*, 2005) have also found extremely deep divergence between the two focal taxa, suggesting that a shared history has influenced all of these loci. This result is striking considering the widespread expectation of genealogical discordance among genomic regions for recently diverged species (Wang *et al.*, 1997; Kliman *et al.*, 2000; Ting *et al.*, 2000; Beltrán *et al.*, 2002; Broughton & Harrison, 2003; Machado & Hey, 2003; Dopman *et al.*, 2005). Therefore, it is unlikely that the pattern of mitochondrial diversity observed here is because of the effects of an unrecognized association with endosymbionts. Even if this were the case, however, linkage disequilibrium with symbiotic strains is not expected to extend to the regions of the genome that control phenotypic variation in wing pattern, and the complete absence of hybrid phenotypes in nature suggests that nuclear introgression is not occurring.

A more plausible alternative explanation for our results might be that reproductive character displacement and enhanced isolation in this system originated at some earlier point in the history of these two species and the current distribution of wing pattern phenotypes resulted from some form of ecological lineage sorting (Smith & Wilson, 2002) rather than as a result of secondary contact. However, this seems implausible given the low levels of intraspecific divergence within both species and the geographic structure of *C. aequabilis* populations. Moreover, no evidence for ecological differentiation has been found between these two species and it is difficult to imagine what form of ecological sorting would maintain the two-wing pattern morphs within *C. aequabilis* in the absence of interspecific interactions with populations of *C. maculata*.

Finally, there is the possibility that sympatric populations of *C. aequabilis* actually represent a distinct species with clinal variation in wing pattern (Fig. 2). In these species-level phylogenies, sympatric *C. aequabilis* forms a distinct genealogical grouping and appears to be closely related to the splendid Jewelwing, *C. amata*. Interestingly, this species of Jewelwing damselfly has evolved a wing pattern that represents the extreme of phenotypes seen among north-eastern populations of *C. aequabilis* and broadly co-occurs with it. Although it is possible that these two forms actually represent a single, reproductively isolated species, such a finding would not substantially alter our conclusion that wing pattern displacement and enhanced isolation in this system post-date divergence and speciation with *C. maculata*.

Although many hypotheses have been promulgated to explain patterns of enhanced isolation in sympatry, the increased mate discrimination and wing pattern displacement in *Calopteryx* may be best explained by selection acting to reduce interference between the mate recognition signals of these nonhybridizing species (Templeton, cited in Howard, 1993; Noor, 1999). This 'noisy neighbour' hypothesis (Otte, 1989; Noor, 1999), a special case of ecological character displacement, predicts a displacement in sexual signals to reduce wasted mating effort between fully isolated taxa. Given the heavy potential costs of increased predation risk, limited resource availability, and male–male competition associated with locating and courting heterospecific females, selection should act strongly in favour of displaced sexual cues and increase mate discrimination in sympatry. For example, interspecific male–male aggression causes negative selection on male secondary sexual characters (wing spots) and leads to displacement of male sexual signals among sympatric populations of the European damselflies *C. splendens* and *C. virgo* (Tynkkynen *et al.*, 2004, 2005).

Interestingly, this pattern is consistent with Coyne & Orr's (2004) prediction that 'noisy neighbour' effects should lead to greater changes in male signals in sympatry, whereas reinforcement should typically result in larger changes in female than male behaviour, physiology or morphology. If a similar mechanism is operating among sympatric populations of *C. maculata* and *C. aequabilis*, then it is possible that the large change in female wing pattern observed in this system is the indirect result of selection on male wing patterns, which are also displaced in sympatry. However, regardless of the exact cause of reproductive character displacement in this system, it is clear that selection acting on sexual signals in sympatry can rapidly drive the evolution of novel phenotypes. In this regard, although selection against hybridization may not always lead to the origins of biological diversity in the sense of completing speciation, it can certainly be viewed as a major creative force in the origins of adaptive phenotypic novelty.

Recognizing that disruptive selection can be caused by a variety of evolutionary mechanisms, Servedio & Noor

(2003) have advocated adopting a broader definition of reinforcement that incorporates the many alternative processes that can produce a pattern of enhanced isolation in sympatry. They have argued persuasively that a broader definition will allow us to assess the overall frequency of the many processes that can act to reduce gene flow between hybridizing taxa in nature. We agree with this view. However, because interest in reinforcement stems largely from its controversial role in the process of speciation, the eventual determination of how important selection against hybridization is as a mechanism of speciation, in general, will require a case-by-case evaluation of the classic criteria of reinforcement outlined by Butlin (1987a) and Howard (1993). Ultimately, we must move away from our historical focus on the pattern of enhanced isolation and strive to distinguish explicitly between the myriad processes that can produce this pattern if we hope to gain a better understanding of how natural selection has influenced divergence between closely related populations.

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