Contrasting hybridization rates between sympatric three-spined sticklebacks highlight the fragility of reproductive barriers between evolutionarily young species

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Abstract

Three-spined sticklebacks (*Gasterosteus aculeatus*) are a powerful evolutionary model system due to the rapid and repeated phenotypic divergence of freshwater forms from a marine ancestor throughout the Northern Hemisphere. Many of these recently derived populations are found in overlapping habitats, yet are reproductively isolated from each other. This scenario provides excellent opportunities to investigate the mechanisms driving speciation in natural populations. Genetically distinguishing between such recently derived species, however, can create difficulties in exploring the ecological and genetic factors defining species boundaries, an essential component to our understanding of speciation. We overcame these limitations and increased the power of analyses by selecting highly discriminatory markers from the battery of genetic markers now available. Using species diagnostic molecular profiles, we quantified levels of hybridization and introgression within three sympatric species pairs of three-spined stickleback. Sticklebacks within Priest and Paxton lakes exhibit a low level of natural hybridization and provide support for the role of reinforcement in maintaining distinct species in sympatry. In contrast, our study provides further evidence for a continued breakdown of the Enos Lake species pair into a hybrid swarm, with biased introgression of the 'limnetic' species into that of the 'benthic'; a situation that highlights the delicate balance between persistence and breakdown of reproductive barriers between young species. A similar strategy utilizing the stickleback microsatellite resource can also be applied to answer an array of biological questions in other species' pair systems in this geographically widespread and phenotypically diverse model organism.

Keywords: ecological speciation, *Gasterosteus aculeatus*, gene flow, introgression, microsatellites

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Introduction

Three-spined sticklebacks (*Gasterosteus aculeatus*) are a powerful evolutionary model system due to the rapid phenotypic divergence of freshwater forms from an ancestral marine colonizer throughout the Northern Hemisphere since the end of the last ice age, 10 000–15 000 years ago (Bell & Foster 1994). The presence of multiple, independently

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derived populations, which are now reproductively isolated from each other, provides excellent opportunities to investigate the mechanisms driving speciation in natural populations. For example, parallel evolution of size-assortative mating between anadromous and stream-resident forms throughout the range of the *G. aculeatus* species complex suggests that reproductive isolation may arise largely as a result of ecological differences and divergent selection on a few phenotypic traits (McKinnon *et al.* 2004). As a first step in understanding the genetics of such phenotypic evolution, parallel inheritance of body shape and lateral plate number has been shown in different

freshwater lineages (Schluter *et al.* 2004). Further insight into the genetic architecture of parallel phenotypic evolution in natural populations is now being gleaned from genetic mapping studies made feasible by the development of a microsatellite linkage map for sticklebacks (Peichel *et al.* 2001). Parallel changes in lateral plate number (Colosimo *et al.* 2004; Cresko *et al.* 2004) and pelvic reduction (Cresko *et al.* 2004; Shapiro *et al.* 2004) appear to be caused by changes at the same genetic loci. Indeed, Colosimo *et al.* (2005) have shown that the Ectodysplasin gene is crucial to stickleback plate morph development, and that parallel evolution of most low-plated freshwater phenotypes has occurred by repeated selection of ancestral marine alleles at this locus. This highlights the potential for adaptive divergence to arise repeatedly from relatively few genetic changes, a phenomenon that may help to explain the rapid evolution of natural stickleback populations (Bell *et al.* 2004).

The development of new genomic and genetic tools for three-spined sticklebacks now holds promise for the identification of the actual genes and mutations responsible for evolutionary change in physiological, behavioural and morphological traits, as well as the processes that drive such change (Kingsley *et al.* 2004). These same genomic resources, however, may also be utilized to address challenging questions in population ecology. For example, understanding the population dynamics at stickleback species boundaries is an essential component of our understanding of the mechanisms driving speciation (Barton & Hewitt 1985). This has been hampered, however, by the difficulty in adequately differentiating genetically between such recently derived species. While many studies have used species diagnostic markers, which greatly increase the power of analyses, to examine hybridization dynamics amongst older species of fish (e.g. Ostberg *et al.* 2004; Rubridge & Taylor 2004; Bettles *et al.* 2005), identifying such markers amongst younger species that share a more recent common ancestry is challenging. Armed with the battery of stickleback genetic markers now available (Peichel *et al.* 2001), however, we are able to efficiently search for highly discriminatory markers, overcoming these limitations and enabling us to explore interactions at the interface of stickleback species pairs.

Species pairs of three-spined stickleback

Several systems of stickleback species pairs have been recognized, where a pair of morphologically and ecologically divergent forms naturally coexists and exhibit varying degrees of reproductive isolation (reviewed in McKinnon & Rundle 2002). Those systems that include a freshwater form are considered of recent origin, having diverged from marine ancestors since the colonization of fresh water after the retreat of the Pleistocene glaciers, about 10 000–15 000

years ago. This includes many parapatric anadromous-freshwater and lake-stream pairs, as well as sympatric benthic-limnetic systems. Evidence amongst the more recently derived forms implicates ecologically based divergent selection in the evolution of both pre- and postzygotic reproductive isolation (reviewed in McKinnon & Rundle 2002), with the benthic-limnetic lake pairs having been studied the most extensively.

Extant benthic-limnetic pairs have evolved independently in three separate water drainages (Taylor & McPhail 1999, 2000) in coastal British Columbia: Enos Lake on Vancouver Island (49°17′N, 124°10′W); Priest and Emily lakes in the Vananda Creek drainage (49°45′N, 124°34′W), and Paxton Lake (49°43′N, 124°30′W) on Texada Island (McPhail 1984, 1992, 1994). Much of their divergence from their marine ancestors may have occurred in sympatry after a brief period of allopatry (about 2000 years) between a first and second marine incursion that brought two waves of colonists into the lakes (known as the double invasion hypothesis, McPhail 1993; Taylor & McPhail 2000). Regardless of the exact sequence of initial events bringing the fish into these lakes, it appears that ecological speciation has been a major factor driving their divergence. Both comparative and experimental work on the group has strongly implicated divergent selection caused by interspecific resource competition (Bentzen & McPhail 1984; Schluter & McPhail 1992; Schluter 1993, 1994, 1995, 2003) and predation (Rundle *et al.* 2003; Vamosi & Schluter 2004) in the origin and maintenance of the divergence of the species pairs.

In each of these sympatric pairs, one member is known as the 'benthic' stickleback, a bottom-dwelling fish foraging mainly on large invertebrates from sediment or on plants in littoral habitats, and the other member is known as the 'limnetic' stickleback, a specialized pelagic zooplankton feeder. This ecological divergence is associated with consistent morphological differences: limnetics tend to be shorter and more slender, with a narrower mouth and more numerous, longer gill rakers (the protruberances along the gill arches that sieve ingested prey and direct fluid movement within the buccal cavity; Sanderson *et al.* 1991), compared with the longer, more robust, wider-mouthed benthics (Schluter & McPhail 1992). During the breeding season, they share the littoral zone, where they frequently encounter one another despite occupying different microhabitats (Bentzen *et al.* 1984). Although strong assortative mating, predominantly based on size, prevents extensive admixture, premating isolation between ecomorphs is not complete, and rare natural hybrids (about 1–2% of adults) have been reported based on their intermediate morphology (McPhail 1984, 1992). There is no evidence of genetic incompatibilities in laboratory-bred hybrids (with the possible exception of benthic backcrosses), which are fully viable and fertile (McPhail 1984, 1992;

Hatfield & Schluter 1999). However, given the role of divergent natural selection and strong size-based assortative mating, these phenotypically intermediate fish can be expected to show a poorer fit to available niches and reduced mating success relative to the parental species. Indeed, experimental evidence suggests that extrinsic, rather than intrinsic, postzygotic reproductive isolating mechanisms are in force, including both ecologically based postzygotic isolation (Schluter 1995; Hatfield & Schluter 1999; Rundle 2002) and sexual selection against hybrid males (Vamosi & Schluter 1999).

In order to better understand the role of these forms of reproductive isolation in the divergence of the species pairs, as well as their influence on reinforcement (Rundle & Schluter 1998), the extent of gene flow and rates of hybridization and introgression between the species in nature must be quantified. Although estimates based on morphological assessment of wild populations suggest a low but persistent level of hybridization between the two species (McPhail 1984, 1992, 1994), these may be underestimates of the amount of intercrossing as the discriminant function analysis used probably does not detect backcrosses (McPhail 1992). Furthermore, although the phenotypic differences between the species have a genetic basis and laboratory-reared hybrids are morphologically intermediate (McPhail 1984, 1992; Hatfield 1997; Peichel *et al*. 2001), the traits exhibit plasticity in an adaptive direction (Day *et al*. 1994), creating wide error margins around estimates of natural hybridization rate based on morphology.

Here we seek unambiguous genetic assessment of hybridization rates between benthics and limnetics. Although previous allozyme (McPhail 1984, 1992) and molecular work (Taylor & McPhail 1999, 2000) indicated that the species are genetically distinct within each lake, the resolution of the genetic markers has been inadequate to accurately assess gene flow. We overcame this constraint by selecting highly discriminatory markers from the large microsatellite resources generated by the creation of the stickleback linkage map. The development of such species diagnostic molecular profiles for the three extant sympatric species pairs of three-spined sticklebacks has enabled us to provide the first quantitative estimates of hybridization and introgression within the species pairs.

Materials and methods

Selection of highly discriminatory microsatellite markers

Sample selection. 'Pure' representatives of each species from each of the three species pairs were required to identify genetic markers that clearly differentiated the two species. Therefore, 48 benthic and 48 limnetic fish were chosen from existing specimen collections from Priest and Paxton lakes (preserved in 95% ethanol) using the following

morphometric analysis. After tissue was removed for DNA extraction, fish were soaked in 10% formalin for 1 week and stained with alizarin red as previously described (Peichel *et al*. 2001). A suite of morphological traits associated with differences between benthics and limnetics were measured: body length, body depth, pelvic spine length, pelvic girdle width, gape width, snout length, and number of gill rakers were assessed as described in Schluter & McPhail (1992); the number of lateral plates and gill raker length were measured according to Lavin & McPhail (1985). For each lake, a two-dimensional multivariate scatter plot produced using nonstandardized Euclidean distances in SPSS version 11 (SPSS Inc.) revealed two distinct clusters (Fig. 1) that corresponded to previous morphological differences used to distinguish benthics from limnetics, highlighting the suitability of these samples for marker selection.

Morphological samples were more limited for Enos Lake. It is suspected that Enos Lake is experiencing a species breakdown (Kraak *et al*. 2001; Taylor *et al*. in press) and ethanol-preserved material prior to extensive introgression
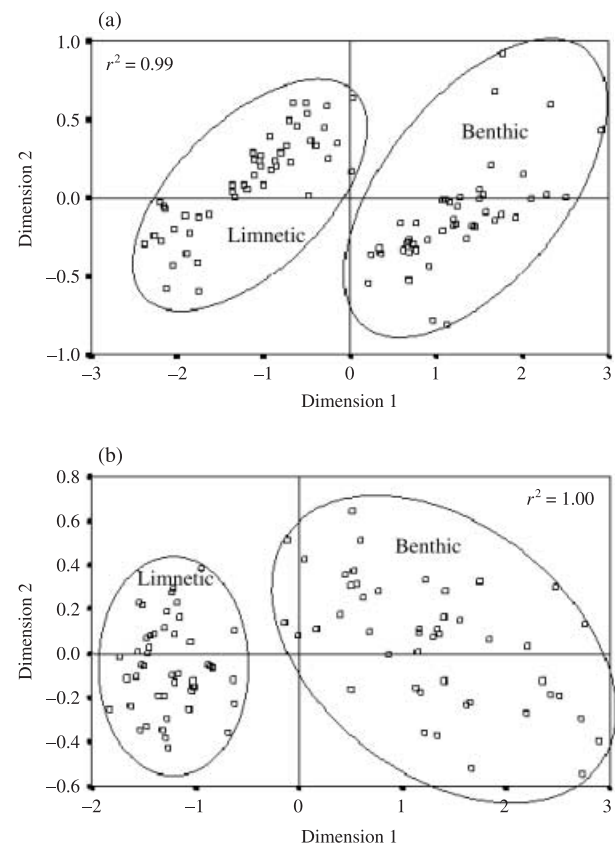
Fig. 1 Two-dimensional multivariate scatter plots of nine morphological traits from 96 three-spined stickleback from (a) Paxton Lake and (b) Priest Lake. The squared correlation in distances (r^2) indicates the proportion of variance of the data that is accounted for by the corresponding distances in the figure.

is scarce. In order to overcome this potentially confounding factor, only the most morphologically and genetically extreme samples from the oldest DNA surveys of this lake were included (benthics: 10 from 1994 and 15 from 1997; limnetics: 11 from 1994 and 15 from 1997), based on analysis from Taylor & McPhail (2000) and Taylor *et al*. (in press).

Screening of microsatellite library. In order to confidently identify hybrids within the lakes (Cornuet *et al*. 1999; Anderson & Thompson 2002), we searched for a suite of species-diagnostic markers for each lake pair. DNA was extracted from the right pectoral fin of these individuals using a QIAGEN DNeasy Tissue Kit. The DNA from each of the 48 benthics and 48 limnetics from Priest and Paxton lakes were pooled at equal concentration into their species and lake types. These four pools of DNAs were screened for microsatellite variation at 288 *Gasterosteus aculeatus*-derived loci using the polymerase chain reaction (PCR) and genotyping procedures outlined in Peichel *et al*. (2001), with the exception that 0.5 ng of each individual sample's DNA was present in the PCR. Those loci showing potentially nonoverlapping allele (NOA) ranges between benthics and limnetics from either lake were explored further by genotyping eight individuals of each species, according to Peichel *et al*. (2001). Those loci still showing a NOA range were verified by genotyping all 96 individuals from the entire lake's panel.

As the DNA samples from Enos Lake were restricted in number and amount available, and were variable in quality, an altered strategy from the pooled DNA approach was used. Initially, four of the most morphologically and genetically differentiated limnetics and benthics were screened for microsatellite variation at 192 *G. aculeatus*-derived loci using the methods outlined in Peichel *et al*. (2001). PCR products were visualized using ethidium bromide on a 2% agarose gel and those loci showing a potentially discriminating pattern between benthics and

limnetics were shortlisted for genotyping of the entire Enos sample panel of 51 individuals on an ABI 3700 sequencer according to Peichel *et al*. (2001). Together, these procedures used to screen sticklebacks from Priest, Paxton and Enos lakes identified nine loci that highly discriminated between benthics and limnetics in one or more of the species pairs (see Results; Table 1).

Assessment of gene flow and hybridization within species pairs

Sample collection and microsatellite genotyping. We collected between 192 and 198 subadult fish from Priest, Paxton and Enos lakes during September and October 2003. Thirty minnow traps distributed approximately evenly along the entire littoral zone shoreline were used in conjunction with dip-netting throughout the same area to ensure lake-wide samples in which the two species were represented approximately equally. No selection was made against indeterminate forms, i.e. fish with ambiguous morphology were not discarded. Fish were sacrificed in MS-222 and preserved in 95% ethanol before DNA extraction. In addition to the suite of nine species diagnostic microsatellite loci, these samples were genotyped at five additional *G. aculeatus*-derived microsatellite loci (*Gac*4, 7, 10 and 14: Taylor 1998; *Cir*51: Rico *et al*. 1993) that have been useful in previous population genetic surveys of these populations (Taylor & McPhail 2000). This not only increased the statistical power of analysis, but also retained consistency for population monitoring purposes. All 14 loci were amplified using the PCR conditions described above except that primer concentration varied from 25 to 500 nM, and different cycling conditions were used for the latter five loci: initial denaturation at 95 °C for 3 min was followed by 5 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min. A subsequent 25 cycles of 92 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s finished with a 7-min extension step

Locus	GenBank accession no.	Species pair	Allele range (base pairs):		Number of discrepancies
			Benthics	Limnetics	
Stn388	BV678141	Paxton	185	199–215	4 L
Stn295	BV678106	Paxton	151	163–185	2 L
Stn142	G72195	Paxton	199–219	179–187	1 B; 1 L
Stn383	BV212282	Paxton	192–208	178–182	1 B; 3 L
Stn387	BV678140	Priest	205–235	165–175	1 B
		Enos	201–239	165–173	10 L
Stn254	BV678079	Priest	249–279	225–227	
		Enos	255–275	225–227	1 B; 9 L
Stn216	BV102494	Enos	195–209	177	1 B; 7 L
Stn386	BV678139	Enos	210–223	233–241	1 B; 10 L
Stn43	G72149	Enos	148–166	132–136	9 L

Table 1 Suite of microsatellites showing nonoverlapping allele ranges between benthics and limnetics in at least one of the species pairs. The sample sizes for the species pair to which the allele range refers are 48 for Priest and Paxton benthics and limnetics, 25 for Enos Lake benthics and 26 for Enos Lake limnetics. Number of discrepancies refers to the number of individuals from the screening panel ('L' suffix for limnetic, 'B' for benthic) which carry an allele from the other species range.

at 72 °C. PCR products from two to three different primer pairs were then pooled and analysed on a CEQ 8000 Genetic Analysis System (Beckman Coulter) with CEQ DNA Size Standard Kit-400 used as internal size standard. Locus *Stn*387 consistently amplified only Priest samples on the CEQ 8000 despite attempted re-optimization of PCR conditions. Therefore, this locus was excluded from statistical analyses.

Statistical analyses

Hybrid identification and assignment. NEWHYBRIDS version 1.1 (Anderson & Thompson 2002) was used to categorize individual fish from each lake into benthics, limnetics or hybrids (F_1, F_2, limnetic or benthic backcross). This program implements a model-based Bayesian method that employs Markov chain Monte Carlo (MCMC) sampling to compute posterior probabilities that individuals in a sample (known to consist of pure individuals and recent hybrids of two species) fall into parental or different hybrid categories. Individuals were assigned to the category with the highest posterior probability. To minimize the effect of the over-dispersed starting values during the Monte Carlo simulation, we simulated 1000 sweeps of the Markov chain before data for the parameter estimation were collected from another 10^6 iterations. Three independent runs of the Markov chain, each of least 10^6 updates, were performed to assure convergence of the chain and homogeneity among runs. Differences in individual posterior probabilities between different runs of the Markov chain for Priest and Paxton lakes never exceeded 1%. Individual Enos values varied up to 4% but individual category assignment never changed.

NEWHYBRIDS assumes that any linkage disequilibrium or deviations from Hardy–Weinberg equilibrium (HWE) expectations are entirely the result of admixing of the 'parent' populations. The fulfilment of these assumptions in our data set was tested using the 'parent' benthic and limnetic subsamples from Priest and Paxton lakes, which excluded individuals identified as hybrids by NEWHYBRIDS. Enos Lake samples were excluded from this test as no 'pure' limnetics were detected from this lake's sample (see Results). Using a Markov chain method in GENEPOP version 3.3 (Raymound & Rousset 2001), the Fisher exact test revealed only 4 out of 300 tests between locus pairs within populations to be in genotypic linkage disequilibrium ($P < 0.05$, using the sequential Bonferroni procedure, Rice 1989), involving three locus pairs in three populations. Weir & Cockerham's estimator *f* (1984) of the inbreeding coefficient, F_{IS}, was tested at each locus within each population using FSTAT version 2.9.3 (Goudet 2001). About half of the tests showed significant deviations from genotypic frequencies expected under HWE [$P < 0.05$ in 27 out of 52 tests using the sequential Bonferroni procedure (Rice 1989)]. Populations had between four and nine significant single locus results. No locus was in Hardy–Weinberg

disequilibrium across all samples, although *Stn*216 was the only one showing no incidence of departure from HWE within any sample. These deviations from HWE are perhaps unsurprising, given the biased method used to select diagnostic markers. Indeed, two of these microsatellites are linked to known morphological quantitative trait loci; *Stn*216 is linked to a plate size modifier (Colosimo *et al.* 2004) and *Stn*43 is on linkage group four, near the plate morph major locus (Peichel *et al.* 2001; Colosimo *et al.* 2004).

The power of many new assignment-based methods has not been widely explored over different evolutionary scenarios or with markers exhibiting various levels of variation. We therefore ran simulations to test the ability of NEWHYBRIDS to detect hybrids given the level of polymorphism in our data set. We generated artificial multilocus hybrid genotypes between benthics and limnetics from Priest and Paxton lakes using HYBRIDLAB (see Nielsen *et al.* 2001), which draws alleles randomly from the observed allele frequency distribution for each population. The 'pure' parental populations used for this comprised individuals whose multilocus genotype was assigned to a parent population with a posterior probability exceeding 0.99 in the NEWHYBRIDS analysis (n = 92, 91, 93 and 74 for benthics and limnetics in Priest and Paxton lakes, respectively). Two different sets of hybrids were constructed for simulation: first, we created 10 F_1 hybrids alone for each species pair; second, we generated 10 each of F_1, F_2, and limnetic and benthic backcrosses, using the 'parents' for the F_1, as well as two simulated groups comprised of 100 F_1 for the latter three categories. These groups of 10 or 40 artificial hybrids, along with the 'parent' samples used to generate them, were subsequently analysed in NEWHYBRIDS, as described above, and the accuracy of their assignment assessed. Each type of simulation was repeated five times for each species pair. Simulations were not run using Enos Lake samples because no 'pure' limnetics from which to construct artificial hybrids were detected from this lake.

Factorial correspondence analysis (FCA) in GENETIX version 4.03 (Belkhir *et al.* 2001) was used to project individuals in microsatellite allele frequency space. By visualizing the relative similarity among all the samples in this way, the relative distribution of the hybrids identified by NEWHYBRIDS was presented. Ten of the 19 hybrids from Priest and Paxton lakes showed a smaller probability in NEWHYBRIDS of belonging to one of the two pure parental forms, in addition to the higher probability of belonging to their designated hybrid class. However, nine had a posterior probability of 1.00 for their hybrid class or had posterior probabilities distributed only amongst hybrid classes. In order to define two populations within each lake for the purpose of further analyses, we calculated which parental species the hybrids were genetically more similar to, using a likelihood-based Bayesian method of assignment (Rannala & Mountain 1997) in GENECLASS2 (Piry *et al.* 2004).

This method employed a Monte Carlo resampling algorithm (Paetkau *et al.* 2004) to compute the probability that each of the 19 hybrids belonged to either 'pure/parental' reference population. Based on 10 000 simulated individuals, we assigned hybrids to the closest one. These Priest and Paxton groupings of benthics and limnetics were used to analyse long-term gene flow. Given that no 'pure' limnetics were detected in Enos Lake, its fish were divided into a benthic and a hybrid group based on the NEWHYBRIDS results.

Long-term gene flow estimation. To complement the information on recent gene flow derived from hybridization rate estimates, we also sought comparative estimates of longer term gene flow (*m*). Such estimators derived from the simple mathematical relationship between gene flow and genetic differentiation [e.g. $F_{ST} = 1/(1 + 4N_e m)$, Wright 1931] have been widely criticized for their unrealistic ecological assumptions, such as constant population size, symmetrical migration and population persistence to enable genetic equilibrium (Whitlock & McCauley 1999). Furthermore, our data set is likely to overestimate F_{ST} (and so underestimate *m*) because of bias derived from using a set of diagnostic markers that were chosen to accentuate the species' differences relative to the average genetic marker.

Coalescence theory, which follows ancestral genealogies of samples opposed to modelling changes of gene frequencies in the entire population, has enabled the relaxation of many of these restrictive assumptions. Accounting for unequal migration rates and population sizes, the maximum-likelihood coalescent program MIGRATE version 2.0 (Beerli & Felsenstein 1999) measures long-term gene flow. We used the microsatellite model to simultaneously estimate the effective population size (N_e) and the proportion of migrants (*m*) for each population. The microsatellite threshold, which specifies the window in which probabilities of change between allelic states are calculated, was set at 15 repeat units to ensure that all allelic ranges were encompassed in the analysis. A mutation rate of 10^{-4} (Feldman *et al.* 1999) was assumed, which seems reasonable given that the most extensive assessment of microsatellite mutation rate in fish yielded an estimate of 1.5×10^{-4} (Shimoda *et al.* 1999). Summing the two unidirectional estimates of N_e gives the total N_e for each species pair, while dividing the sum of the two $N_e m$ estimates by total N_e calculates the total *m*. Approximately 95% confidence intervals for estimates of N_e and *m* were generated from a summary of profile likelihood percentiles of all parameters.

Results

Species diagnostic molecular profiles

The screening of 288 microsatellites identified 84 and 103 loci with potentially NOA (nonoverlapping allele) ranges

between benthic and limnetic pools of DNA from Paxton and Priest lakes, respectively. Further screening of eight benthics and eight limnetics at 84 (Paxton) and 18 (Priest) of these loci, revealed four and two markers, respectively, that showed NOA ranges between benthics and limnetics. This pattern was verified by genotyping all individuals from the entire lake's panel, with only a few exceptional instances of individuals carrying an allele that was from the range of the other species (Table 1). These exceptions were assumed to be a signature of introgression, although the possibility of incomplete assortment of ancestral polymorphism cannot be excluded. The alternative strategy designed to conserve the historical Enos Lake DNA resources found 23 potentially discriminatory loci from the 192 microsatellites screened. Of these, five distinguished between benthics and limnetics upon further analysis (Table 1).

Hybridization and long-term gene flow within species pairs

Out of 198 individuals from Priest Lake, NEWHYBRIDS categorized 92 as pure benthics ($P \geq 0.99$) and 97 as pure limnetics ($P = 0.62–1.00$; Fig. 2). Ninety of these limnetics had a posterior probability exceeding 0.99. The remaining seven with posterior probabilities < 0.99 also had a lower posterior probability of belonging to a hybrid category ($P = 0.01–0.38$). Nine hybrids yield a hybridization rate for Priest Lake species pair of 4.5%. All of the hybrids were categorized as F_2 ($P = 0.67–1.00$), although seven of them also had a lower posterior probability of belonging to another hybrid category ($P = 0.02–0.33$). In contrast, only three had a posterior probability ($P = 0.04−0.24$) of belonging to one or other of the parental populations, with six having a probability 0.00 of being either pure benthic or limnetic. That is, two-thirds of the hybrids have probability 1.00 of being hybrids of some sort. GENECLASS2 assigned three hybrids to the limnetic reference population and six to the benthic one.
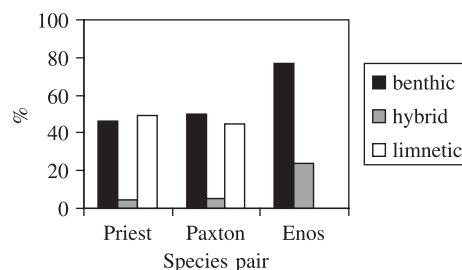
Fig. 2 Categorization of three-spined sticklebacks sampled from Priest (*n* = 198), Paxton (*n* = 192) and Enos lakes (*n* = 192) in 2003 using a model-based Bayesian method implemented by NEWHYBRIDS (Anderson & Thompson 2002).

In Paxton Lake, 96 out of 192 individuals were categorized as pure benthics ($P = 0.79$–1.00) and 86 as pure limnetics ($P = 0.74$–1.00; Fig. 2). Ninety-three of these benthics and 74 of these limnetics had a posterior probability exceeding 0.99. The remaining three benthics and 12 limnetics with posterior probabilities < 0.99 also had a lower posterior probability of belonging to a hybrid category ($P = 0.01$–0.18). Ten hybrids yield a hybridization rate for Paxton Lake species pair of 5.2%. Eight of them categorized as F_2 ($P = 0.47$–1.00) and two as benthic backcrosses ($P = 0.72$ and 0.85), although nine of them also had a lower posterior probability of belonging to another hybrid category (three with $P \leq 0.10$, six with $P > 0.10$). In contrast, six showed generally lower probabilities of belonging to one or other of the parental populations (four with $P < 0.10$, two with $P > 0.10$), and four had a probability 0.00 of being either pure benthic or limnetic. That is, 80% of the hybrids have probability greater than 0.90 of being hybrids of some sort. GENECLASS2 assigned half of them to each parental reference population.

While 146 out of 192 individuals from Enos Lake were categorized as pure benthics ($P = 0.49$–1.00), no pure limnetics were recognized (Fig. 2). Furthermore, a smaller portion was assigned with high posterior probabilities compared to Priest and Paxton lakes; only 56% of those categorized as pure benthics had a posterior probability greater than 0.99, compared to 100% for Priest benthics and 97% for Paxton benthics. All 46 hybrids were categorized as F_2 ($P = 0.45$–1.00), although 40 of these also had a lower posterior probability of belonging to one ($n = 35$) or two ($n = 5$) other hybrid categories ($P \leq 0.29$): only one hybrid had a lower probability of being F_1; seven had a lower probability of being a limnetic backcross; and 36 had a lower probability of being a benthic backcross. Fewer hybrids showed any chance of belonging to one or other of the parental populations; 25 out of 46 hybrids had a lower posterior probability of being pure benthic (11 with $P \leq 0.10$, 14 with $P \geq 0.10$) whilst only one had a probability of being pure limnetic ($P = 0.04$). That is, 70% of the hybrids have a probability greater than 0.90 of being hybrids of some sort.

These contrasting levels of hybridization detected within the three lakes are visualized by a two-dimensional FCA (Fig. 3). Although the two axes describe only 7.5% and 8.1% of variability within Priest and Paxton species pairs, respectively, the distinction of two clusters with few intermediate (hybrid) fish is clear. This visualization agrees with the low level of hybridization detected within these lakes, as well as the high posterior probabilities assigned to the vast majority of 'pure' samples by NEWHYBRIDS. In contrast, Enos Lake individuals form one diffuse cluster, reflecting the high level of hybridization and introgression of the limnetic form into that of the benthic within this lake. A greater variability amongst the hybrids relative to the benthics is consistent with their having a wider array

of genotypic classes from multiple generations of hybridization and introgression.

The robustness of NEWHYBRIDS to deviations from ideal model conditions was tested by looking at assignment patterns of known hybrids. Five repeats of simulations including 10 F_1 hybrids along with the 'parent' samples resulted in all samples being correctly assigned with very high posterior probabilities ($P > 0.99$). The simulations that included 10 hybrids of each of the hybrid categories F_1, F_2, and limnetic and benthic backcrosses, as well as the 'parent' samples, also resulted in correct assignment of all F_1 (Table 2, $P > 0.77$ with some also showing a lower probability of belonging to other hybrid, but not parental, categories). While there were very few instances of mis-assigned parents (< 1% of parents from each species pair were misassigned as a backcross), there were more mis-assignments of the second-generation hybrids: 30% and 20% of F_2 from Priest and Paxton lakes, respectively, were wrongly assigned to another hybrid category while 19% and 28% of backcrosses from these lakes were wrongly assigned to another, usually parental, category (Table 2). Only some backcrosses had a posterior probability ($n = 37$ from both species pairs) of belonging to one or other of the parental populations, with the vast majority of simulated hybrids (82% for each species pair) having a probability 0.00 of being either pure benthic or limnetic, i.e. these hybrids have probability 1.00 of being hybrids of some sort.

Using the populations of benthics and limnetics (or hybrids in the case of Enos Lake) within each lake defined by NEWHYBRIDS and GENECLASS2, MIGRATE estimated N_e to be remarkably consistent across species and lakes, at approximately 1000 individuals per population (Table 3). In contrast, long-term gene flow estimates (m) varied four-fold among lakes (Table 3): while migration was relatively symmetrical within Priest and Paxton lakes, an overall estimate for the Priest Lake species pair was less than half that for the Paxton Lake one; Enos Lake sticklebacks exhibited migration levels that were more than four times greater than those found in Priest Lake, and nearly two times greater than those found in Paxton Lake.

Discussion

Development of species diagnostic molecular profile from targeted exploration of genome linkage map

Two strategies that we explored proved effective at targeting diagnostic markers. Using selected samples to screen a large collection of microsatellite loci, we minimized the time and resources expended to identify a suite of micro-satellites that can unambiguously distinguish between benthics and limnetics in each of the extant sympatric species pairs of three-spined sticklebacks in British Columbia. This was a successful strategy even when baseline samples
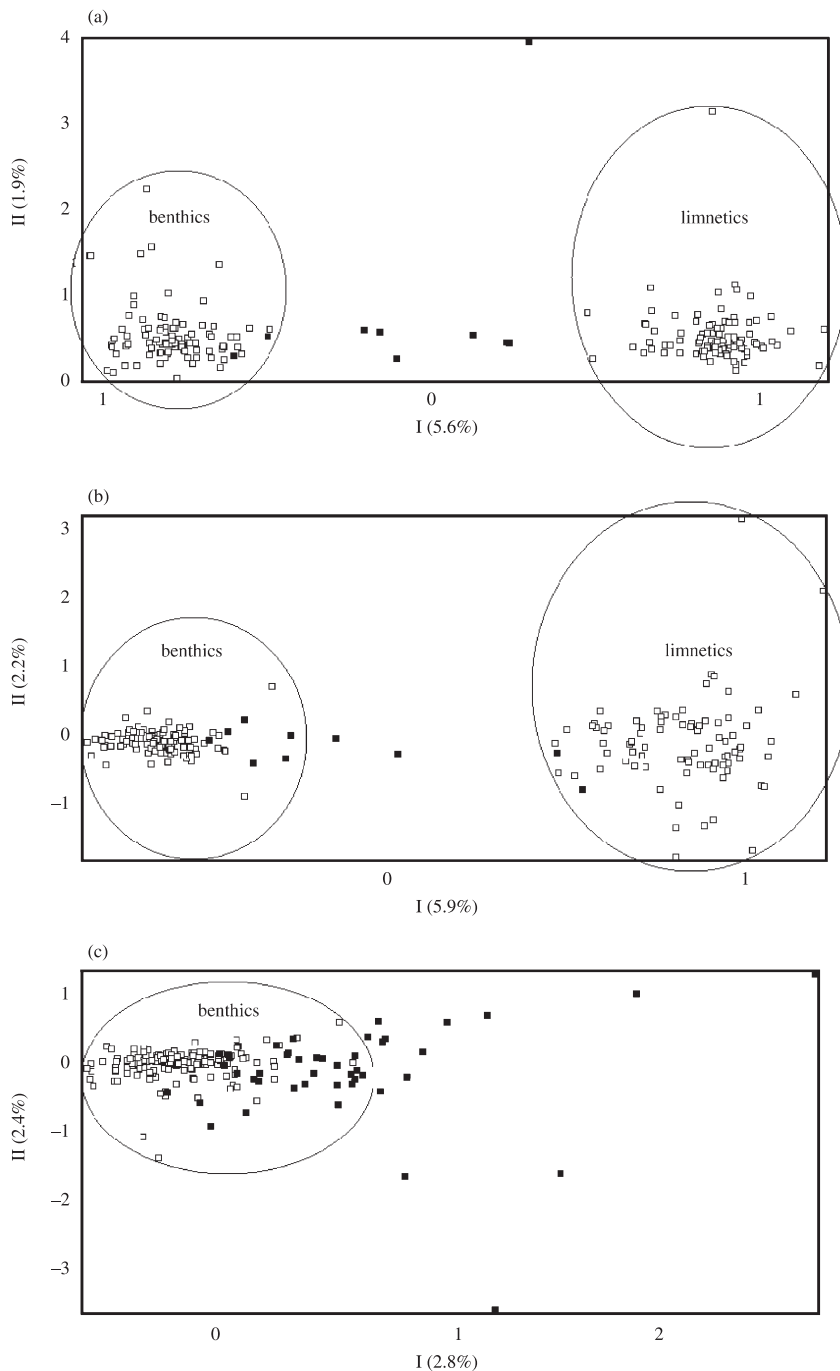
Fig. 3 Two-dimensional Factorial correspondence analysis illustrating relationships among the multilocus genotypes of individual three-spined sticklebacks from (a) Priest, (b) Paxton and (c) Enos lakes. I and II are the first and second principal factors of variability, respectively. Large circles encompass individuals categorized as 'pure' benthic or limnetic by NEWHYBRIDS, and filled symbols represent hybrids.

were limited in number, as was the case for the Enos Lake species pair.

This approach selected markers that showed accentuated differences between species relative to the average marker. These patterns of allele frequency and range differences between benthics and limnetics within species pairs may simply be the outcome of genetic drift. Another possibility, however, is that they may be linked to loci under selection, with hitch-hiking on nearby loci subject to

selective sweeps having driven the current differences in allele ranges and frequencies (Maynard Smith & Haigh 1974). Indeed, two of the microsatellites from the species diagnostic molecular profile (*Stn*43 and *Stn*216) are linked to known morphological quantitative trait loci (Peichel *et al*. 2001; Colosimo *et al*. 2004). Both these and four other microsatellites from the species diagnostic molecular profile show extremely restricted allele ranges (one to three alleles) in one member of the species pair. It is conceivable

Table 2 Number of misassigned individuals during NEWHYBRIDS simulations. Categories of samples of known origin include: limnetic (L) or benthic (B) parent, F_1, F_2, and limnetic (LBx) or benthic (BBx) backcross from Priest (Pr) and Paxton (Pa) lakes. Total sample sizes of each category run during five repeated simulations for each species pair included in parenthesis

Sample origin	Number and category of misassignments for species pair:	
	Priest	Paxton
Parents:	3 BBx	6 BBx
B (460 Pr, 455 Pa) and L (465 Pr, 370 Pa)		
F_1 (50)	0	0
F_2 (50)	15 BBx	8 BBx
		2 F_1
LBx (50) and BBx (50)	6 L	15 L
	9 B	3 B
	3 F_1	10 F_1
	1 F_2	

that this pattern is the product of selection via hitch-hiking. While demographic bottlenecks could also produce such a pattern, they would do so across the entire genome rather than be restricted to specific regions (Schlötterer 2003). Two of the loci were identified from the screening to have NOA ranges in two of the three species pairs (*Stn*387 and *Stn*254 for both Priest and Enos lakes), which also argues against the sole role of genetic drift in producing these patterns. Genetic drift is unlikely to produce repetitive shifts in the same direction under a specific environmental setting, given the independent origin of each pair (Taylor & McPhail 1999, 2000). Instead, this pattern of a locus being discriminatory in more than one species pair is suggestive of parallel evolution (Schluter & Nagel 1995). This speculation, however, awaits a more rigorous statistical investigation as other processes, such as genomic variation in

recombination and mutation rate, could also generate the observed patterns.

Regardless of the processes driving these differences in microsatellite allele ranges between benthics and limnetics, biasing marker selection towards those that most clearly distinguished between them has enabled us to overcome previous limitations of genetically distinguishing between such evolutionarily young species. As codominant markers, these microsatellites can now be used not just as simple diagnostic markers but can serve as powerful population genetic tools. Many pertinent questions about the ecological and genetic basis of adaptive divergence and speciation can now be addressed in sticklebacks, which serve as excellent models for these studies (e.g. Coyne & Orr 2004). The utility of sticklebacks is demonstrated in this study by our exploration of the interactions between species through a quantification of hybridization rates and levels of gene flow within each species pair.

Hybridization rates and gene flow within each species pair

The genetic assessment of hybridization rates within each lake revealed two contrasting scenarios. Priest and Paxton lakes exhibited a remarkably similar, relatively low level of natural hybridization of about 5%, with no significant bias in the direction of introgression. In contrast, our study provides further evidence for a continued breakdown of the Enos Lake species pair into a hybrid swarm (see Taylor *et al.* in press), with pronounced biased introgression of limnetics into the benthic population.

The results for Priest and Paxton lakes support earlier estimates of low hybridization rates (1–2%) within species pairs based on morphological criteria (McPhail 1984, 1992). Although our estimates are slightly higher than these earlier ones, McPhail (1992) recognized the potential for underestimating hybridization rates based on morphology due to the poor ability to detect backcrosses. The NEWHYBRIDS

Table 3 Long-term gene flow estimates between benthics (B) and limnetics (L) in Priest and Paxton lakes, and between benthics and hybrids (H) in Enos Lake. Population estimates calculated using a maximum-likelihood coalescent method implemented by MIGRATE (Beerli & Felsenstein 1999) are summed to estimate the total effective population size (N_e); the total proportion of migrants (m), highlighted in bold print, is calculated by dividing the sum of the two $N_e m$ estimates by the total N_e. Approximate 95% confidence intervals are given in parenthesis for population estimates of N_e and m

Lake	N_e			m		
	L or H	B	Total	L or H	B	Total [$N_e m$(L or H) + $N_e m$(B)/N_e(Total)]
Priest	915	1334	2249	0.00092	0.00062	0.84182 + 0.82799/2249 = **0.00074**
	(873–930)	(1272–1356)		(0.00083–0.00095)	(0.00056–0.00064)	
Paxton	908	1160	2068	0.00205	0.00156	1.86092 + 1.80840/2068 = **0.00177**
	(864–924)	(1110–1178)		(0.00192–0.00209)	(0.00147–0.00159)	
Enos	926	1342	2268	0.00417	0.00259	3.86246 + 3.47533/2268 = **0.00324**
	(868–947)	(1295–1358)		(0.00398–0.00424)	(0.00247–0.00263)	

methodology enabled the detection of two generations of hybrids (F_1, F_2, and limnetic or benthic backcrosses). Additionally, this study looked at the hybridization rate amongst subadult fish, and a reduction in the number of hybrids found amongst sexually mature adults may be expected if the ecologically based postzygotic isolation that has been detected experimentally (Schluter 1995; Hatfield & Schluter 1999) is in force within the natural populations.

The accuracy of the NEWHYBRIDS analysis is supported, first by the generally very high posterior probabilities with which individuals were assigned within Priest and Paxton lakes. Levels of potential misassignment between hybrid and parental groups were low, with fewer than 4% of individuals from Priest Lake being assigned with less than 0.95 probability, and fewer than 9% in Paxton Lake. In addition, these potential misassignments were evenly distributed amongst the parent and hybrid groups (three and five fish from parent vs. hybrid groups in Priest Lake species pair, and nine vs. eight for the same groupings in Paxton Lake). Second, this program assigned samples of known origin with generally very high probabilities during simulations, and was found to be robust to violation of the assumption of HWE prior to hybridization (which was approximated by testing for HWE in parental data subsets). There were very few instances of parents being misassigned as hybrids (less than 1%) but there was a higher rate of the opposite scenario, with 33 second-generation hybrids being wrongly assigned to a parental category (8% of all simulated hybrid assignments). Therefore, any deviation of hybridization rate estimates from the true value is likely to be an underestimate.

Generally, lower posterior probabilities were obtained for Enos Lake individuals compared to Paxton or Priest Lake fish. Although the lack of baseline samples (ethanol-preserved material prior to extensive introgression) makes it difficult to verify, this is likely the outcome of introgression that has occurred beyond a second generation of hybrids (Taylor *et al.* in press) reducing the assignment power of NEWHYBRIDS. As the number of generations over which introgression has been occurring increases, the number of possible genotype frequency classes to which an individual may belong increases exponentially and distinguishing between them becomes increasingly difficult, with a prohibitive amount of data required (Boecklen & Howard 1997; Anderson & Thompson 2002).

Our estimates of long-term gene flow support this idea of gene exchange between benthics and limnetics in each species pair. Given that the markers used accentuate differences between species relative to the average neutral marker, these estimates may underestimate true levels of gene flow. Nevertheless, the relative comparison between lakes gives insight into patterns of gene flow. In agreement with the estimates of hybridization rates, gene flow was highest within Enos Lake, although a long-term migration rate of 0.3% is 80 times lower than the current hybridization rate estimate of 24%. Similarly, long-term gene flow estimates within Priest and Paxton lakes are over an order of magnitude less (61 and 29 times lower, respectively) than current hybridization estimates. With the lowest levels of gene flow recorded in Priest Lake, the Paxton Lake pair has a value intermediate between the other two species pairs.

Evolutionary implications

The contrasting scenarios found between species pairs raise the question as to what processes are controlling the rates of hybridization and levels of gene flow. The findings within Priest and Paxton Lake species pairs support experimental evidence that strong assortative mating is playing a significant role in limiting hybridization (Ridgway & McPhail 1984; Nagel & Schluter 1998). Our demonstration of a low background level of hybridization, however, shows that this premating reproductive isolation is incomplete.

Despite this hybridization, however, there is no evidence of extensive introgression of these hybrids within Priest and Paxton lakes. Indeed, there is over an order of magnitude of difference between the hybridization rate estimates, which reflect recent gene flow, and the lower long-term estimates of gene flow. These long-term estimates may underestimate true levels of gene flow to a certain degree since biased markers that accentuated species differences were used. Nevertheless, congruence in the magnitude of N_e and m between our MIGRATE estimates and those derived from alternative methods applied to independent data sets supports the validity of our results and the conclusion that levels of long-term gene flow are, indeed, much reduced compared to current hybridization rates. First, N_e estimates of a similar magnitude as those derived from MIGRATE analysis are obtained when the commonly assumed N_e/N_c ratio of 0.1 is applied to mark–recapture population census size estimates (B. Matthews, M. Nomura & D. Schluter, unpublished) from Enos Lake [benthics: N_e = 3700 (95% CI 3000–4700) and limnetics: N_e = 2200 (95% CI 1200–7700) from 1999 and 2000] and Paxton Lake [benthics: N_e = 2900 (95% CI 2300–3900) and limnetics: N_e = 5900 (95% CI 3500–10200), from 2005]. Second, applying Wright's (1931) infinite island model [$F_{ST} = 1/(1 + 4N_em)$] to previous F_{ST} estimates yields total N_em estimates (Taylor & McPhail 2000; 1.892, 1.847 and 1.892 for Priest, Paxton and Enos lake species pairs, respectively) that are similar in magnitude to those derived from MIGRATE (1.67, 7.337 and 3.618 for Priest, Paxton and Enos lake species pairs, respectively). These F_{ST} estimates, derived from a population genetic survey of six (seemingly) neutral microsatellites that conform to Hardy–Weinberg expectations (Taylor & McPhail 2000), represent the only other gene flow estimates for the species pairs to date.

This trend of lower longer term gene flow supports experimental work suggesting that selection is acting against hybrids. Field enclosure experiments found that reduced F_1 hybrid growth rates in both parental habitats is likely due to reduced foraging efficiency (Schluter 1995; Hatfield & Schluter 1999). The ecological basis of this postzygotic isolation was confirmed by a similar experiment using backcrosses which controlled for intrinsic factors (Rundle 2002). Sexual selection against hybrid males has also been implicated by the reduced mating success of F_1 hybrid males in their preferred nesting habitat compared to limnetics, the parental species sharing the same nesting preference (Vamosi & Schluter 1999). Collectively, this work supports the view that postzygotic reproductive isolation via reduced hybrid fitness is important in maintaining distinct gene pools in sympatry. Indeed, this has been suggested by experimental work supporting the role of reinforcement in the divergence of the species pairs (Rundle & Schluter 1998).

The detection of only F_2 hybrids and backcrosses within each species pair is unexpected, given that F_1 hybrids are, of course, essential to their production. It is feasible that F_1 hybrids may occur at lower frequency relative to other, more abundant hybrid categories, and so have evaded detection in this survey. Although there is no evidence to support intrinsic selection against any hybrid class [with the possible exception of benthic backcrosses (McPhail 1984, 1992; Hatfield & Schluter 1999)], the pattern of hybrid abundance observed here could reflect extrinsic selection directed primarily against F_1 hybrids. Indeed, if divergent selection against hybrids plays an important role in maintaining species integrity, then phenotypically intermediate individuals (F_1) would be expected to fare worse than those hybrids that are more parental-like (second generation hybrids; Hatfield 1997); the latter would be better able to exploit parental niches. Indeed, reciprocal field enclosure experiments show just this, with F_1 hybrids showing a significant growth disadvantage relative to the parent adapted to the environment (Hatfield & Schluter 1999), while neither backcross differed significantly from the parent from which it was mainly derived (Rundle 2002). Although misassignment of hybrid category cannot be excluded, the NEWHYBRIDS simulations consistently assigned F_1 hybrids correctly with high probabilities, corroborating this hybrid assignment pattern as a real biological phenomenon. This view is also supported by a review of hybrid fitness which consistently found significant variation in the relative fitness of hybrid classes (Arnold & Hodges 1995). Indeed, some experimental studies have directly demonstrated lower F_1 hybrid viability relative to other hybrid classes (e.g. Reed & Site 1995). Furthermore, indirect evidence of this comes from other empirical studies that have also observed a low level of F_1 hybrids relative to post-F_1 hybrid categories in natural hybrid populations (e.g. Arnold 1994; Redenbach & Taylor 2003; Ostberg et al. 2004).

The forces driving the demise of natural hybrids in Paxton and Priest lakes are still unknown. The species diagnostic molecular profile that we have described here can now be used to tackle this question. For the first time, these genetic tools will enable us to assess hybridization rates temporally across the various life history stages of the stickleback, overcoming limitations of morphological methods to distinguish between immature benthics and limnetics. A decrease in the relative number of hybrids throughout the stickleback life cycle would provide compelling evidence for ecological selection against hybrids playing a significant role in postzygotic isolation, while a consistent proportion of sexually mature hybrids would support a role for sexual selection against hybrids in reinforcement. While not defining precise mechanisms of selection against hybrids, assessing their existence and relative contributions within natural populations would give valuable insight into their role in speciation, an area where empirical tests are lacking (Rundle & Nosil 2005).

Whatever the mechanism of selection, the prerequisite conditions for it have now been altered in Enos Lake, such that postzygotic isolating mechanisms are no longer effective at maintaining divergence between the species. The balance between hybridization and selection has tilted towards increased levels of gene flow, resulting in a breakdown of the species pair into a hybrid swarm. The collapse of reproductive isolating mechanisms is likely due to environmental change within the lake. An account of the possibilities accompanies a full description of the demise of this species pair elsewhere (Taylor et al. in press). This breakdown has been asymmetrical, with biased introgression of the limnetic form into that of the benthic. The cause of this directionality awaits investigation, i.e. do limnetic females now mate preferentially with benthic or hybrid males, or are limnetic males managing to mate successfully with benthic or hybrid females? Patterns of mitochondrial DNA inheritance in hybrid lines have tested for directionality in other fish from this region (Redenbach & Taylor 2003; Ostberg et al. 2004; Bettles et al. 2005). Unfortunately, the utility of this marker for this purpose in the evolutionarily young benthic–limnetic system is limited by a lack of clear distinction between the mitochondrial DNA of the two forms within each lake (Taylor & McPhail 1999; J. L. Gow, unpublished), caused either by historical introgression or incomplete lineage sorting. Circumstantial evidence suggesting that limnetic females mating preferentially with benthic or hybrid males may be the predominant mode of hybridization includes: (i) the known role of visual cues in stickleback mate choice (Ridgway & McPhail 1984); (ii) female perceptual sensitivity to red light diverging according to habitat differences in light environment, and male nuptial colour being tuned to this female perceptual

sensitivity (Boughman 2001); and (iii) suspected (although unsubstantiated) increased turbidity in Enos Lake. Clarification of the mechanism behind the observed directionality may be best addressed by mating trials conducted under the altered environmental conditions.

The breakdown of the species pair within Enos Lake highlights the delicate balance between persistence and breakdown of reproductive barriers between young species, where prezygotic isolation and extrinsic postzygotic isolation are typically thought to evolve before intrinsic postzygotic isolation (Coyne & Orr 2004). This collapse via elevated hybridization following anthropologically induced environmental change in lake conditions (Taylor *et al.* in press) highlights a serious threat to freshwater fish faunas (e.g. Seehausen *et al.* 1997; Bettles *et al.* 2005). The demise of the benthic–limnetic species pair in Enos Lake is not the first recorded; the Hadley Lake species pair on Lasqueti Island became extinct following human habitat disturbance. In this instance, the fish were exterminated by an introduced non-native catfish, *Ameiurus nebulosus* (Hatfield 2001). Intriguingly, there is also some evidence of historical introgression within Paxton Lake species pair; the higher level of long-term gene flow but the same level of hybridization estimated in this species pair compared to that of Priest Lake could be indicative of a recovery from historical introgression. Such a phenomenon would correlate with a known history of human-induced, major environmental change. For over 20 years, from the late 1950s to the late 1970s, the lake level varied greatly due to annual draw down for quarry-mining purposes (Larson 1976; McPhail 1992). Furthermore, 5000 coho salmon (*Oncorhynchus kisutch*) were introduced into the lake during this period and became significant stickleback predators before their extinction 5 years later (Larson 1976; McPhail 1992). The higher proportion of intermediate morphological forms during, rather than after, this period of disturbance (McPhail 1992) supports the hypothesis that such environmental change may have triggered increased hybridization rates.

If this was indeed the case, then the subsequent recovery of the species pair after the termination of the human disturbances should be heartening to conservation efforts for the Enos Lake species pair. Given the sensitivity of these highly endemic young species to environmental change, as well as their importance as scientific models for the study of adaptive divergence and speciation (see Coyne & Orr 2004; Rundle & Nosil 2005), the benthic–limnetic species pairs are now listed as endangered. The species diagnostic molecular profile developed here is now serving as a tool to monitor the population status of these endangered species (COSEWIC 2004). They can also aid in more proactive conservation management by helping to select the most 'benthic-like' and 'limnetic-like' Enos Lake fish for use in captive breeding programmes designed to artificially produce offspring for use in possible re-introductions.

In conclusion, species diagnostic marker profiles can be applied to answer an array of biological questions in populations of this geographically widespread and phenotypically diverse model organism. Given that the approach we used successfully identified diagnostic markers for evolutionarily young species (about 13 000 years old), a similar methodology would likely uncover discriminatory markers useful to assessing gene flow in other species pairs of sticklebacks which have diverged over longer periods, such as the sympatric Japan Sea system, or over a similar time frame, including parapatric freshwater-anadromous and lake-stream systems (McKinnon & Rundle 2002; Hendry & Taylor 2004). Such tools could also be beneficial to experimental approaches exploring ways in which selection, gene flow and adaptive divergence interact in natural populations over multiple generations.

Acknowledgements

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