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Nested biological variation and speciation

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The modes of speciation that are thought to have contributed most to the generation of biodiversity require population differentiation as the initial stage in the speciation process. Consequently, a complete understanding of the mechanisms of speciation requires that the process be examined not just after speciation is complete, or nearly so, but also much earlier. Because reproductive isolation defines biological species, and it evolves slowly, study of the process may require a prohibitive span of time. Even if speciation could be observed directly, selection of populations in the process of speciation is typically difficult or impossible, because those that will ultimately undergo speciation cannot be distinguished from those that will differentiate but never assume the status of new biological species. One means of circumventing this problem is to study speciation in taxa comprising several sibling species, at least one of which exhibits extensive population differentiation. We illustrate this approach by exploring patterns of population variation in the post-glacial radiation of the threespine stickleback, *Gasterosteus aculeatus*. We focus on lacustrine populations and species within this complex, demonstrating parallel axes of divergence within populations, among populations and among species. The pattern that emerges is one of parallel relationships between phenotype and fitness at all three hierarchical levels, a pattern that facilitates exploration of the causes and consequences of speciation and secondary contact. A second outcome of this exploration is the observation that speciation can be the consequence of a cascade of effects, beginning with selection on trophic or other characteristics that in turn force the evolution of other population characteristics that precipitate speciation. Neither of these conclusions could have been reached without comparative studies of wild populations at several hierarchical levels, a conclusion reinforced by a brief survey of similar efforts to elucidate the process of speciation. We address the issues most likely to be resolved using this approach, and suggest that comparisons of natural variation within taxa at several hierarchical levels may substantially increase our understanding of the speciation process.

Keywords: *Gasterosteus aculeatus*; threespine stickleback; population differentiation; sympatric; allopatric; reinforcement

1. INTRODUCTION

Widespread species typically comprise mosaics of genetically differentiated populations that are often, but not always, linked by gene flow. The pattern of geographic variation is reflective of spatial variation in patterns of natural selection and gene flow, in combination with the effects of sampling drift, and is expected given the dynamic nature of evolutionary processes. Over the last half century, population geneticists have made extensive use of this genetic structuring within species to explore the mechanisms of microevolutionary change, often with success (for reviews, see Endler 1977, 1986). However, studies of differentiated populations have rarely been incorporated into research on speciation even though population differentiation is envisioned as the first stage in speciation in the most widely favoured speciation models (Verrell 1998).

The speciation models that invoke geographic variation as the earliest step in the generation of biodiversity comprise a set of models that differ with respect to the level of gene flow among the sites at which differentiation is occurring. The geographical or allopatric model described by Mayr (1963) is at one extreme, allowing for no gene flow among the differentiating populations. The populations are assumed to evolve independently of one another, as a consequence of natural selection and sampling drift. Reproductive isolation between the populations, which we use to define species for the purposes of this discussion, evolves as an incidental outcome of genetic changes in the populations. The remaining models in this group, the 'divergence-with-gene-flow' models (*sensu* Rice & Hostert 1993), allow varying levels of gene flow, and differing spatial patterns of divergent selection (reviewed by Maynard Smith 1966; Endler 1977; Felsenstein 1981). Reproductive isolation between the subpopulations is assumed to evolve gradually, largely as a consequence of divergent selective regimes. At one end of the continuum, geographically separated subpopulations experience dramatically different selective regimes and little gene flow (parapatry). At the other end is sympatric speciation, in which disruptive selection within a single population results in reproductive isolation between two resultant subpopulations.

In this general family of models, as in the geographical model, reproductive isolation is a by-product of genetic divergence between subpopulations. If this isolation is incomplete when secondary contact occurs (geographical model, and the parapatric end of the 'with-gene-flow' continuum), the populations may interbreed with one of two outcomes. A single gene pool may result, or reinforcement may complete the speciation process, producing two biological species. Reinforcement is said to occur when heterotypic matings produce low-fitness offspring, thereby selecting for positive assortative mating and prezygotic isolation (Dobzhansky 1937). In recent years this mode of speciation has proven particularly controversial, although several recent studies have lent support to its existence (Coyne & Orr 1989, 1997; Howard 1993; Butlin 1995; Noor 1995; but, see also Rice & Hostert 1993).

A complete understanding of the most common mechanisms of speciation requires that the process be examined before reproductive isolation evolves, if population differentiation is integral during the initial stages of speciation. The problem becomes one of distinguishing differentiated populations that will ultimately become biological species (*sensu* Mayr 1942), from those that will not. Even if populations likely to speciate could be identified, there remains the problem that reproductive isolation evolves so slowly that direct observation may be impossible. Evaluation of the degree of reproductive isolation between population pairs, in combination with knowledge of geographic distributions and genetic distances, has offered insight into the rates of evolution of pre- and post-zygotic isolation, and into the role of reinforcement in speciation (Coyne & Orr 1989, 1997; Tilley *et al.* 1990). However, the causes of divergence and reproductive isolation cannot be examined in this strictly comparative manner. For cause to be inferred, laboratory manipulation or *in situ* studies of naturally occurring divergent populations are needed.

Here we suggest that studies of divergent natural populations *in situ* are essential if we are to understand the causes of speciation, especially if we wish to understand the role of local adaptation and sexual selection in promoting reproductive isolation. We are not the first to make this suggestion. Pleas for such research have appeared repeatedly (e.g. Mayr 1963; Lewontin 1974; Endler 1989; McPhail 1994; Verrell 1998), but have gone largely unheeded. We suspect this is because of the difficulty of selecting appropriate populations and the time-consuming nature of the research. One means of circumventing these problems is to select a taxon for study that comprises two or more sibling species, which exhibit population differentiation that mirrors the differences between the species. The presence of variation within single populations, which parallels the variation among populations and species, provides an especially strong framework for making inferences about speciation. When such parallel variation exists, comparisons of the relationship between fitness and phenotype across the levels can be used to infer the cause of at least one speciation event within the group.

Freshwater fishes in postglacial lakes offer some of our best opportunities for evaluating the speciation process through comparison at several hierarchical levels. They typically reside in lakes that are depauperate in species, and that are comparatively young, as indicated by the dates of the most recent glacial recession in the regions of the northern hemisphere affected by the last glacial advance (McPhail & Lindsey 1986; Bell & Foster 1994; McPhail 1994; Schluter 1996). Closely related species are sometimes found within lakes where they exhibit morphological and trophic differences that parallel those observed within and across populations (reviews in Schluter & McPhail 1993; McPhail 1994; Robinson & Wilson 1994; Skúlasson & Smith 1995; Smith & Skúlasson 1996; Bell & Foster 1994; Schluter 1996; Bell & Andrews 1997). The example we offer, that of the threespine stickleback species complex, is unusual even among postglacial fishes. Within this complex there exists compelling evidence of repeated parallel evolution of allopatric ecotypes, and of sympatric species pairs which the allopatric ecotypes resemble (McPhail 1993, 1994; Schluter & McPhail 1992, 1993; Bell & Foster 1994; Schluter & Nagel 1995). There is also at least one case of parallel within-population polymorphism (Cresko & Baker 1996). All appear to have been drawn from a common marine ancestor that is generally intermediate between the ecotypes (Schluter & McPhail 1992; McPhail 1993; Walker 1997), and much of the lacustrine diversification can be interpreted in an ecological context, including the evolution of the species pairs.

2. THE THREESPINE STICKLEBACK SPECIES COMPLEX

The threespine stickleback fish, *Gasterosteus aculeatus*, has undergone a remarkable endemic radiation in freshwater habitats in recently deglaciated regions of north-western North America, Scotland and other less well-studied regions of the northern hemisphere. Restricted primarily to marine, brackish and coastal freshwater habitats in temperate and arctic regions, this small fish ($\leqslant 10$ cm standard length (SL)) must have been absent from glaciated parts of its range at the last glacial maximum (for reviews, see Bell & Foster 1994; McPhail 1994). As the glaciers began to recede 22 000 years ago (less in some areas; McPhail 1994), marine and/or anadromous stickleback colonized the new freshwater habitats formed after isostatic rebound, giving rise not only to a diverse array of freshwater populations (figure 1), but also to new species (McPhail 1984, 1993, 1994; Schluter & McPhail 1993).

The radiation of threespine stickleback in recently deglaciated freshwater habitats is particularly intriguing because plesiomorphic character states can be inferred with unusual assurance. This is because the morphology of marine and anadromous sticklebacks has changed little in the last 11 million years, as indicated by fossil remains (Bell 1977, 1994), and because these two life history types differ little in morphology across their ranges (Bell & Foster 1994). Similarly, there appears to be little differentiation of behaviour across the ranges of these forms (Foster 1995; see below). Allozyme and mtDNA sequence data indicate that there exist two major clades of threespine stickleback. A set of populations ranging from Japan, along the coasts of Russia and Alaska, and south to the Queen Charlotte Islands of British Columbia form a sister group to all other *G. aculeatus*, which in turn are separated into monophyletic groups in the Atlantic and Pacific basins (Haglund *et al.* 1992; Buth & Haglund 1994; Orti *et al.* 1994). The clades are morphologically cryptic, and the dynamics of the interactions between them are not well-explored where the two overlap. The species pairs we discuss here are out of the range of the Northern Pacific clade, which cannot therefore have contributed to their evolution (Buth & Haglund 1994; Orti *et al.* 1994; Taylor *et al.* 1998; Thompson *et al.* 1998).

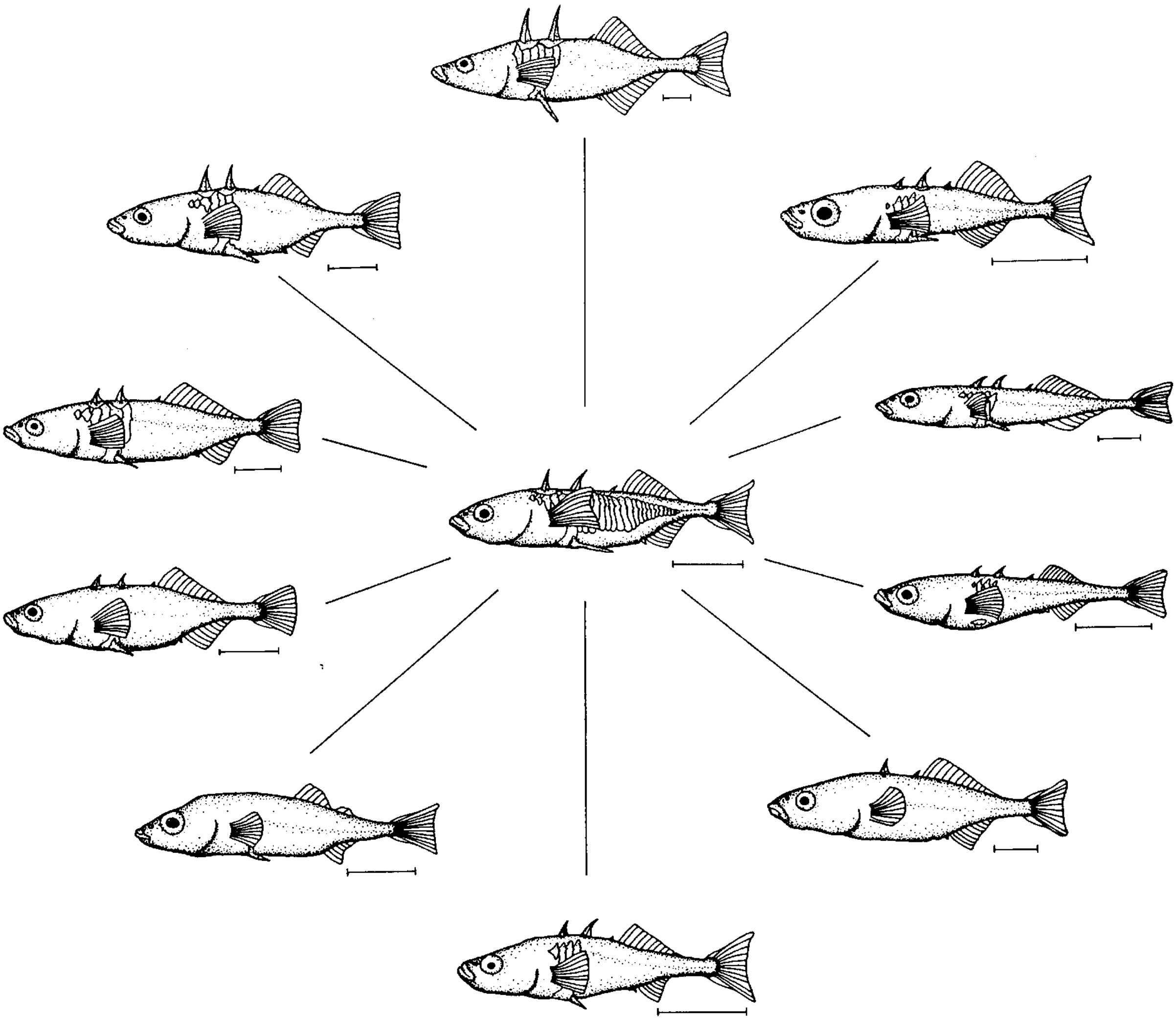

Figure 1. Morphological variation among North American populations of *Gasterosteus aculeatus*. The central specimen is an anadromous fish from Birch Cove, Cook Inlet, Alaska, USA. Those around the periphery are from freshwater populations that have probably been derived independently from marine ancestors, represented by the Birch Cove individual. Details of the freshwater populations are in Bell & Foster (1994). (Reproduced from Bell & Foster (1994) with permission). Scale bars represent 1 cm.

A final attribute of this radiation that makes it especially useful for evolutionary study is the existence of iterative, or replicate, parallel evolution. In separate drainages, and possibly even on a finer scale, aquatic habitats have been colonized by marine or anadromous populations and have independently given rise to similar, apparently adaptive, derived phenotypes (Bell 1988; Foster *et al*. 1992; McPhail 1993; Schluter & McPhail 1993; Bell & Foster 1994; Walker 1997). In several instances, similar sympatric or parapatric species pairs have also evolved (McPhail 1984, 1993, 1994; Schluter & Nagel 1995; Thompson *et al*. 1998). These are of three types: anadromous and stream resident sticklebacks; lake and stream resident sticklebacks; and sympatric lacustrine benthic and limnetic forms (*sensu* McPhail 1984; see below). We will focus on the latter because they are best-studied and can best be used to illustrate the hierarchical approach to the study of speciation.

(a) *The lacustrine species pairs*

In 1984, McPhail first described pairs of *G. aculeatus* species in six small lakes in the Strait of Georgia region of British Columbia, Canada. Each of these lakes possessed one species specialized for feeding on plankton (limnetic form, *sensu* McPhail (1984)) and one specialized for feeding on bottom-dwelling invertebrates, or benthos, in the littoral zone (benthic form, *sensu* McPhail (1984)). In limnetic fish, the mouth is narrow and the snout long (enhancing sucking action), the eye is large and the fish have long, closely-spaced gill rakers. Benthic fish are typically larger and deeper-bodied than limnetics, have larger mouths and smaller eyes, and have short, widely-spaced gill rakers that prevent escape of prey but do not impede water movement (figure 2; Bentzen & McPhail 1984; McPhail 1984, 1992, 1993, 1994; Schluter & McPhail 1992).

The sympatric species pairs in the Enos and Paxton lakes have been studied intensively by McPhail, Dolph Schluter and their colleagues. The morphological differences between the forms are heritable and all available evidence suggests that they represent distinct gene pools (McPhail 1984, 1992, 1993, 1994). Although morphological evidence suggests that about 1% of the adults in both lakes probably are hybrids, the percentage has remained stable over 25 years in Paxton Lake and there is

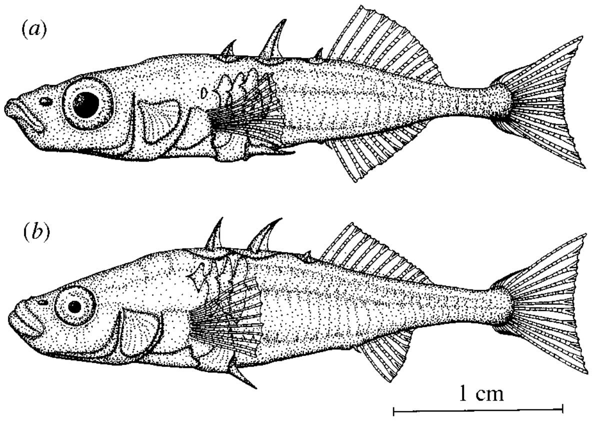

Figure 2. Representative individuals from the Enos Lake species pair which includes a limnetic (*a*) and a benthic (*b*) species. From Hart & Gill (1994).

no evidence of ecologically significant gene flow (McPhail 1984, 1992). There exists little, if any, viability reduction in hybrids or backcrosses (McPhail 1992; Hatfield 1995). The only known disadvantage to F_1 hybrids is reduced foraging efficiency in the wild (McPhail 1994; Hatfield 1995). A mating disadvantage was not detected (Hatfield & Schluter 1996), but the sequential choice design that was used can fail to detect significant assortative mating (Verrell 1990), and the head-up choice criterion that was used is not a reliable criterion of mate choice in threespine stickleback (Hay & McPhail 1975; McPhail 1994). Thus, the possibility of a hybrid mating disadvantage is not fully resolved.

McPhail (1993, 1994) has suggested that the most likely explanation for the evolution of these species pairs is that the lakes were invaded twice by the marine or anadromous ancestor, the first invasion giving rise to the benthic form and the second to the limnetic. He used several lines of evidence to create this model. The species pairs appear to be restricted to a small area in British Columbia that was subjected to a second minor marine incursion about 2000 years after the lakes would have initially become isolated from marine waters (Mathews *et al*. 1970). Also, all of the lakes with species pairs are at an elevation that would have permitted a second invasion without submergence. The strength of this model is that it accounts for the restricted geographic range of the pairs.

The sequence of events McPhail envisions has the benthic form evolving from the marine/anadromous ancestor first, because the lakes which contain the benthic/limnetic pairs are physiogeographically similar to other lakes in the region that contain only benthic populations. After 2000 years of evolution a second invasion by the morphologically intermediate, planktivorous ancestor would have given rise to a population poorly designed to compete with the benthic already extant in the lake. Further divergence could thus have been a consequence of trophic character displacement (McPhail 1993, 1994; see also Schluter & McPhail 1992).

The competing hypotheses are, of course, (i) sympatric speciation, and (ii) secondary contact between benthic and limnetic forms that evolved once and then spread, or that evolved multiple times and then came into secondary contact. Sympatric speciation is unlikely given the very restricted distribution of the species pairs (McPhail 1993, 1994; see below). Secondary contact between limnetic and benthic forms is unlikely because lakes of a size and depth to have favoured the evolution of limnetics are not found on two of the three islands on which species pairs occur. Even less likely is a single event producing the divergent forms, because they are so widespread geographically in allopatry, and freshwater stickleback are unlikely to migrate readily between drainages or through the marine environment.

The two most likely explanations for the origins of the species pairs are thought to be double invasion and sympatric speciation (McPhail 1993, 1994; Taylor *et al.* 1998). One way to discriminate these models would be to use molecular markers to resolve relationships among the limnetic, benthic and ancestral forms. The expectation under the double invasion model would be that the closest relative of each species in each lake would be the ancestral form, and that the limnetic species should have diverged more recently from the ancestor than the benthic species. Under sympatric speciation the pairs within each lake would be one another's closest relatives. Unfortunately, results to date have proven equivocal. Mitochondrial DNA analysis is compatible with independent sympatric origin of the species pairs; benthic and limnetic species within a lake are more similar to each other than they are to their counterparts in the other lakes. The problem is that if isolation was incomplete on the second invasion, resultant gene flow could produce the same mtDNA pattern as that expected with sympatric speciation (Taylor *et al*. 1998).

The double invasion hypothesis remains the most plausible of the explanations given the geographical distribution of the species pairs (McPhail 1993, 1994) and the geological history of the region in which they occur (Mathews *et al*. 1970; McPhail 1993). The mtDNA results suggest that speciation was not complete when the second invasion occurred, and may have been reinforced via strong disruptive selection despite gene flow. Given that 1% of the individuals in each of the Paxton and Enos lakes appear to be hybrids, low levels of gene flow may still occur (McPhail 1994; Taylor *et al*. 1998).

(b) *Parallel divergence in allopatry*

In British Columbia, Canada and the Cook Inlet region of Alaska, USA, population differentiation has occurred along the limnetic–benthic continuum such that populations in deep, oligotrophic lakes tend to be specialized for feeding on plankton in open water, whereas those in small, shallow lakes are specialized for foraging on large benthic invertebrates in the littoral zone (benthic populations; McPhail 1984, 1994; Lavin & McPhail 1985, 1986; Schluter & McPhail 1992). The morphological differences among the ecotypes parallel those exhibited by the species pairs. As in the species pairs, the divergent suites of characters adapt the ecotypes to their respective environments (Hagen & Gilbertson 1972; McPhail 1984; Lavin & McPhail 1985, 1986), and are clearly related to differences in trophic habitat even among lakes within a single drainage (Lavin & McPhail 1985). Each form is more efficient at foraging on the resource for which it is specialized than it is on the other (Lavin & McPhail 1986; Ibrahim & Huntingford 1988; Schluter 1993, 1995).

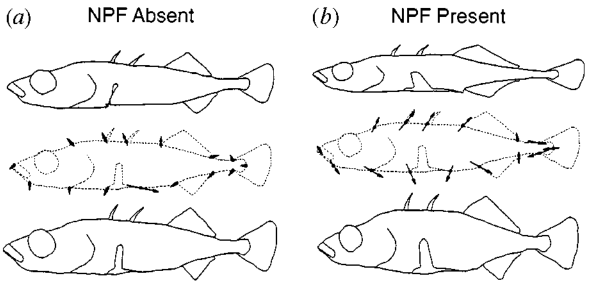

Figure 3. Extreme hypothetical figures based on regression vectors (magnified five-fold; (*a*) and (*b*), middle row) reflecting direction and relative magnitude of change in landmark locations per unit change in RLA from allopatric populations of four types. RLA stands for relative littoral area, or the proportion of the lake surface area for which the euphotic zone extends to the lake bottom, is a measure of the relative amounts of benthic and limnetic foraging habitat. The shapes of the figures represent the expected body shape for stickleback from deep lakes without native predatory fishes (NPF; (*a*) top row), shallow lakes without native predatory fishes ((*a*) bottom row), deep lakes with native predatory fishes ((*b*), top row), and shallow lakes with native predatory fishes ((*b*), bottom row). From Walker (1997).

The differences between the species pairs are more extreme than those found among populations in small, shallow lakes in southern British Columbia like those in which the species pairs are found (Lavin & McPhail 1985, 1986; Schluter & McPhail 1992; McPhail 1993). Allopatric, limnetic ecotypes from some of the large, oligotrophic lakes of southern British Columbia may approach the phenotypic extremes observed in sympatry (Lavin & McPhail 1985, 1986; personal observation), but the possibility has not yet been examined. The morphological differences between the ecotypes are based on genetic differences in all cases examined to date (Hagen 1973; Lavin & McPhail 1987; McPhail 1994; Hatfield 1995).

Although ecotypic differentiation in threespine stickleback seems to be driven primarily by available prey type and foraging habitat, predators may also influence the pattern of differentiation across populations (figure 3; Walker 1997). In Cook Inlet, Alaska, where most lakes are relatively small and shallow, populations exposed to native predators have evolved more extreme benthic and limnetic morphotypes than have those in otherwise equivalent lakes devoid of native predators. This is a consequence of direct effects on body shape associated with ability to evade predators, and indirect effects on elements of head shape associated with trophic characteristics.

Benthic populations differ from limnetic populations with respect to several aspects of behaviour as well. Fish from limnetic populations feed high in the water column in large groups, whereas those from benthic populations search stubstrata in the littoral zone for large benthic invertebrates. Benthic foraging is the predominant, but not exclusive, mode of feeding by non-territorial adults on the breeding grounds in three British Columbia populations at Cowichan, Crystal and Hotel lakes, and four Cook Inlet populations at Big Beaver, Bruce, Stephan and Willow lakes that have been well-studied (Foster 1988, 1994*a*,*b*, 1995; Hyatt & Ringler 1989). In all of these lakes, groups foraging actively on the bottom are routinely observed. They range in size from a few individuals to several hundred, and routinely attack nests guarded by males. If the nests contain embryos or yolk-sac fry, they are cannibalized, and the nests are destroyed. Because males cannot aggressively defend their nests against groups of conspecifics, they typically exhibit a complex, conspicuous diversionary display in response to the approach of conspecifics. If effective, the groups follow the male away from the nest, and attempt to feed elsewhere, following his apparent lead.

This suite of foraging and defensive behaviour patterns appears to be ancestral within the stickleback radiation. Although marine and anadromous stickleback possess trophic structures and body forms that range from intermediate between sympatric ecotypes to relatively limnetic (Schluter & McPhail 1992; McPhail 1993), on the breeding grounds, group foraging on benthos is typically observed, and diversionary displays are employed in response to the approach of such groups. Cannibalism of young in nests is typically prevalent as well. This behavioural repertoire has been observed in an anadromous tidepool population that breeds in salt-marsh tidepools along the St Lawrence Estuary (Whoriskey & FitzGerald 1985, 1994), in two anadromous and one marine population in the Cook Inlet region of Alaska (Rabbit Slough, Anchor River and Glacier Spit, respectively), and in anadromous populations in the Swy Y Lana and Francis Peninsula lagoons in British Columbia, Canada (Foster 1995, unpublished data).

In contrast, non-territorial adult stickleback in four limnetic populations that have been well-studied feed nearly exclusively on plankton during the breeding season (Foster 1994*a*,*b*, 1995). Three populations, those in Garden Bay, North and Sproat lakes, are in British Columbia, while the fourth, that in Lynne Lake, is near Cook Inlet. Benthic foraging groups, if present, are small and loosely formed. Males attack them directly, typically causing them to disperse. Cannibalism of young in nests has never been observed, males court females in large planktivorous foraging groups rather than performing diversionary displays, and we have never been able to elicit diversionary displays by any means in any of the populations (Foster 1988, 1994*a*,*b*, 1995). Thus, males in these populations appear to have lost the ancestral transition from juvenile plankton-foraging to benthic-foraging by adults, and to have lost ancestral diversionary display repertoires.

The differences in foraging behaviour and in the incidence of group cannibalism between populations of the two ecotypes appear also to have precipitated the evolution of differences in courtship behaviour (figure 4; Foster 1994*a*, 1995). In limnetic populations in both British Columbia and Alaska, males are more likely to initiate courtship than are benthic males and they typically do so with a conspicuous zig-zag dance. Even if not used in the initial stages of courtship, the zig-zag dance is more likely to appear in courtship between limnetics than benthics (figure 4). In contrast, in benthic populations, a female typically initiates courtship by assuming a position dorsal to the male, and appressing her underside to his back. This typically initiates a behaviour called dorsal pricking in which both circle or meander forward slowly, while the male repeatedly jerks backward, pricking the female's underside with erect dorsal spines. Although dorsal

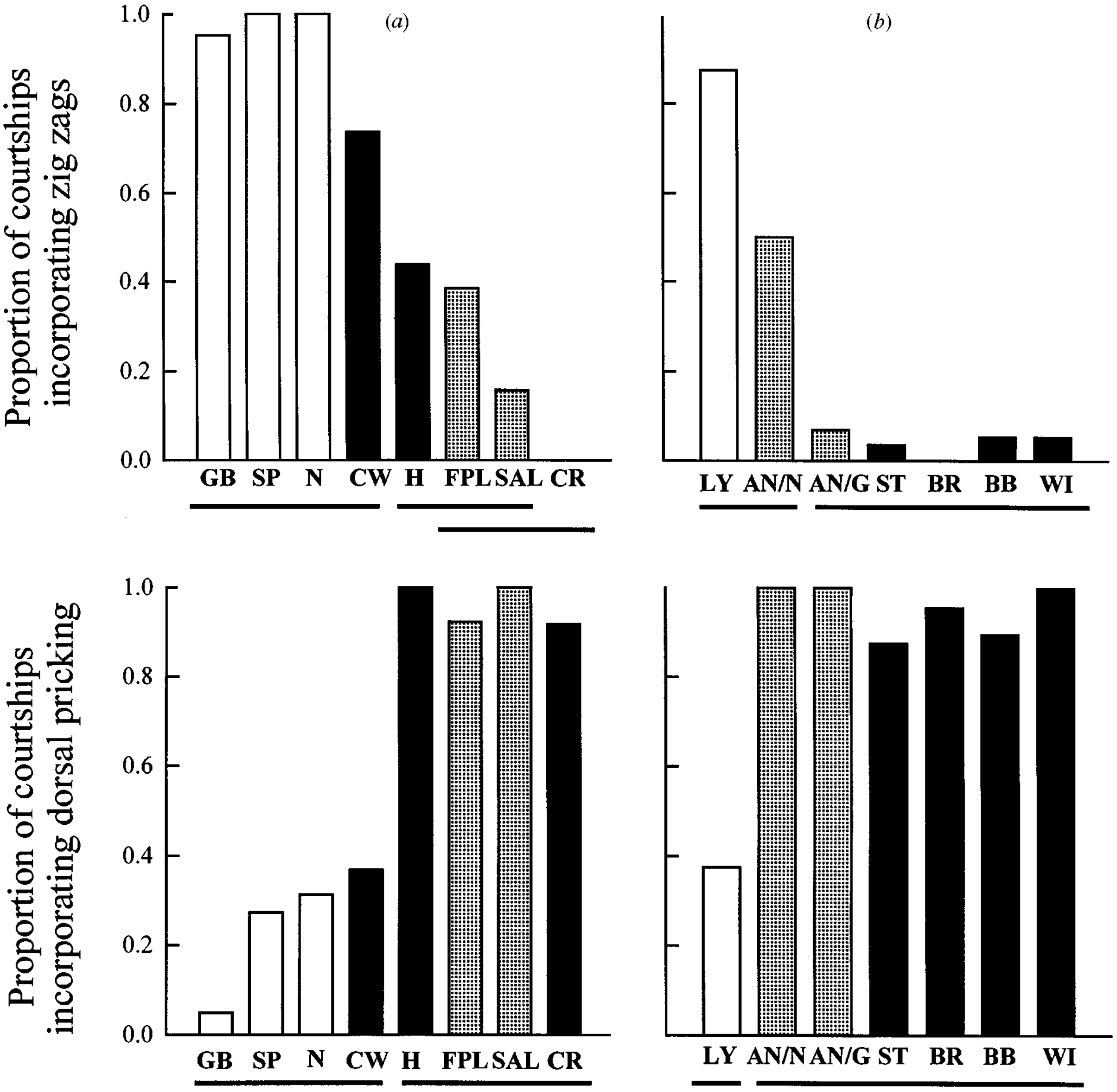

Figure 4. Proportions of courtship interactions incorporating the zig-zag dance and dorsal pricking in 14 populations of threespine stickleback *Gasterosteus aculeatus* in (*a*) southern British Columbia, Canada, and (*b*) Cook Inlet, Alaska, USA. Open bars designate limnetic, non-cannibalistic populations; dark bars designate benthic, cannibalistic populations; and shaded bars, anadromous populations. The Alaskan Anchor River data are from two years, 1992 (AN/N), in which no cannibalistic foraging groups were present in the pools and 1995, (AN/G) in which large, cannibalistic groups were routinely observed. The data sets represented in each figure displayed significant heterogeneity (*G*-test, $p<0.001$ all cases). Within histograms, bars connected by horizontal lines did not differ ($p>0.05$, STP contrast procedure of Sokal & Rohlf (1981)). Canadian populations: CR, Crystal Lake; CW, Cowichan Lake; FPL, Francis Peninsula Lagoon; GB, Garden Bay Lake; H, Hotel Lake; N, North Lake; SAL, Swy A Lana Lagoon; SP, Sproat Lake. Alaskan populations: AN, Anchor River; BB, Big Beaver Lake; BR, Bruce Lake; LY, Lynne Lake; ST, Stephan Lake; WI, Willow Lake. Data are from at least 16 independent courtships at each site.

pricking has historically been interpreted as an indication that the male is not yet ready to lead the female to his nest (for review, see Rowland 1994), in benthic populations it can be the only behaviour that precedes showing of the nest entrance to the female, and it may directly precede this action (Foster 1994*a*, 1995).

Courtship behaviour in anadromous and marine populations appears to be most like that observed in benthic populations, although in some cases the characteristics of courtship are intermediate (figure 4). In Anchor River, natural annual variation offered us insight into the extent of plasticity in an anadromous population (figures 4*b*,*d*). In 1992 adult stickleback were present in the tidepools in very low densities only, and no foraging groups were ever observed. Although dorsal pricking was common in both years, the representation of zig-zag dancing was much higher in 1992 when groups were absent. The zig-zag frequency in 1992 overlapped with that observed in Lynne Lake, Alaska, but not with the three extreme limnetic populations from Canada ($p<0.05$, standard test procedure of Sokal & Rohlf (1981)) when data from the Canadian limnetic populations were included in the analysis of the Alaskan data (overall heterogeneity, $G=202.132$, $p\ll 0.001$). These analyses suggest considerable

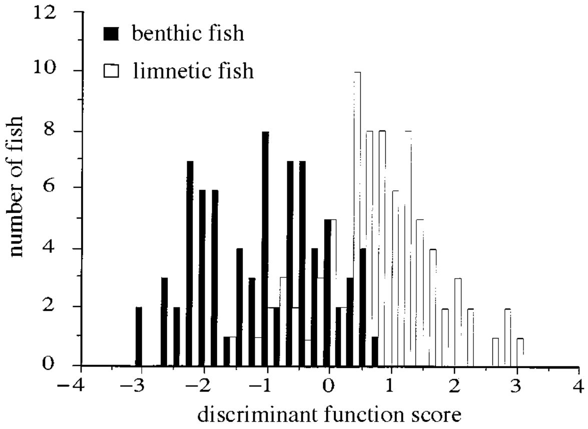

Figure 5. Histogram of canonical discriminant function scores of benthic and limnetic morphotypes of threespine stickleback in Benka Lake, Alaska. The two different foraging types are distinct in multivariate morphological space (Wilk's lambda$=$0.573; $F_{5,153}$ 22.77; $p<0.0001$). Standard length, mass, gill raker number and length, snout length, and eye diameter all contributed significantly to the discrimination; only body depth did not.

plasticity in courtship, but also suggest that the range within marine populations is not as great as that seen across lacustrine benthic and limnetic populations.

Although the differences in courtship behaviour between the ecotypes do not include a behaviour unique to one ecotype that might act as a recognition signal, these differences in behaviour may contribute to reproductive isolation between the species pairs. Only in Enos Lake has reproductive behaviour been studied in detail (Ridgway & McPhail 1984). In this lake, nearly complete assortative mating occurs in nature although heterotypic courtship is often initiated but then aborted. In the laboratory, forced heterotypic courtships were also initiated but usually aborted, and consistent differences that paralleled those of allopatric ecotypes were observed. In particular, the zig-zag dance was more often displayed by limnetic than benthic males, and it more often was the initial approach used by limnetic males. The responses of females to heterotypic versus homotypic males also differed. The failure of limnetic males to have lost the diversionary display, unlike their allopatric counterparts, is not surprising given that the two species are often nearest neighbours during breeding in Enos Lake, and encounters between the two types are common.

(c) *Parallel divergence within populations*

Within-population differentiation along the benthic–limnetic axis of resource use is proving extremely common in lacustrine fishes, presumably because of the consistent availability of both a plankton-rich open-water habitat and a structurally complex littoral zone in which large, benthic invertebrates are typically abundant. The result is often a pronounced resource polymorphism, incorporating differences in both body form and trophic morphology, enabling fish to forage most efficiently either on plankton in the water column or on benthic macroinvertebrates in the littoral zone (Robinson & Wilson 1994; Skúlasson & Smith 1995; Smith & Skúlasson 1996). Given the existence of both the sympatric limnetic and benthic species pairs, and the extensive parallel ecotypic differentiation across populations, threespine stickleback might be expected to exhibit within-population polymorphism as well. Yet, this pattern seems to be extraordinarily rare.

Although threespine stickleback do exhibit variation in trophic characteristics within populations, and this variation can be associated predictably with food type (Schluter & McPhail 1992), in only one lake (Benka) have two distinct morphotypes been described within a population (figure 5; Cresko & Baker 1996). The two types were detected because *in situ* observations suggested that individuals feeding on plankton were of a different body form than those feeding on benthos. Collections of fish feeding on each of the two resources confirmed this expectation, and, as in the case of the more strongly divergent species pairs and population ecotypes, each morphotype performs better on the resource for which it is specialized than it does on the alternative resource (W. A. Cresko, unpublished data).

In this instance, secondary contact is unlikely to have been responsible for the co-ocurrence of the two morphotypes because Benka is a relatively high elevation lake (160 m above sea level) and it is 125 km from the ocean. Thus, a secondary marine incursion is unlikely to have occurred. There are presently no surface connections to other bodies of water and Benka Lake is surrounded by a ridge approximately 5–20 m high, making connections in the recent past unlikely. Thus, the most plausible explanation for this divergence is that it has arisen *in situ*.

If the Benka Lake morphs arose via disruptive selection, the question immediately arises as to why similar polymorphic populations have not been detected elsewhere. In our view, the most likely explanation is that traditional collecting techniques bias in favour of bottom-feeding fish, rather than those which feed in deeper, open water and are unlikely to enter traps or be captured by seine net in shallow water. Additional collections in lakes possessing both fairly extensive limnetic zones and areas of deeper water should address the issue effectively.

3. HIERARCHICAL VARIATION AND SPECIATION IN THREESPINE STICKLEBACK

The reason we find threespine stickleback especially exciting for the study of speciation is that differentiation across the same resource axis can be examined in polymorphic populations, among limnetic and benthic ecotypes that have evolved in allopatric populations, and in nearly fully formed species pairs that exhibit no postzygotic barriers to hybridization. Although we would not argue that the relationships between phenotype and fitness need be the same at all of these levels and in all situations, in this case they do seem to be. Consequently, differentiation on both sides of the speciation boundary can be examined in an explicitly ecological context, and experiments can be designed to test specific aspects of individual models of speciation (e.g. Schluter 1994).

Because most data support the anadromous form as ancestral to freshwater populations, and that the ancestral type has changed little for at least 11 000 years (Bell 1994), experiments likely to replicate the first contact between anadromous and benthic forms can be performed, testing the predictions of McPhail's model for the evolution of the species pairs. The decision to use a benthic population

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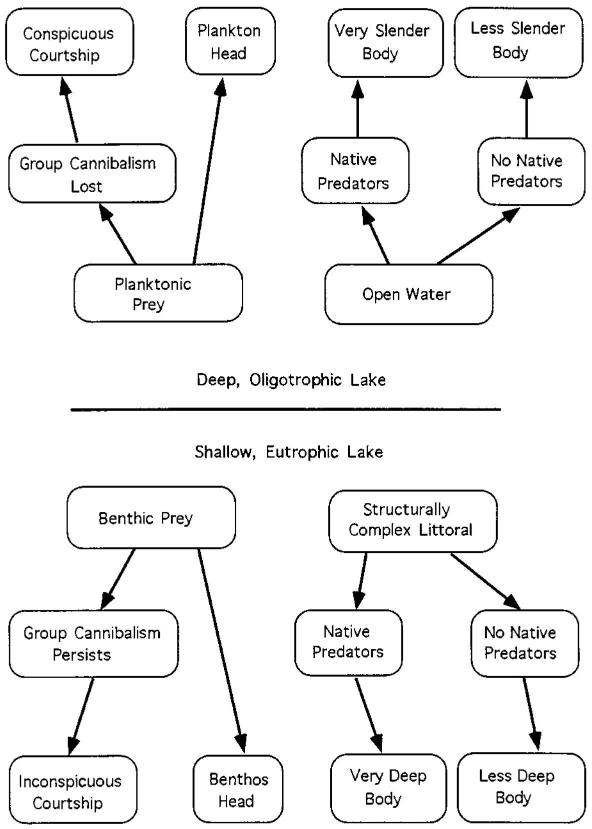

Figure 6. Evolutionary consequences of planktivory (limnetic form) and feeding on benthos (benthic form) for phenotypes of lacustrine threespine stickleback *Gasterosteus aculeatus*. The phenotypes indicated may be of significance in assortative mating between the benthic–limnetic lacustrine species pairs. See text for further details.

based on McPhail's inference of evolutionary history, rather than a limnetic (as used by Schluter (1994) in his study of character displacement) is not trivial. Our work suggests that marine/anadromous and benthic stickleback have more similar courtship behaviour than do marine/anadromous and limnetic stickleback. Thus, this choice could have a profound effect on our interpretation of the early stages of the speciation event that produced the species pairs. Intriguingly, Ridgway & McPhail's (1984) study of courtship and assortative mating in the Enos Lake species pair suggests that the behavioural differences between the species parallel those observed between allopatric benthic and limnetic populations. If McPhail's hypothesis is correct, as seems likely, the limnetic must have evolved more conspicuous courtship in the presence of benthic foraging groups, which is a somewhat surprising insight.

Sympatric speciation remains a plausible alternative given that, in many lacustrine fish, limnetic and benthic forms coexist as a resource polymorphism, and a polymorphic population of threespine stickleback has recently been described (Cresko & Baker 1996). If more polymorphic populations are discovered when appropriate collecting techniques are applied, additional support will be lent to the hypothesis. However, the sympatric speciation hypothesis cannot explain the restricted distribution of the species pairs. Given the relative ease of detecting two such dissimilar forms within a lake, and the extensive collections already made in British Columbia and Alaska without detecting other species pairs, we think it unlikely that the restricted distribution of the pairs is a sampling artefact. Thus, in our opinion, the double invasion hypothesis remains the most likely explanation for the evolution of the species pairs. It is this scenario that we feel should be replicated and tested experimentally as a means of adding to our knowledge of the speciation process.

Perhaps the most robust insight from the work on threespine stickleback is that natural selection has played a major role in the evolution of these species pairs. Not only are ecological differences among the lakes implicated as factors driving speciation, but their action has involved a complex cascade of effects (figure 6). Presumably, differences in the availability of prey types has selected directly on trophic characteristics and head shape, and secondarily on elements of body form as indicated by Walker's (1997) comparative study of allopatric ecotypes. To the extent that trophic characteristics or elements of body form are criteria of mate choice, thereby contributing to reproductive isolation, the effect has been direct. However, differences in foraging behaviour include differences in the prevalence of group cannibalism, which in turn have influenced the nature of courtship behaviour in benthic and limnetic ecotypes according to a pattern that parallels courtship differences in the Enos Lake species pair. Because heterotypic matings are initiated, but rarely completed, differences in courtship behaviour as well as body form are implicated as criteria of choice.

Walker's (1997) work also suggests a possible role for predation in the divergence that has led to speciation. Apparently, in Alaska at least, morphological divergence of the ecotypes from the ancestral form is greatest when predatory fish are native to the lakes (NPF). In this case, the primary effect is on elements of body form essential for efficient hydrodynamic performance in the two different foraging habitats, and the effect on head shape and trophic characteristics is secondary. This work on the allopatric ecotypes suggests the possibility that the presence of predatory fish might enhance the likelihood that a first invasion would produce an ecotype sufficiently different from the ancestor that speciation would result in the event of a secondary marine incursion. Predatory fish must not be essential to the process, however, as Paxton Lake benthics often lack a pelvic girdle and lateral plates (McPhail 1994), a morphology indicative of the absence of native predatory fishes (Hagen & Gilbertson 1972; Bell *et al.* 1993).

The pattern that emerges is one in which speciation has been driven by natural selection. The mechanism has not necessarily involved direct selection on traits involved in mate choice and reproductive isolation, but instead appears to have involved indirect cascades of effects. Although we still need to unambiguously discriminate the traits that are involved in mate choice and isolation, video image modification now provides us with the appropriate tools (McDonald *et al.* 1995; McKinnon 1995; Rowland 1995; Rowland *et al.* 1995) and we should be able to develop a far better understanding of the causes of speciation in the benthic–limnetic species pairs. Without *in situ* studies and the opportunity to compare cases of parallel divergence at lower hierarchical levels we could not have achieved even this level of understanding.

4. POPULATION VARIATION AND SPECIATION: INSIGHTS FROM OTHER TAXA

Natural selection has long been invoked as a primary cause of both geographic variation (Gould & Johnston 1972; Endler 1977, 1986) and speciation (Mayr 1942; Dobzhansky 1951). Because genetic changes that occur in isolated populations can include changes that reduce the reproductive compatibility of their members (for review, see Verrell 1998), reproductive incompatibility can, in theory, evolve in allopatry as a consequence of natural selection. Although there exist a number of examples in which sexual signals have been shown to have differentiated in response to local selection (for recent reviews, see Verrell 1997; Wilczynski & Ryan 1997), and selection is implicated in the evolution of reproductive isolation in some fully formed species (e.g. Gerhardt 1983; Stratton & Uetz 1983, 1986), we know of no examples that unambiguously link local adaptation to reproductive isolation. Two studies do, however, deserve special attention.

The first is a set of studies that have examined patterns of reproductive isolation between species of *Drosophila* that are incompletely reproductively isolated in the laboratory, and among populations within species. The approach was pioneered by Dobzhansky and his colleagues in an effort to understand the early stages of speciation in the group (for review, see Chatterjee & Singh 1989). Recently, Coyne & Orr (1989, 1997) have correlated the data on reproductive isolation between species with electrophoretic genetic distance between the pairs. A wide range of levels of incompatibility existed among the 171 pairs comprising their final data set, ranging from incompatibility to near-complete compatibility. Their analyses indicated that: (i) both prezygotic and postzygotic isolation increase with divergence time; (ii) prezygotic isolation increases more rapidly than postzygotic isolation, but this is due entirely to disproportionately strong prezygotic isolation between species in sympatry; and (iii) hybrid sterility and inviability evolve at similar rates but appear much earlier in divergence in hybrid males, than in hybrid females.

Taken together, the first two findings indicate that prezygotic isolation does evolve in allopatry, but also that reinforcement can enhance prezygotic isolation in sympatry when hybrids are unfit. The latter finding is particularly important because of the controversial nature of reinforcement, and because surveys of character variation (e.g. ethological, ecological and genetic) in nature are likely to offer the only strong insight into this problem other than perturbation followed by direct observation. Because the process may be slow, direct observation, whether in nature or as a consequence of an experimental perturbation, may be problematic. The difficulty with survey studies is, of course, one of finding taxonomic groups that include a diverse array of populations (or species) that exhibit appropriate variation in the degree of reproductive isolation.

Survey studies of this kind are particularly useful in addressing problems involving patterns and rates of evolutionary change, but are unlikely to be able to detect cause of the divergence in allopatry. Especially when the study taxa disperse as widely as do *Drosophila*, detection of selective causes of differentiation are unlikely. On the other hand, surveys do have the potential to detect a role of selection if the divergent population or species are slow-moving or are confined to habitat patches in which the differentiation is likely to have occurred. Although degree of isolation has been examined across few populations of stickleback (e.g. Schluter & Nagel 1995), similar survey methods could yield insights into the relative roles of lake habitat characteristics and native predatory fish as determinants of degree of reproductive isolation between ecotypes. However, the method can also suggest the absence of a strong role for selection as is the case in the next example.

Geographic variation in the sexual behaviour of the dusky salamander, *Desmognathus ochrophaeus*, and associated patterns of reproductive isolation across populations have been particularly well-studied (Houck *et al.* 1985, 1988; Verrell & Arnold 1989; Tilley *et al.* 1990). The dusky salamander is distributed across mountaintops of the eastern United States from New York to northern Georgia. The disjunct populations exhibit considerable variation in body size, life history and colour pattern (Tilley 1973). In the southern Appalachian Mountains there also exist considerable genetic differences among populations, as indicated by geographic variation in allozyme frequencies (maximum Nei's distance of 0.46; Tilley *et al.* 1978). However, there is no consistent relationship between genetic differentiation and morphological or structural characteristics in the populations, a pattern similar to that observed across populations of other species of salamanders (Wake 1981).

Populations of the dusky salamander also exhibit a complete range of ethological compatibilities, ranging from full compatibility to complete incompatibility (Houck *et al.* 1988; Verrell & Arnold 1989; Tilley *et al.* 1990). A survey in which levels of ethological isolation between populations from 11 southern Appalachian sites (31 crosses total) were interpreted in the context of genetic differentiation (26 protein loci) and geographic distance, demonstrated that geographic distance was the best predictor of the degree of reproductive isolation. Presumably this is because distance, rather than ethological isolation, disrupts gene flow among the populations. The ethological basis of mating failure in heterotypic matings usually involved a failure of the male to initiate courtship or a failure later in the courtship sequence (Verrell & Arnold 1989). Unlike the case for the lacustrine pairs of stickleback, there is little to suggest that natural selection has contributed substantially to the divergence in *D. ochrophaeus* (Tilley *et al.* 1990; Verrell & Arnold 1989; P. A. Verrell, personal communication).

5. WHAT QUESTIONS SHOULD WE BE ASKING?

One of the fundamental problems in evolutionary biology has been to develop a predictive and comprehensive theory for the process of speciation. This has also proven one of the most frustrating of all efforts, possibly because speciation may occur through a family of processes that depend on, among other things, the genetic system of the organism or the specific ecological context of the process. Although theory has enhanced our understanding of what can and cannot happen, it tells us little of what really does happen. For that purpose, we need to examine the process under natural conditions, or to glean insights from experimental perturbations.

Following a comprehensive review of laboratory experiments on speciation, Rice & Hostert (1993) concluded that there exists strong theoretical and empirical evidence that speciation can occur in the face of gene flow, particularly when geographic distance holds gene flow to relatively low levels and the individuals in the geographically disparate areas are subject to 'strong, discontinuous, and multifarious directional selection'. They emphasized pleiotropy or hitchhiking as the cause of reproductive isolation, suggesting that it had most often been driven by strong, multifaceted directional selection, but that it also could be a consequence of sampling drift, or of natural or sexual selection along a single axis. If this view is correct, much of speciation has occurred in an implicitly geographical context, just not one quite as restrictive as that envisioned by Mayr (1942) and Dobzansky (1951), and comparisons of carefully selected, divergent populations may offer insights into the causes of speciation in nature.

Perhaps then we should begin by asking questions that would help us to better understand the causes of speciation under the sets of circumstances that theory and laboratory research indicate are most likely to culminate in speciation. Here we focus on questions that can be addressed through population comparison, especially when variation at more than one hierarchical level can be exploited. For example, we might ask:

1. What are the characteristics or processes that result in incompatibility between partially reproductively isolated populations, and can their divergence be linked to divergence in selective regimes?
2. If differences in selective regimes are implicated, can we demonstrate causative roles experimentally (via selection experiments)?
3. Is multifarious selection more likely to lead to speciation than is selection along a single axis?
4. Does selection most often act directly on the characteristics or processes that yield incompatibility, or is the effect most often indirect, acting through a cascade of effects?

These questions are, of course, all related to the role of selection in generating differences among populations. These are essential questions to ask, not only because of the evidence afforded by the Rice & Hostert (1993) review, but also because local adaptation has for so long been viewed as a primary cause of speciation (e.g. Mayr 1942), and we have so little direct evidence of its action in this regard. Adaptive radiations and cases in which parallel divergence occurs at several hierarchical levels are most likely to offer informative answers to questions related to selection. The quality of the inference will depend on our assurance that the populations still reside at the sites where reproductive divergence has occurred. Land-locked postglacial populations of threespine stickleback and mountaintop populations of the dusky salamander offer excellent examples. The answers were different in the two cases, with strong evidence of a role for ecotypic differentiation in the first, and little in the second.

Other questions that have been addressed more often using population comparisons are those that do not invoke natural selection directly. These involve the roles of reinforcement and gene flow. Often, but not always, they require information about population histories that is hard to acquire. New coalescent methods may help in this regard. For example, the coalescent may be useful in discriminating levels of gene flow at the present and during periods in which reproductive isolation was evolving between now partially interconnected populations (for reviews, see papers in Harvey *et al.* (1996)). We could then ask, with a greater degree of assurance in the answer:

5. Can we find cases in which reproductive isolation has evolved under natural conditions, despite gene flow between the diverging subpopulations?
6. What are the relationships among spatial patterns of selection and gene flow that prohibit or facilitate the evolution of reproductive isolation?
7. What is the role of reinforcing selection in speciation?

Although Coyne & Orr (1989, 1997) offer a strong answer to the latter question, the answer applies only to a single taxonomic group. It is not the same answer reached in the Rice & Hostert (1993) review of laboratory research on speciation. We see little reason to expect that speciation patterns or causes should be the same in all groups. Indeed, the restriction of speciation by polyploidy to certain groups provides evidence that this is not the case. Until we have gathered strong empirical evidence regarding the patterns and causes of speciation in many taxa, we seem to have little hope of generating a theory that is either comprehensive or predictive. We do see our hope of ever achieving this goal as one that lies in the painstaking acquisition of data.

A final set of questions pertaining to speciation that can readily be addressed using crosses between incompletely isolated populations or species, are those involving time. In these cases, time frames for events related to speciation are based on the magnitudes of protein or DNA divergence between the units being crossed. The power of such methods for exploring relative rates at which pre- and postzygotic isolation evolve, and for examining the consequences of secondary contact, are apparent in the comparative analyses of Coyne & Orr (1989, 1997) and Tilley *et al.* (1990). Here again, time-consuming data collection may offer us our best window into the evolutionary processes that end in speciation.

In conclusion, we feel that a comprehensive knowledge of the speciation process can only be achieved through detailed, *in situ* studies of populations that are partially, but incompletely, reproductively isolated. Such research programmes should incorporate experiments designed to test specific mechanisms of divergence. We suggest that the study of taxonomic groups that exhibit extensive parallel variation at multiple hierarchical levels may offer especially good windows into the process of speciation, particularly with respect to the role of selection. Perhaps we will not need a time machine (*sensu* Rice & Hostert 1993) after all.

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