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Evolution of intrinsic post-zygotic reproductive isolation in fish

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The comparative approach is important in investigating the evolution of reproductive isolation. Yet this method has not been extended to teleost fish, the most diverse vertebrate lineage. Many experimental interspecific crosses have been performed in aquacultural research, and some of the resultant data is analysed in relation to mitochondrial cytochrome *b* divergence to investigate the evolution of intrinsic postzygotic isolation. Such isolation was found to increase gradually, suggesting that hybrid unfitness is usually due to the gradual accumulation of deleterious epistatic interactions among species. Hybrid sterility evolved more rapidly than inviability, although both forms of hybrid fitness are probably important in isolating natural populations. Because of general difficulties in characterising fish genetic sex-determination systems, and because of the plethora of systems potentially represented in any given cross, too few crosses are currently available to evaluate the generality of Haldane's rule in fish.

Introduction

Speciation is a typically slow process whose genetical basis requires substantial effort to elucidate. Furthermore, taxa differ in their modes and rates of speciation (Mayr 1942). Thus, comparative approaches are an important means of identifying general phenomena characterising speciation (e.g., Coyne & Orr 1989, 1997), and potentially of discerning the relative importance of different speciation mechanisms (e.g., Presgraves 2002).

Comparative analyses considering the evolution of intrinsic post-zygotic reproductive isolation (RI) relate the magnitude of RI (as assayed by hybrid sterility and inviability) to genetic divergence. Several notable patterns emerge from these analyses. Hybrid sterility tends to evolve sooner than hybrid inviability in taxa as divergent as Lepidoptera (Presgraves 2002), *Drosophila* (Coyne & Orr 1989, 1997) and birds (Price & Bouvier 2002). This is probably because sex- and reproduction-related genes evolve more rapidly than viability-related ones (Singh & Kulathinal 2000). Also evident is Haldane's rule: "when, in the F_1 offspring of two different animal races, one sex is absent, rare, or sterile, that sex is the heterozygous [heterogametic] sex (Haldane 1922)." Haldane's rule is very widespread and has been documented in both groups with heterogametic males (e.g., Mammalia, Amphibia, Reptilia), and those with heterogametic females (Diptera, Orthoptera, Heteroptera, Lepidoptera, birds) (Coyne & Orr

1989, 1997, Laurie 1997, Orr 1997, Presgraves 2002, Price & Bouvier 2002). Haldane's rule is probably a composite phenomenon reflecting both faster male evolution (through sexual selection) and the recessivity of x-linked genes generating hybrid unfitness (the Dominance theory) (Laurie 1997, Orr 1997).

Various mechanisms can induce intrinsic post-zygotic RI; for example, founder-effect speciation (Carson & Templeton 1984), differences in ploidy between related taxa (Stebbins 1950), and microbe-induced isolation (Werren 1998). However, the Dobzhansky-Muller model has the greatest general explanatory power. Dobzhansky (1936) and Muller (1940) considered that hybrid sterility and inviability result as pleiotropic by-products of independent evolution in allopatric populations. In the Dobzhansky-Muller (D-M) model, alleles that enhance fitness on the normal genetic background may lower fitness when brought together in hybrids with alleles from another taxa. Thus, RI results from the accumulation of deleterious epistatic interactions in hybrids. Empirical and theoretical studies implicate natural selection in the accumulation of D-M incompatibilities (e.g., Coyne *et al*. 1997, Turelli *et al*. 2001). Theoretical analyses indicate that D-M incompatibilities are rare and that the severity of hybrid unfitness and the number of loci underlying incompatibilities should increase as the square of time separating two taxa (Orr 1995, Orr & Turelli 2001). So, the D-M model predicts an approximately regular rate in the acquisition of RI, in contrast to other post-zygotic RI mechanisms that typically induce speciation instantaneously (e.g., founder-effect speciation, ploidy differences, etc.). Comparative studies may therefore be used to evaluate the relative importance of different isolation mechanisms.

Fish are the most diverse vertebrate lineage, yet RI evolution in fish has not been considered within a comparative context. Many experimental crosses have involved teleost fish taxa for aquacultural research (for example, Schwartz (1981) lists over 1800 studies), and these constitute a large and important, but unexploited resource. Here, I examine a small subset of these crosses in relation to mitochondrial cytochrome *b* divergence, and investigate whether general trends are apparent in the development of intrinsic post-zygotic RI. Equivalent data for sexual isolation are relatively lacking, so this isolation mechanism is not considered here.

Material and methods

Data on hybrid unfitness were obtained from interspecific crosses largely described in Argue and Dunham (1999) and Ryabov (1981) (*see* Appendix). Consideration was limited to F_1 viability and fertility as in equivalent comparative studies of other organisms. Thus, although many studies have documented gross hybrid breakdown only in later hybrid generations (Edmands 2002), due to break-up of parental gene combinations through recombination, these were discarded. Analysis was limited to studies that had examined fertility and viability of both sexes. Asymmetries in the strength of post-zygotic RI between reciprocal crosses often exist (e.g., Tiffin *et al*. 2001). However, reciprocal crosses were usually not distinguished in the source literature, so they are generally not distinguished here; in the occasional instances when reciprocal cross data was available, crosses were averaged across. Similarly, different races of a particular species can be isolated to differing degrees with the same taxon (Argue & Dunham 1999); although any such instances identified in the literature happened to be discarded from this analysis because of phylogenetic non-independence, this caveat remains potentially important. The magnitude of RI was coded from 0 (both sexes viable and fertile) to 4 (both sexes inviable). A code of "2" means that both sexes are viable but sterile, such that effectively complete intrinsic RI exists (*see* Fig. 1 for isolation index classification).

Mitochondrial cytochrome *b* sequences were obtained from GenBank (accession numbers are given in the Appendix) and aligned using ClustalX (Thompson *et al*. 1994). Pairwise distance estimates were estimated using the Kimura-2-parameter implemented in Mega v. 2.0 (Kumar *et al*. 2001). The cytochrome-*b* gene, unlike allozymes, is unlikely to be affected by selection on traits conferring RI (Fitzpatrick 2002), and is therefore an appropriate basis for genetic dis-

tance estimation. However, mitochondrial DNA exhibits rate variation between different lineages (Rand 1994), and is generally only useful for lineages separated by < 10 myr because of saturation properties (Meyer 1994). These caveats are considered further in the Discussion. A further caveat that may be only occasionally important is that interspecific introgessive hybridisation involving mitochondrial DNA may lead to underestimation of overall genetic divergence (Smith 1992).

In ensuring phylogenetic independence of the cross data crosses were not averaged across (e.g., by following Felsenstein's (1985) phylogenetically independent contrasts method) because of current uncertainties in phylogenetic relationships amongst higher-order taxa. Instead, two alternative approaches were adopted: (1) following Price and Bouvier (2002), a list of crosses was prepared wherein each species was represented in only one cross; (2) in a more robust analysis based on the database generated by (1), only one cross per family was considered. Following phylogenetic corrections, 37 (method 1) and 17 (method 2) crosses from 17 families were available for comparative analysis (Appendix).

Much ancillary information exists regarding the maximum genetic distance allowing partial reproductive compatibility. This data derives from molecular genetic analyses of introgressive hybridisation involving wild populations (where post-F_1 hybrids are formed), backcrossing experiments that examine the fertility of only one sex from the F_1 generation, and from studies that certified F_1 fertility but were excluded from the comparative analysis because of phylogenetic non-independence (using method 1). Such data was examined to determine if incomplete isolation extended beyond the range detected in the comparative analysis. 32 additional crosses were compiled for this analysis from Verspoor and Hammar (1991), Argue and Dunham (1999) and Sakaizumi *et al*. (1992) (data available from author upon request). Again, genetic distances were based on cytochrome *b* sequences obtained from GenBank. Morphological analyses of introgressive hybridisation were not considered because of occasional inconsistencies in their findings with molecular work (Verspoor & Hammar 1991), and because the unknown genetic and environmental determinants of most traditional morphological markers constrain their reliability in detecting post-F_1 hybrids (Campton 1987).

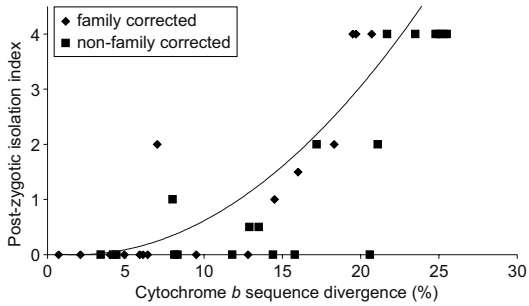

Fig. 1. Plot of intrinsic post-zygotic reproductive isolation against mitochondrial cytochrome *b* distances (Kimura-2-parameter corrected). Isolation index is as follows: 0, both sexes fertile; 0.5, one sex fertile, the other sex some individuals recorded as fertile; 1, one sex fertile, one sex viable but infertile; 1.5, one sex sometimes fertile, one sex viable but infertile; 2, both sexes viable but infertile; 2.5, one sex viable but infertile, one sex sometimes viable; 3, one sex viable, one sex missing; 3.5, one sex sometimes viable, one sex missing; 4, both sexes missing. All data derives from method 1 for controlling phylogenetic dependence, wherein species were only considered in one cross, irrespective of familial membership. The family-corrected subclass considers only one cross per family (method 2). The line of best fit through the family-corrected data is a second-order polynomial ($y = 0.0081x^2 - 0.007$).

Results

In the comparative analysis only six out of 17 congeneric crosses showed F_1 fitness reduction (*see* Appendix). But, most heterogeneric crosses (13 out of 19) showed fitness reduction. Post-zygotic isolation only became evident after a sequence divergence of 7%, and the maximum divergence at which no isolation was reported was 20.6%. The magnitude of unfitness was positively related to sequence divergence (Fig. 1). Moreover, the rate of increase of isolation appeared to increase gradually with time; for instance, the line of best fit for the data from method 2 (only one cross considered per family) was a second-order polynomial forced through the origin (Fig. 1). Those crosses that

resulted in F_1s with reduced sterility (isolation index = 0.5–2) were between taxa with an average sequence divergence of 14.3% (method 1) and 14.0% (method 2). The equivalent averages between taxa that produced inviable F_1s (index = 2.5–4) were 23.1% and 21.2% (methods 1 and 2 respectively). Means for inviability and sterility were significantly different using Mann-Whitney tests (method 1: $U = 48$, $n = 19$; $p < 0.001$; method 2: $w = 10$, $n = 8$; $p < 0.05$).

Analysis of the ancillary information indicated that the number of instances of partial reproductive compatibility declined rapidly with sequence divergence (data not shown). Partial reproductive compatibility occurred at a maximum sequence divergence of 18.9% (*Oryzias curvinotus* × *O. latipes*; Sakaizumi *et al.* 1992). Thus, incomplete isolation does not extend beyond the range detected in the comparative analysis (20.6%; Appendix).

Discussion

This comparative analysis has ascertained that several patterns of RI evolution that have been characterized in various taxa exist in fish too. For instance, as in *Drosophila* (Coyne & Orr 1989, 1997) and Lepidoptera (Presgraves 2002), intrinsic post-zygotic isolation increases gradually. This pattern is consistent with the relatively greater importance of the D-M model in describing the evolution of RI. Otherwise, alternative models of RI evolution (e.g., frequent microbe-induced speciation, polyploid speciation, etc.) would have regularly generated significant isolation at low sequence divergences; a pattern not observed here. Interestingly, the observation that the rate of increase in hybrid unfitness seems to increase with time is predicted from theoretical treatments of the D-M model (specifically, these analyses indicate that the severity of hybrid unfitness should increase as the square of time separating two taxa; Orr 1995, Orr & Turelli 2001). Equivalent comparative studies of other organisms have not detected this pattern, and have instead generally inferred linear relationships. This difference is largely due to the delayed acquisition of RI in fish, which only becomes evident after a sequence divergence of 7%; in contrast, RI in other groups is apparent relatively much sooner. This disparity has several possible explanations, including differences in the relative importance of speciation mechanisms (mechanisms that typically induce speciation instantaneously may be more prevalent in non-fish groups). Alternatively, my data may be biased by non-random sampling introduced by aquacultural practices (*see* below).

Hybrid sterility evolves more rapidly than inviability in fish as well. These forms of unfitness have different genetic bases as suggested by the fact that hybrid sterility mutations tend to affect one sex only, whereas most lethal mutations kill both sexes (Wu & Davis 1993). Male sex- and reproduction related genes often evolve more quickly than those of females (e.g., Hollocher & Wu 1996); a pattern evident in the more rapid evolution of male sterility in male-heterogametic taxa (Orr 1997). Nevertheless the evolutionary rate of such genes in both sexes has been sufficient on average to induce complete hybrid sterility before complete inviability in *Drosophila* (Coyne & Orr 1997) and fish (method 1: Mann-Whitney test: $U = 92$, $n = 14$, $p < 0.05$; insufficient crosses from method 2 exist for a comparable analysis).

Because fish sex chromosomes are typically homorphic (morphologically similar) (Ohno 1974), it is frequently difficult to characterise fish sex-determination systems. Thus, although ~1700 fish species have been cytogenetically characterised, only ~10% possess cytogeneti-

Table 1. Crosses involving male (XY) or female (ZW) heterogametic species.

Family	Species 1	Species 2	Sex-determination system	Notes on F_1s
Cyprinidae	*Carassius auratus*	*Cyprinus carpio*	XX-XY	Males sterile; females sterile
	Rhodeus ocellatus	*Tanakia limbata*	ZZ-ZW	Male only broods
	Rhodeus ocellatus	*Tanakia lanceolata*	ZZ-ZW	Male only broods
Poeciliidae	*Poecilia sphenops*	*P. latipinna*	ZZ-ZW	No hybrid unfitness

cally distinct sex chromosomes (Devlin & Nagahama 2002, Arkhipchuk 1995). Of this fraction, both male (XY) and female (ZW) heterogametic systems have been detected, although the additional presence of autosomal influences in many such systems means that the number that can be directly considered in relation to Haldane's rule is reduced. I know of only four crosses involving species that possess the same heterogametic system (Table 1). Of these, three conform to Haldane's rule. These numbers are too small to evaluate the generality of Haldane's rule in fish, which is a nearly ubiquitous pattern in the early stages of RI evolution in other groups. So, more cytogenetic work in fish is clearly advantageous.

Introgressive hybridisation is prevalent amongst interspecific fish populations (Scribner *et al*. 2001), leading Thorgaard and Allendorf (1988) to suggest that hybrid fish may be less susceptible to severe developmental incompatibilities than interspecific hybrids from other vertebrate classes showing comparable levels of genetic divergence. The mean divergence of completely isolated taxa in this study was 23.1%, although this may be an underestimate because saturation of cytochrome *b* sequences can become evident around 20% divergence (Meyer 1994). Assuming a molecular clock of 2% myr^{-1}, this value equates to ~11.6 myr. Similarly, the maximum divergence for partial reproductive compatibility was 20.6% (~10.3 myr). Only the former value appears substantially larger than equivalent values for other lineages (excepting birds) (Table 2). Although necessarily crude, these figures don't provide strong support for Thorgaard and Allendorf's (1998) hypothesis. Nevertheless, amongst the five organismal groups for which comparative data currently exists, it seems that total post-zygotic isolation evolves most slowly in birds and fish. Also noteworthy is that the mean divergence time for completely isolated fish taxa (~11.6 myr) substantially exceeds that between 108 putatively allopatric sister species pairs in Avise *et al*. (1998) (~2.05 myr), and the mean time for speciation between putatively allopatric clades (~1.1–2.9 myr species^{-1}; data recalibrated assuming a molecular clock of 2% myr^{-1}) estimated by McCune and Lovejoy (1998). Both these latter studies used cytochrome *b* sequences.

Here, I examined the evolution of intrinsic post-zygotic isolation. However, because sexual isolation would act first to isolate taxa, the general relevance of post-zygotic isolation to speciation remains somewhat controversial. To evaluate its relevance one must examine the number of species pairs that experience natural range overlap and suffer intrinsic hybrid unfitness. From my data set, two out of the 19 species pairs possessing an intrinsic isolation index ≥ 0.5 have naturally overlapping ranges (*Barbus meridionalis–B. barbus*, and *Alburnus alburnus–Leuciscus cephalus*) (Scribner *et al*. 2001). However, the proportion of species pairs likely to satisfy these criteria is probably greater because I have not considered extrinsic post-zygotic unfitness that becomes apparent under natural environments (for example, between different sympatric ecotypes of the three-spined stickleback, *Gasterosteus aculeatus*; Hatfield and Schluter (1999)). Thus, intrinsic postzygotic RI is probably likely to play an important role in isolating natural fish populations. Similar conclusions were drawn for Lepidoptera (Presgraves 2002) and *Drosophila* (Coyne & Orr 1997).

My results are unlikely to have much reflected rate variation in mitochondrial DNA between lineages (e.g., the rate is strongly reduced amongst salmonids; Smith 1992) because similar trends generally resulted from both methods that controlled for phylogenetic dependence. However, of potentially greater importance are biases in how hybridisation experiments are selected and published (Edmands 2002). The aquacul-

Table 2. Estimated mean age for completely isolated taxa, and maximum ages for partial fertility in various taxonomic groups.

Taxonomic group	Mean age of total post-zygotic isolation (myr)	Maximum age for partial reproductive compatibility (myr)
Drosophila[1]	~5	~7.8
Birds[2]	~11.5	~16.8
Lepidoptera[3]	~3.5	~7.5
Amphibians[4]	~4.2	~9

Data obtained from comparative studies in: [1]Coyne & Orr 1997; [2]Price & Bouvier 2002; [3]Presgraves 2002; [4]Sasa *et al.* 1998. Estimates were generated from allozyme data using a molecular clock of 2% myr^{-1}.

tural industry is interested in developing hybrid strains that show heterosis, and sometimes sterility (to prevent genetic contamination of natural populations and to enhance individual growth) and sex-ratio distortions (to regulate stock population growth) (Bartley *et al*. 2001). Thus, the aquacultural industry may preferentially select and publish crossing experiments that yield hybrids with such traits, causing published crosses to be a non-random sample of those possible. However, a large proportion of crosses produced wholly inviable offspring (e.g., five out of the 17 (29%) crosses used in method 2), suggesting that these biases may not be especially strong. Because biases may operate on multiple hybrid traits (e.g., heterosis, sterility, partial inviability), effects of their interactions on the comparative analysis are potentially complex, but not necessarily detrimental.

In summary, the evolution of intrinsic postzygotic RI in fish shows several phenomema identified in other taxa. Specifically, hybrid sterility evolves more rapidly than inviability, and RI evolves gradually, consistent with the relatively greater importance of the D-M model in causing RI. It is likely that such intrinsic isolation plays an important role in isolating natural populations, either directly or indirectly through promoting sexual isolation (reinforcement; Noor 1999). Because of general difficulties in characterising fish genetic sex-determination systems, and because of the plethora of systems potentially represented in any given cross, too few crosses are currently available to evaluate the generality of Haldane's rule in fish.

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Appendix. Cross data for comparative analysis. Cytochrome *b* sequences were obtained from GenBank. * = cross used in analyses involving only one cross per family.

Family	Species 1 (cyt. *b* accession no.)	Species 2 (cyt. *b* accession no.)	Sequence divergence (%)	Hybrid unfitness score	Reference
Acipenseriformes: Acipenseridae	*Acipenser ruthensus* (AF283733)	*Huso huso* (AF283745)	4.9	0*	1
Beloniformes: Adrianichthyidae	*Oryzias latipes* (AB084753)	*O. luzonensis* (AB084755)	19.7	4*	2
Cyprinodontiformes: Aplocheilidae	*Aphyosemion bualanum* (AF002303)	*A. exiguum* (AF002304)	5.9	0*	1
	Aphyosemion cognatum (AF002324)	*A. christyi* (AF002322)	8.3	0	1
Cypriniformes: Cobitidae	*Misgurnus anguillicaudatus* (AF051868)	*Cobitis biwae* (AB039347)	18.3	2*	3
Cypriniformes: Cyprinidae	*Cyprinus carpio* (Y09469)	*Zacco temminckii* (AF309084)	25.0	4*	4
	Richardsonius balteatus (AY096011)	*Mylocheilus caurinus* (AF117169)	13.5	0.5	1
	Notemigonus crysoleucas (U01318)	*Scardinius erythrophthalmus* (Y10444)	15.1	2	1
	Rutilus rutilus (AF090772)	*Abramis brama* (Y10441)	12.9	0.5	1
	Barbus meridionalis (AF112130)	*B. barbus* (AY013479)	8	1	1
	Leuciscus schmidti (AY026396)	*Schizothorax pseudoaksaiensis* (AF180827)	24.8	4	4
	Gobio gobio (AJ388431)	*Chondrostoma nasus* (AJ388454)	23.5	4	4
	Biwia zezera (AF309507)	*Henigrammocypris rasborella* (AF375863)	21.7	4	4
	Carassius auratus (AF045966)	*Zacco platypus* (AF309085)	25.3	4	4
	Hypophthalmichthys molitrix (AF051866)	*Aristichthys nobilis* (AF051855)	8.1	0	1
	Puntius conchonius (AY004751)	*Rhodeus ocellatus* (AF051876)	25.5	4	4
	Alburnus alburnus (AJ388428)	*Leuciscus cephalus* (LCE389571)	17.2	2	1
	Cyprinella lutrensis (U01319)	*C. venusta* (AF261218)	15.8	0	1
Cyprinodontiformes: Fundulidae	*Fundulus olivaceus* (U77123)	*F. notatus* (L31598)	6.4	0*	1
Cyprinodontiformes: Poeciliidae	*Xiphophorus helleri* (AF404301)	*X. maculatus* (XMU06515)	4.0	0*	1

Continues

Appendix. Continued.

Family	Species 1 (cyt. *b* accession no.)	Species 2 (cyt. *b* accession no.)	Sequence divergence (%)	Hybrid unfitness score	Reference
Perciformes: Centrarchidae	*Lepomis macrochirus* (AY115976)	*L. cyanellus* (AY115973)	20.6	0	1
	Pomoxis annularis (AY115990)	*P. nigromaculatus* (AY115992)	2.1	0*	1
	Micropteris salmoides (AY116000)	*M. dolomieu* (AY115998)	11.8	0	1
Perciformes: Cichlidae	*Oreochromis mossambicus* (X81565)	*O. niloticus* (AF550011)	0.7	0*	1
	Sarotherodon galilaeus (AF375618)	*Oreochromis aureus* (AF375617)	3.4	0	1
Perciformes: Moronidae	*Morone saxatilis* (AF240746)	*M. chrysops* (AF240745)	14.5	1*	1
Perciformes: Percidae	*Perca flavescens* (AF045357)	*Stizostedion vitreum* (AF386602)	20.7	4*	5
Perciformes: Serranidae	*Epinephelus marginatus* (AJ420205)	*E. aeneus* (AJ420206)	19.5	4*	6
Perciformes: Sparidae	*Acanthopagus latus* (AF539743)	*Sparidentex hasta* (AF240734)	12.8	0*	6
	Sparus auratus (AJ319809)	*Pagrus major* (NC_003196)	21.1	2	1
Pleuronectiformes: Pleuronectidae	*Pleuronectes platessa* (AY164472)	*Platichthys flesus* (AF113179)	6.1	0*	1
Salmoniformes: Salmonidae	*Oncorhynchus mykiss* (D58401)	*O. clarkii* (AY032633)	4.4	0	1
	Oncorhynchus keta (AF165078)	*O. gorbuscha* (AF165077)	4.2	0	1
	Salmo trutta (D58400)	*S. salar* (AF202032)	7.0	1*	1
	Oncorhynchus masou (D58402)	*Salvelinus fontinalis* (D58399)	14.4	0	1
Siluriformes: Clariidae	*Clarias fuscus* (AF416885)	*C. gariepinus* (AF475153)	16.0	1.5*	7
Siluriformes: Ictaluridae	*Ictalurus furcatus* (AF484159)	*I. punctatus* (AB069646)	9.5	4*	1

1 = Argue & Dunham 1995; 2 = Sakaizumi *et al.* 1992; 3 = Suzuki 1973; 4 = Ryabov 1981; 5 = Wiggins *et al.* 1983; 6 = Bartley *et al.* 2001; 7 = Smitherman *et al.* 1996.