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## Zebrafish hybrids suggest genetic mechanisms for pigment pattern diversification in *Danio*

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**Abstract** Pigment patterns of *Danio* fishes are a tractable system for assessing the developmental genetic bases for the evolution of adult form in vertebrates. These pigment patterns include multiple horizontal melanophore stripes in the zebrafish *D. rerio*, a complete absence of stripes in *D. albolineatus*, a few broad stripes in *D. kerri*, and a combination of stripes and spots in *D. nigrofasciatus*. Here we assess the genetics of pigment pattern development and evolution using interspecific hybrids. We first reconstruct the phylogenetic relationships of these species by analyzing mitochondrial 12S and 16S rDNA sequences. We find a clade comprising several small species of danio, and within this clade a sister taxon relationship between *D. rerio* and *D. nigrofasciatus*. We also find that the large bodied *D. dangila* is more closely related to the clade of small danios than other large bodied species. As a first step in evaluating the genetics of pigment pattern diversification in the group, we then examine the phenotypes of interspecific hybrids. Adult pigment patterns of hybrids between *D. rerio* and other danios are in many respects more similar to *D. rerio* than the heterospecific danio, demonstrating that alleles of pigment pattern genes in other species typically are recessive to *D. rerio* alleles. Furthermore, hybrids between two additional striped species (*D. kerri*, *D. nigrofasciatus*) and *D. albolineatus* suggest that striped patterns are dominant or semi-dominant over an absence of stripes. Together, these analyses support a model in which pigment pattern differences between *D. rerio* and other

species result from gain-of-function alleles in *D. rerio*, or loss-of-function alleles in other danios. Finally, because several *D. rerio* pigment pattern mutants resemble heterospecific danios, we use interspecific complementation tests to assess potential roles for these loci in pigment pattern diversification. Crosses between other danios and most *D. rerio* pigment pattern mutants develop stripes, similar to control hybrids with wild-type *D. rerio*. These complementation phenotypes allow us to exclude most of these loci as having major effect roles in generating pigment pattern differences between species. In contrast, hybrids between *fms* mutant *D. rerio* and *D. albolineatus* fail to develop stripes, similar to *D. albolineatus*. This non-complementation phenotype identifies changes in *fms*, or the pathway in which it acts, as candidates for contributing to the evolutionary loss of stripes in *D. albolineatus*.

**Keywords** Evolution · Neural crest · Phylogenetic systematics · Pattern formation · Melanophore

### Introduction

The genes and cellular behaviors underlying differences in adult form remain largely unknown, particularly among vertebrates. Yet, a knowledge of the mechanistic bases for phenotypic variation will be essential for a more complete understanding of the evolution of morphology. One approach to addressing this problem is to search for genetic and developmental differences that underlie differences in the expression of a trait between closely related species, as mechanistic differences observed at shallow phylogenetic levels are more likely to be causally related to trait variation than mechanistic differences that may have accumulated between species at deeper phylogenetic levels. Adult pigment patterns of fishes in the genus *Danio* are particularly amenable to genetic and developmental analyses because these patterns differ dramatically across closely related species,

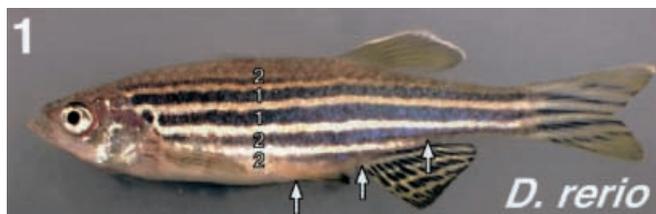
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because pigment patterns are accessible to observation and experimentation, and because one species, the zebrafish *D. rerio*, is a commonly used model organism with a variety of resources that can be applied towards understanding the evolution of developmental genetic mechanisms (Meyer et al. 1995; Parichy 1996; Fang 1997a, 1998; McClure 1999).

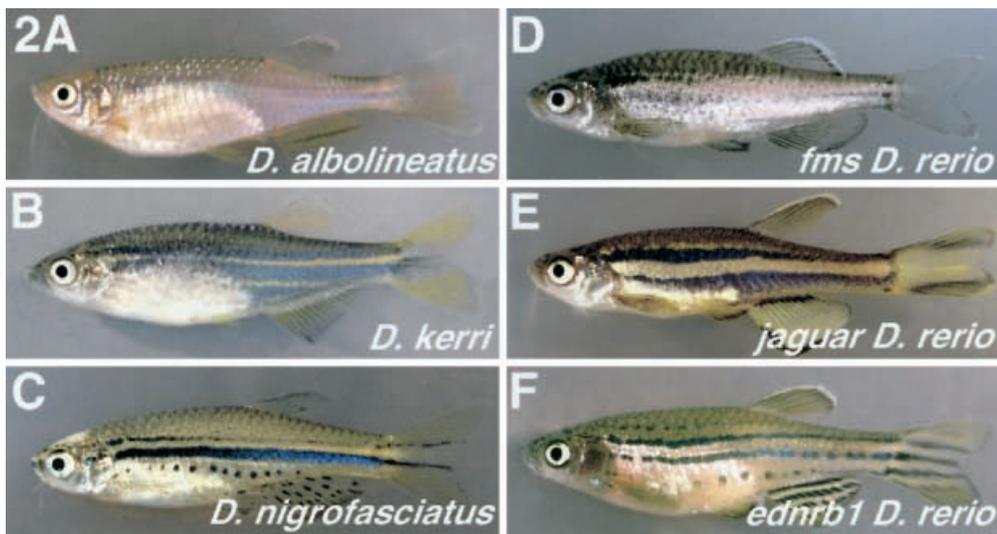
In danios and other ectothermic vertebrates, several classes of pigment cells are derived from embryonic neural crest cells, including black melanophores, yellow or orange xanthophores, silvery iridophores, and red erythrophores (Fujii 1993; Bagnara 1998; Reedy et al. 1998). Pigment patterns result from the numbers and spatial arrangements of these differently colored pigment cells, and these patterns may have roles in mate choice, camouflage, and schooling behavior (e.g. Endler 1978;



**Fig. 1** Adult *Danio rerio* exhibit several well-defined, horizontal dark stripes with intervening, light interstripe regions. Primary melanophore stripes (1) are the first stripes to develop and arise during metamorphosis. Secondary melanophore stripes (2) arise during later juvenile and adult stages as the fish grow. Arrows indicate locations used for determining melanophore and stripe numbers (see text)

Houde 1997). Examples of *Danio* pigment patterns are shown in Figs. 1–3. *D. rerio* exhibit four to five horizontal dark stripes that include melanophores and iridophores, with light interstripe regions that include xanthophores and iridophores (Fig. 1). In contrast, *D. albolineatus* lack stripes and instead exhibit a dispersed pattern of melanophores, and xanthophores or erythrophores (Fig. 2A); *D. kerri* have three broad stripes of melanophores that are defined by light interstripe regions (Fig. 2B); and *D. nigrofasciatus* exhibit melanophore stripes dorsally and melanophore spots ventrally (Fig. 2C). Finally, in addition to the small bodied species (3–5 cm total length) shown in Figs. 1 and 2, the genus also includes large bodied species (12–15 cm total length) such as *D. aequipinnatus* and *D. dangila*. The former exhibits three broad horizontal melanophore stripes posteriorly defined by light interstripe regions, as well as light vertical stripes, spots, or horizontal stripes anteriorly (Fig. 3A); *D. dangila* exhibits a pattern of wavy light stripes and spots (Fig. 3B; also see Fang 1997a, b).

Although little is known of pigment pattern formation in most danios, the development of the adult pigment pattern in *D. rerio* is becoming increasingly well understood (Kirschbaum 1975; Milos and Dingle 1978; Johnson et al. 1995; McClure 1999; Parichy et al. 2000a, b). In this species, pigment pattern metamorphosis transforms an early larval pigment pattern comprising stripes of melanophores along the dorsal and ventral myotomes and yolk mass into the horizontal striped pattern of the adult. During an initial phase, a population of early metamorphic (EM) melanophores arises dispersed over



**Fig. 2** Examples of small danios (A–C) and *D. rerio* pigment pattern mutants (D–F). **A** *D. albolineatus* exhibit a dispersed arrangement of melanophores on the body and thus lack stripes. In addition to iridophores this species also exhibits widely dispersed xanthophores and reddish cells that may be erythrophores (Bagnara 1998; McClure 1999). **B** *D. kerri* exhibit three broad melanophore stripes that are defined by lighter, narrow interstripe regions. **C** *D. nigrofasciatus* exhibits melanophore stripes dorsally and a series of melanophore spots ventrally. **D** *fms* mutant *D. rerio* lack distinct

stripes, owing to a defect in melanophore and xanthophore development (Parichy et al. 2000b). Shown is *fms*<sup>4e1</sup>; pigment patterns of other *fms* alleles are indistinguishable. **E** *jaguar* mutant *D. rerio* have fewer and broader stripes than wild-type *D. rerio*. Shown is *jaguar*<sup>5</sup>. **F** *ednrbl* (*rose*) mutant *D. rerio* exhibit melanophore stripes dorsally and spots ventrally. Shown is the weak allele *ednrbl*<sup>3e3</sup>; more severe (*ednrbl*<sup>b140</sup>) or null alleles (*ednrbl*<sup>3e1</sup>) typically exhibit only a single melanophore stripe dorsally, and spots ventrally

**Table 1** Zebrafish pigment pattern mutants identify candidate genes for contributing to interspecific pigment pattern variation. Mutants and corresponding references are indicated, as well as the overall mutant phenotype and, when known, adult pigment cell populations known to be deficient in mutant individuals (*EM* early

metamorphic melanophores, *LM* late metamorphic melanophores, *SM* scale melanophores, *irid.* iridophore, *xanth.* xanthophore). Species indicated are those tested for non-complementation with the candidate mutant (*al*, *D. albolineatus*, *ke*, *D. kerri*, *ni*, *D. nigrofasciatus*)

Mutant	Phenotype	Species
<i>ednrb 1 (rose)</i> <sup>a,b</sup>	Melanophore stripes dorsally, spots ventrally (LM, irid.)	<i>al</i> , <i>ni</i>
<i>fms (panther)</i> <sup>c</sup>	Disrupted stripes anteriorly; stripes absent posteriorly (LM, xanth.)	<i>al</i> , <i>ke</i> , <i>ni</i>
<i>fritz</i> <sup>d</sup>	Disrupted stripes or spots	<i>al</i> , <i>ni</i>
<i>jaguar</i> <sup>e,f</sup>	Fewer and broader melanophore stripes	<i>al</i> , <i>ke</i> , <i>ni</i>
<i>kit (sparse)</i> <sup>b,c,g,h</sup>	Reduced melanophore number and fewer melanophore stripes (EM, SM)	<i>al</i> , <i>ke</i> , <i>ni</i>
<i>leopard</i> <sup>b,i-k</sup>	Melanophore spots; nearly uniformly distributed melanophores in strong alleles (LM)	<i>al</i> , <i>ni</i>
<i>nacre (mitf)</i> <sup>l</sup>	Melanophores absent (EM, LM, SM)	<i>al</i> , <i>ni</i>
<i>ocelot</i> <sup>e</sup>	Broken melanophore stripes	<i>al</i>
<i>primrose</i> <sup>e</sup>	Melanophore stripes dorsally, spots ventrally (LM, irid.)	<i>al</i> , <i>ni</i>
<i>puma</i> <sup>d</sup>	disrupted stripes	<i>al</i>

<sup>a</sup> Parichy et al. 2000a

<sup>b</sup> Johnson et al. 1995

<sup>c</sup> Parichy et al. 2000b

<sup>d</sup> Parichy, unpubl. data

<sup>e</sup> Johnson, unpubl. data

<sup>f</sup> Johnson et al. 1996

<sup>g</sup> Parichy et al. 1999

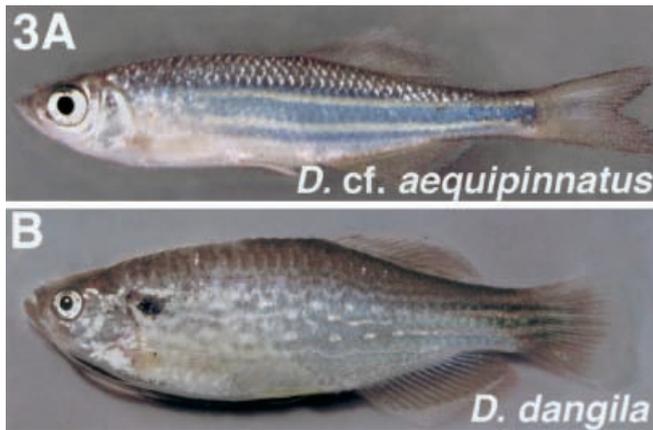
<sup>h</sup> Rawls and Johnson 2000

<sup>i</sup> Asai et al. 1999

<sup>j</sup> Haffter et al. 1996

<sup>k</sup> Kirschbaum 1975

<sup>l</sup> Lister et al. 1999



**Fig. 3A, B** Examples of large danios. **A** *D. aequipinnatus* exhibit three broad melanophore stripes posteriorly, and light spots or stripes anteriorly. **B** *D. dangila* exhibit wavy stripes and light spots

the flank in regions not previously occupied by these cells. Subsequently, EM melanophores migrate into the positions of adult stripes and, simultaneously, a second population of late metamorphic (LM) melanophores differentiates in the positions of these adult stripes. These events result in the formation of a juvenile/early adult pigment pattern by around 28 days that comprises two stripes of melanophores (here referred to as “primary” melanophore stripes) and a lighter interstripe region. During later development, additional stripes (“secondary” melanophore stripes) form as the fish grow (Fig. 1).

Analyses of pigment pattern mutants in *D. rerio* also have started to reveal the genetic bases for adult stripe development. Several loci have been identified for which mutants lack one or another population of pigment cells comprising the adult pigment pattern (Table 1). For example, *kit* (formerly *sparse*) mutant *D. rerio* fail to

develop EM melanophores, as well as dermal scale melanophores that normally contribute an overall dark cast to the dorsum of the fish (Johnson et al. 1995; Parichy et al. 1999). In contrast, *fms* mutants lack both LM melanophores and xanthophores (Fig. 2D; Parichy et al. 2000b), whereas *endothelin receptor b1* (*ednrb1*, formerly *rose*; Fig. 2F; Johnson et al. 1995; Parichy et al. 2000a) and *primrose* (S.L. Johnson, unpublished data) mutant *D. rerio* fail to develop LM melanophores and iridophores.

Intriguingly, the pigment patterns of several *D. rerio* mutants resemble the naturally occurring pigment patterns of other danios. For example, *fms* and *leopard* mutants lack stripes due to varying degrees of melanophore dispersion, similar to *D. albolineatus* (Fig. 2A, D); *jaguar* mutants have fewer and broader stripes, similar to *D. kerri* (Fig. 2B, E); and *ednrb1* and *primrose* mutants exhibit stripes dorsally and spots ventrally, similar to *D. nigrofasciatus* (Fig. 2C, F). These observations raise the possibility that the same genes or cell populations affected in zebrafish pigment pattern mutants also might contribute to pigment pattern differences between *D. rerio* and other danios. Moreover, even loci for which *D. rerio* mutants do not closely resemble other danios represent a priori candidates for contributing to interspecific pigment pattern differences, as differences in genetic background and allelic strength may result in different phenotypes across species. As a single example, *nacre/microphthalmia* mutant *D. rerio* lack stripes due to a complete absence of melanophores (Lister et al. 1999); a weak allele in another species might only reduce melanophore numbers and in so doing change the pattern of these cells, as compared to *D. rerio*.

In this study, we use interspecific hybrids to evaluate the genetic bases for pigment pattern variation among danios. We first reconstruct phylogenetic relationships for the taxa used in this analysis, and assess these relationships in light of hybrid viability and fertility. We then

show that hybrids between several species and *D. rerio* exhibit pigment patterns similar to that of *D. rerio*, consistent with a model in which pigment pattern differences result from gain-of-function alleles in *D. rerio* relative to other species (or loss-of-function alleles in other species relative to *D. rerio*). Finally, we test interspecific roles for pigment pattern genes identified as *D. rerio* mutants by crossing these mutants to hetero-specific danios; these analyses exclude most of these loci from having major effect roles in promoting species differences, but identify one locus, *fms*, as a potential contributor to the loss of stripes in *D. albolineatus*. These studies thus represent an initial step in elucidating the developmental genetics of pigment pattern variation in *Danio*.

## Materials and methods

### Fish stocks and genetic crossing

Wild-type *D. rerio* used in these analyses were strain AB or its derivative, C32 (Streisinger et al. 1981; Nechiporuk et al. 1999). *D. rerio* pigment pattern mutants were maintained in these backgrounds, with the exception of *fms*<sup>4blue</sup> which is derived from the pet trade and maintained in its original genetic background for the purpose of identifying modifiers of *fms* activity (see below). *D. albolineatus* and *D. kerri* were provided by M. McClure (McClure 1999), *D. nigrofasciatus* were derived from stocks collected in Myanmar and provided by K. Yap, and *D. dangila* were provided by D. Nopany. M. Halpern and S. Fisher provided *jaguar*<sup>c5</sup> and deficiency *jaguar*<sup>c7/+</sup> *D. rerio* (Fisher et al. 1997) and J. Lister and D. Raible provided *nacre*<sup>w2</sup> *D. rerio*. All mutant alleles tested for non-complementation in interspecific hybrids were recessive, with the exceptions of *jaguar*<sup>c5</sup> and *jaguar*<sup>b230</sup>, which are semi-dominant in *D. rerio* (see Results). All fertilizations were performed in vitro according to standard procedures (Westerfield 1993). Typically 10–50 hybrids representing 4–10 families were examined for each species and allelic combination, though strain availability and differential survivorship across species precluded examination of such large numbers for some hybrid combinations. For example, mutant *D. rerio* × *D. kerri* hybrids in some families were weak and failed to reach maturity. Nevertheless, at least 5 individuals representing at least 2 families were examined for all hybrid combinations reported here. When possible, crosses were performed with both male and female *D. rerio*, though hybrid phenotypes were not seen to differ depending on sex of parents. Hybrid pigment patterns were evaluated upon reaching adult stages, 2–3 months following fertilization.

### Phylogenetic analysis and sequencing

To assess phylogenetic relationships among danios used in this study we isolated DNA by standard methods from two individuals each of *D. dangila*, *D. nigrofasciatus*, *D. albolineatus*, *D. cf. aequipinnatus*, and *Rasbora trilineata*. We then used universal primers to amplify segments of mitochondrial 12S rDNA (H1478, 5'-TGACTGCAGAGGGTGACGGGCGGTGTGT-3'; L1091, 5'-AAAAAGCTTCAAACCTGGGATTAGATACCCCACTAT-3'; Kocher et al. 1989) and mitochondrial 16S rDNA (16Sar-L, 5'-CGCCTGTTTATCAAAAACAT-3'; 16Sbr-H, 5'-CCGGTCTGAACTCAGATCACGT-3'; Palumbi et al. 1991). Sequences were aligned using CLUSTAL-W, inspected by eye and edited as necessary, then analyzed with PAUP 4.0b4a for Macintosh (Swofford 2000). Maximum likelihood analyses employed a general time reversible model for character state transformations with rate matrix estimation and among site variation modeled with

a gamma distribution estimated from the data. Trees reconstructed using parsimony were evaluated by bootstrapping with 1,000 replicates. To examine the primary structure of the *fms* gene (see below), we amplified overlapping fragments of the *fms* open reading frame from oligo-dT-primed cDNAs produced from at least two individuals each of *D. rerio*, *D. nigrofasciatus*, *D. albolineatus*, *D. kerri*, and *D. dangila* (primer sequences available on request; GenBank accession numbers: AF240639, AF324481, AF324478, AF324480, AF324479). All sequencing was performed using BigDye dye terminator chemistry on an ABI377 automated sequencer.

### Quantitative methods

To evaluate quantitative differences in pigment patterns between *D. rerio* and *D. nigrofasciatus* we assessed melanophore numbers and positions along each of three dorsoventral “transects” on the flank: at the anterior margin of the pelvic fins, and at the anterior and posterior margins of the anal fin (Fig. 1). We assessed the number of stripes according to the mean number of discrete regions of high melanophore density that occurred along each of these transects. Thus, spots that might be present along one transect but not another are represented as fractions <1. Additionally, we determined the number of melanophores within each stripe or spot and used these values to calculate the total numbers of melanophores comprising early developing “primary” melanophore stripes versus later developing “secondary” melanophore stripes. We measured the size of fish by the dorsoventral height of the flank at each transect position, and by the distance from the anterior of the jaw to the caudal peduncle. All fish were similarly sized when evaluated, and because preliminary analyses using these measures of size as covariates did not reveal significant effects on patterning, they are not included in analyses presented below. Statistical analyses were performed using JMP for Apple Macintosh (SAS Institute, Cary, N.C.).

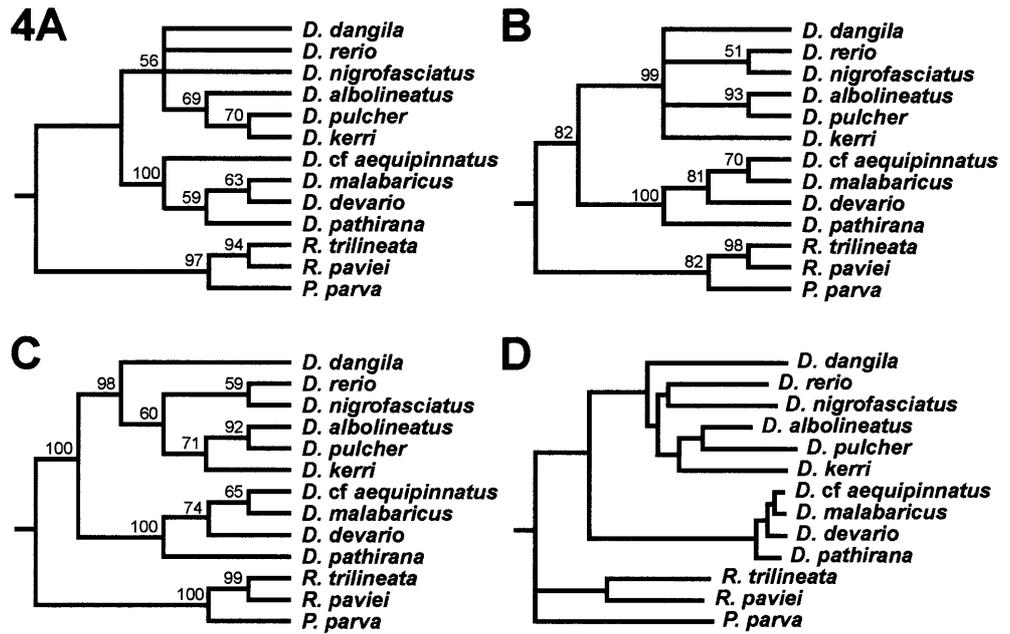
## Results

### Phylogenetic relationships and pigment pattern evolution within *Danio*

As an initial step in assessing the genetic bases for pigment pattern evolution in *Danio*, we evaluated the phylogenetic placement of *D. nigrofasciatus* and *D. dangila*, as these taxa have not been included in previous phylogenetic reconstructions. Sequencing segments of mitochondrial 12S and 16S rDNA and analyses of sequences from GenBank yielded a total of 780 alignable nucleotides (nt) of which 162 nt were phylogenetically informative (12S, 52 nt; 16S, 110 nt). Preliminary analyses using maximum parsimony suggested only marginal discordance between data sets for reconstructed phylogenetic relationships (partition homogeneity test, 1,000 replicates:  $P=0.10$ ; Fig. 4A, B). In analyses combining 12S and 16S data sets, equivalent phylogenetic reconstructions were obtained by maximum likelihood, maximum parsimony (Fig. 4C), and distance methods (Fig. 4D). These analyses all revealed a clade consisting of *D. albolineatus*, *D. pulcher*, and *D. kerri*, and a separate less strongly supported clade comprising *D. rerio* and *D. nigrofasciatus*. Although an earlier phylogenetic analysis identified separate clades of small danios (e.g. *D. rerio*, *D. kerri*) and large danios (e.g. *D. pathirana*, *D. cf. aequipinnatus*; Meyer et al.

**Fig. 4A–D** Phylogenetic relationships of danios. **A, B** Maximum parsimony 50% majority rule consensus trees based on 12S rDNA (**A**) and 16S rDNA (**B**) sequences. **C** Maximum parsimony tree derived from combining 12S and 16S rDNA datasets. Numbers above branches indicate percentage bootstrap support. **D** Neighbor-joining tree of combined 12S and 16S rDNA data sets.

*R. Rasbora*, *P. Pseudorasbora*. GenBank accession numbers for 12S and 16S rDNA sequences for each taxon are (top to bottom): AF322656, AF322661; NC002333, NC002333; AF322657, AF322662; AF322658, AF322663; U21373, U21382; U21372, U21381; AF322659, AF322664; U21376, U21384; U21375, U21370; U21377, U21385; AF322660, AF322665; U21553, U21554; U21378, U21386



1995), our analyses indicate that the large bodied *D. dangila* is more closely related to the clade of small danios than other large bodied species. An analysis of character evolution using the hypothesis of phylogenetic relationships in Fig. 4C, D suggests most parsimoniously that a pattern including at least some horizontal stripes is ancestral for the clade including *D. rerio*, *D. nigrofasciatus*, *D. albolineatus*, and *D. kerri*. The increased number of stripes in *D. rerio*, the reduction in stripe number and presence of ventral spots in *D. nigrofasciatus*, and the absence of stripes in *D. albolineatus*, are all evolutionarily derived.

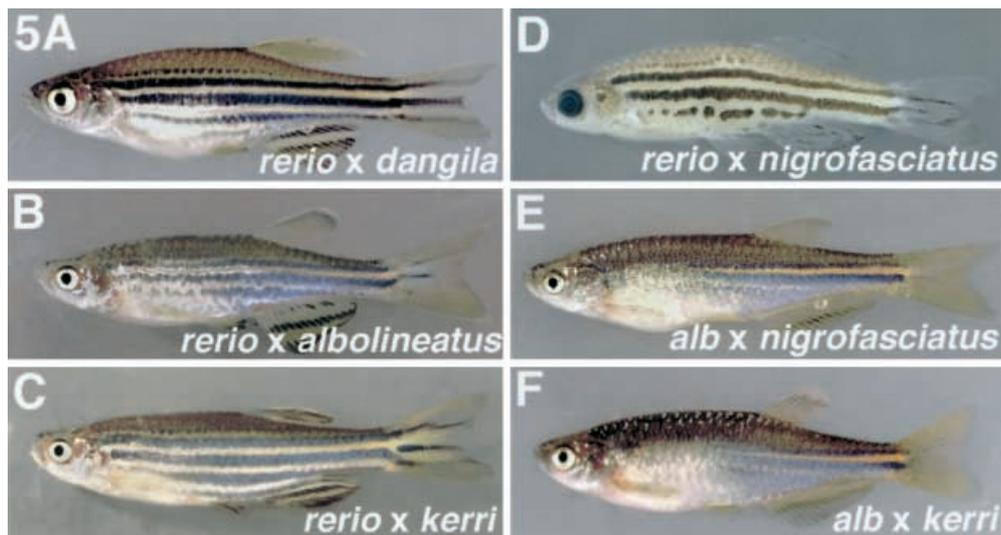
#### Hybrid phenotypes in *Danio*

As a first step in evaluating the genetic bases for stripe evolution and loss, we assessed patterns of dominance and recessivity in hybrids between *D. rerio* and other danios. Interspecific hybrids also provided an independent test of phylogenetic relationships among danios. These analyses revealed that hybrids between *D. rerio* and either *D. cf. aequipinnatus* or the minnow *Tanichthys albonubes* were not viable past 3 days (data not shown) consistent with a relatively large phylogenetic distance separating these taxa (also see Meyer et al. 1995). In contrast, hybrids between *D. dangila* and *D. rerio* were vigorous through adult stages, and reached sizes intermediate to the two parental species (D.M. Parichy, unpublished data). Crosses between *D. rerio* and either *D. albolineatus*, *D. kerri*, or *D. nigrofasciatus* also produced viable adult hybrids. Finally, only crosses between *D. rerio* and *D. nigrofasciatus* produced fertile hybrids, though backcross progeny often were not viable, likely due to chromosomal rearrangements between parental species (D.M. Parichy, unpublished data). Thus,

the pattern of hybrid viability and fertility is concordant with phylogenetic relationships inferred by rDNA sequence analysis.

Pigment patterns of adult hybrids between *D. rerio* and *D. dangila*, *D. albolineatus*, and *D. kerri* typically resembled *D. rerio* more closely than the heterospecific parent. *D. rerio* × *D. dangila* hybrids lacked the wavy stripes and spots present in *D. dangila*, and instead exhibited regular horizontal stripes (Fig. 5A). Likewise, *D. rerio* × *D. albolineatus* hybrids exhibited melanophore stripes rather than a uniform pattern (Fig. 5B), though these stripes tended to have somewhat irregular borders as compared to *D. rerio*. Finally, *D. rerio* × *D. kerri* hybrids typically exhibited four to five melanophore stripes (Fig. 5C), as in *D. rerio*, rather than three melanophore stripes as in *D. kerri*. Thus, alleles of pigment pattern genes in *D. dangila*, *D. albolineatus*, and *D. kerri* typically are recessive to *D. rerio* alleles.

Hybrids between *D. rerio* and *D. nigrofasciatus* developed pigment patterns that included characteristics of each parental species (Fig. 5D). To evaluate these pigment patterns more thoroughly, we counted the numbers of stripes, and numbers of melanophores within stripes, in *D. rerio*, *D. nigrofasciatus*, and the hybrids between these species. Mean numbers of melanophore stripes differed significantly among parental species and hybrids, with hybrids exhibiting on average a stripe number marginally closer to *D. nigrofasciatus* than *D. rerio* (Fig. 7A). Additionally, we compared the numbers of melanophores present in juvenile/early adult primary melanophore stripes, and later adult secondary melanophore stripes. Melanophores were more numerous within primary melanophore stripes of *D. rerio* than *D. nigrofasciatus*, and numbers of primary stripe melanophores in *D. rerio* × *D. nigrofasciatus* hybrids did not differ significantly from *D. rerio*, but were significantly greater



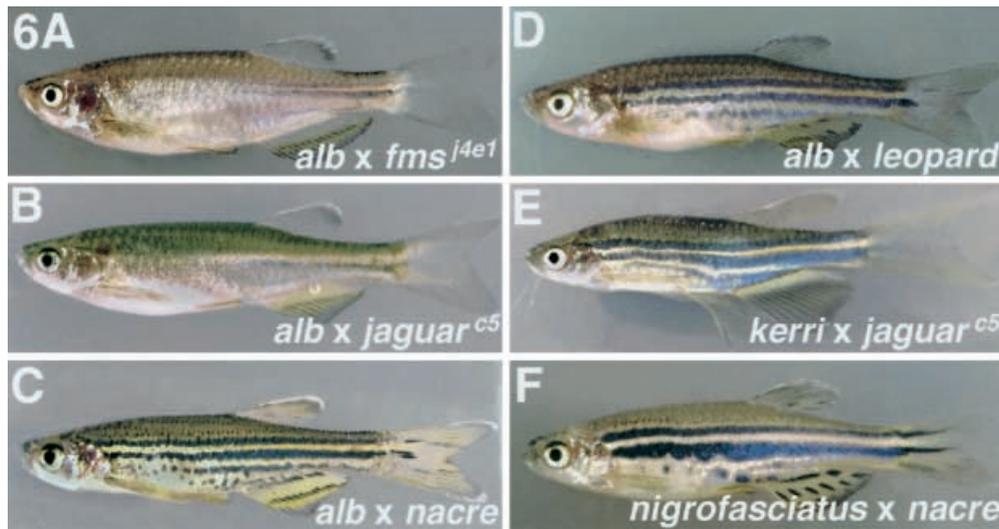
**Fig. 5A–F** Hybrids reveal patterns of dominance and recessivity among danio pigment patterns. **A** *D. rerio* × *D. dangila* hybrids exhibit multiple distinctive horizontal stripes (compare to Figs. 1, 3B). **B** *D. rerio* × *D. albolineatus* hybrids develop horizontal melanophore stripes (Figs. 1, 2A). **C** *D. rerio* × *D. kerri* hybrids exhibit an increased number of melanophore stripes and these stripes are more clearly defined as compared to *D. kerri* (Figs. 1, 2B). **D** *D. rerio* × *D. nigrofasciatus* hybrids exhibit intermediate pigment patterns as compared to parental species, with an increased number of stripes, as well as numbers of melanophores in primary and secondary melanophore stripes as compared to *D. nigrofasciatus* (Figs. 1, 2C). Shown is a fixed specimen. **E** *D. albolineatus* × *D. nigrofasciatus* hybrids develop melanophore stripes that are most distinctive posteriorly (Fig. 2A, C). **F** *D. albolineatus* × *D. kerri* hybrids exhibit melanophore stripes, though these stripes typically are somewhat less distinctive than those exhibited by *D. kerri* (Fig. 2A, B)

than in *D. nigrofasciatus* (Fig. 7B). Melanophores comprising secondary stripes also were more numerous in *D. rerio* than *D. nigrofasciatus*. In contrast to primary stripe melanophores, however, numbers of secondary stripe melanophores in *D. rerio* × *D. nigrofasciatus* hybrids were more similar to *D. nigrofasciatus* than *D. rerio* (Fig. 7C). Thus, alleles of genes controlling primary stripe melanophore number in *D. nigrofasciatus* appear to be largely recessive to *D. rerio* alleles, whereas alleles of genes controlling the numbers of stripes, and melanophores within secondary stripes, are co-dominant between species.

Hybrids between *D. rerio* and other species suggest that a pattern of stripes is dominant over a pattern lacking stripes. To further test the generality of this result, we also crossed *D. albolineatus* to *D. nigrofasciatus* and to *D. kerri*. These hybrids were viable but infertile. Hybrids between *D. albolineatus* and *D. nigrofasciatus* exhibited a pattern intermediate between parental species: stripes formed posteriorly, but were increasingly diffuse anteriorly (Fig. 5E). Hybrids between *D. albolineatus* and *D. kerri* also developed intermediate patterns, exhibiting stripes that were less distinctive than those of *D. kerri* (Fig. 5F). Together, phenotypes expressed by hybrids indicate that a pattern of stripes is dominant or semi-dominant over the *D. albolineatus* pattern, which lacks stripes.

#### Analysis of candidate genes for heterospecific pigment patterns

Our finding that alleles of pigment pattern genes in other species frequently are recessive to *D. rerio* alleles suggests that genetic complementation tests could be used to ask whether genes isolated as pigment pattern mutants in *D. rerio* might be the same genes that contribute to interspecific pigment pattern differences. In standard genetics, the complementation test is used to determine whether two mutations affect different genes or the same gene. If a cross between two mutants yields phenotypically wild-type offspring, the mutations are said to complement and are inferred to affect different genes. If the cross yields phenotypically mutant offspring, the mutations are said to non-complement and are inferred to affect the same gene. In an interspecific context, complementation tests have been used to ask whether mutants in a tester species correspond to the same affected genes in another, closely related species (Long et al. 1996; Sucena and Stern 2000). With respect to danio pigment patterns, crosses between *D. rerio* mutant for pigment pattern genes and heterospecific danios should thus provide an efficient means to rapidly exclude or include candidate genes for future analysis: hybrids that resemble control hybrids between wild-type *D. rerio* and the heterospecific danio (i.e. exhibit a complementation phenotype) would suggest there is no difference in the activity of the candidate gene between species; hybrids that resemble the mutant *D. rerio* or heterospecific parent (i.e. exhibit a non-complementation phenotype) suggest there may be an interspecific difference in the activity of the candidate gene. Such a difference might reflect an evolutionary change in the primary structure of the gene that affects its function, or changes in its spatial and temporal regulation. To test potential roles for these previously isolated pigment pattern genes, we crossed *D. rerio* pigment pattern mutants (Table 1) to *D. albolineatus*, *D. kerri*, and *D. nigrofasciatus*, and compared the phenotypes of the resulting hybrids to control hybrids between wild-type *D. rerio* and each of these species (shown in Fig. 5B–D).



**Fig. 6A–F** Hybrids between *D. rerio* pigment pattern mutants and other danios suggest and exclude candidate genes and mechanisms for pigment pattern diversification. **A** *D. albolineatus* × *fms*<sup>4e1</sup> mutant *D. rerio* lack stripes and exhibit a pigment pattern more similar to *D. albolineatus* (Fig. 2A) than control hybrids (Fig. 5B). **B** *D. albolineatus* × *jaguar*<sup>c5</sup> mutant *D. rerio* also lack stripes (see text). **C** *D. albolineatus* × *nacre*<sup>w2/mitf</sup> mutant *D. rerio* develop stripes similar to control hybrids. **D** *D. albolineatus* × *leopard*<sup>l1</sup> mutant *D. rerio* also develop stripes. **E** *D. kerri* × *jaguar*<sup>b230</sup> mutant *D. rerio* exhibit fewer stripes that are more similar to *D. kerri* (Fig. 2B) than control hybrids (Fig. 5C; see text). **F** *D. nigrofasciatus* × *nacre*<sup>w2/mitf</sup> mutant *D. rerio* do not exhibit pigment patterns significantly different from control hybrids (Figs. 5D, 7).

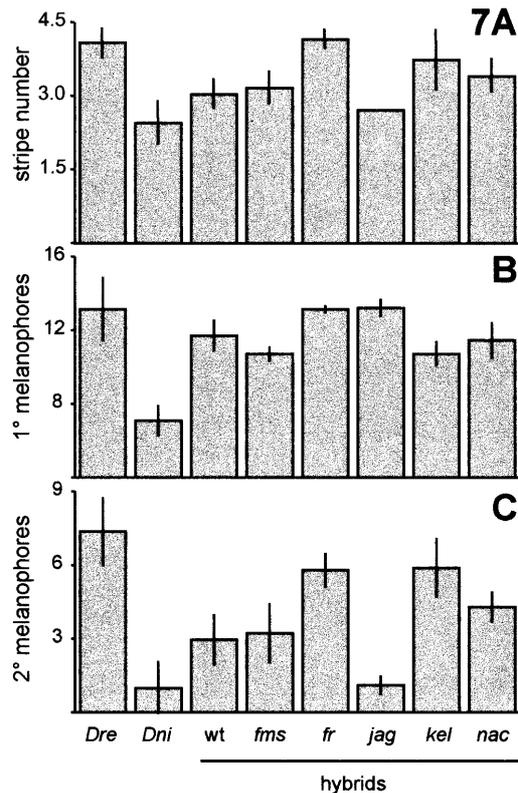
#### *D. albolineatus* candidate genes

Hybrids between mutant *D. rerio* and *D. albolineatus* typically developed stripes similar to control hybrids between wild-type *D. rerio* and *D. albolineatus* (Fig. 6C, D; compare to Fig. 5B). In contrast, hybrids between *fms*<sup>4e1</sup> *D. rerio* and *D. albolineatus* lacked stripes and resembled *D. albolineatus* more closely than the control hybrids (Fig. 6A). These findings raise the possibility that *fms*, which encodes a type III receptor tyrosine kinase expressed by pigment cell precursors (Parichy et al. 2000b), may differ in its activity between *D. albolineatus* and wild-type *D. rerio*, identifying *fms*, or the pathway in which it acts, as a candidate for contributing to the evolutionary loss of stripes in *D. albolineatus*. To further assess this possibility, we tested two additional alleles, *fms*<sup>4e3</sup> and *fms*<sup>4blue</sup>. Whereas hybrids between *fms*<sup>4e3</sup> (like *fms*<sup>4e1</sup>) *D. rerio* and *D. albolineatus* lacked stripes, hybrids between *fms*<sup>4blue</sup> *D. rerio* and *D. albolineatus* resembled control hybrids. As all three mutant alleles tested are predicted to exhibit severe loss of function (Parichy et al. 2000b) but *fms*<sup>4blue</sup> represents a different genetic background, these results suggest that modifier loci may be present that influence whether or not stripes form. Comparison of *fms* open reading frames in *D. albolineatus*, *D. kerri*, *D. nigrofasciatus*, *D. dangila*, and *D. rerio* failed to identify gross lesions (e.g. deletions,

premature stop codons, transposable element insertions) in *D. albolineatus*, but did reveal several unique amino acid substitutions not present in other species (phylogenetic reconstructions based on *fms* sequences were concordant with those from rDNA sequences; data not shown). Finally, crosses between *jaguar*<sup>c5</sup> or *jaguar*<sup>b230</sup> *D. rerio* and *D. albolineatus* also lacked stripes (Fig. 6B), though this phenotype may reflect a dominant effect of these *jaguar* mutations (see below).

#### *D. kerri* candidate genes

Hybrids between *fms*<sup>4blue</sup> or *kit*<sup>b5</sup> mutant *D. rerio* and *D. kerri* exhibited pigment patterns that were not grossly different from control hybrids between wild-type *D. rerio* and *D. kerri*. In contrast, hybrids of *D. kerri* with either *jaguar*<sup>c5</sup> or *jaguar*<sup>b230</sup> each resulted in a reduction in stripe number as compared to control hybrids (Fig. 6E; compare with Fig. 5C), yielding a pigment pattern resembling that of *D. kerri* or *jaguar* *D. rerio* (Fig. 2B, E). As both of these *jaguar* alleles are semidominant, and exhibit broken stripes when heterozygous in *D. rerio*, we asked whether the pigment patterns in these hybrids resulted from a dominant effect of the *D. rerio* *jaguar* mutation in the hybrid background, or a difference that might be associated with the *D. kerri* *jaguar* orthologue. To distinguish between these possibilities, we crossed *D. kerri* to heterozygous carriers of a recessive deficiency, *jaguar*<sup>c7</sup>. Although homozygous *jaguar*<sup>c7</sup> *D. rerio* die prior to development of an adult pigment pattern, trans-heterozygous *jaguar*<sup>c7</sup>/*jaguar*<sup>c5</sup> *D. rerio* are indistinguishable from *jaguar*<sup>c5</sup> homozygotes. Hybrids resulting from crosses between *D. kerri* and *jaguar*<sup>c7</sup>/*D. rerio* did not exhibit fewer stripes as compared to control hybrids. To verify that hybrids carrying the deficiency survived to adult stages, we used PCR to test for the presence of microsatellite marker z4396, which is centromeric to the *jaguar*<sup>c7</sup> deficiency, and the presence or absence of microsatellite markers z13822 and z7381 (Shimoda et al. 1999; see <http://zebrafish.mgh.harvard.edu>),



**Fig. 7A–C** Quantitative analyses of pigment patterns in *D. rerio*, *D. nigrofasciatus*, and hybrids between these species. Parental species: *Dre*, *D. rerio*, *Dni*, *D. nigrofasciatus*. Hybrid offspring: *wt*, wild-type *D. rerio* × *D. nigrofasciatus*, *fms*, *fms*<sup>4blue</sup> *D. rerio* × *D. nigrofasciatus*, *fr*, *fritzj*<sup>101e1</sup> *D. rerio* × *D. nigrofasciatus*, *jag*, *jaguar*<sup>c5</sup> *D. rerio* × *D. nigrofasciatus*, *kel*, *kit*<sup>b5</sup> *ednrb*<sup>140</sup> *leopard*<sup>d1</sup> *D. rerio* × *D. nigrofasciatus*, *nac*, *nacre*<sup>w2</sup> *D. rerio* × *D. nigrofasciatus*. **A** Mean stripe number is significantly greater in *D. rerio* as compared to *D. nigrofasciatus* ( $F_{1,18}=87.48$ ,  $P<0.0001$ ), and the intermediate stripe number in hybrids between *wt D. rerio* and *D. nigrofasciatus* differs significantly from both parental species ( $F_{2,26}=50.75$ ,  $P<0.0001$ ; Tukey-Kramer comparisons, all  $P<0.05$ ). Among hybrids between mutant *D. rerio* and *D. nigrofasciatus*, none exhibited a stripe number significantly less than that observed in control hybrids between *wt D. rerio* and *D. nigrofasciatus* (Tukey-Kramer comparisons, all  $P>0.05$ ). **B** Mean primary melanophore number is significantly greater in *D. rerio* than *D. nigrofasciatus* ( $F_{1,18}=99.63$ ,  $P<0.0001$ ), and hybrids between *wt D. rerio* and *D. nigrofasciatus* exhibit an intermediate number of primary melanophores not significantly different from *D. rerio*, but significantly greater than *D. nigrofasciatus* ( $F_{2,26}=66.01$ ,  $P<0.0001$ ; Tukey-Kramer comparisons,  $P>0.05$ ,  $P<0.05$  respectively). Hybrids between mutant *D. rerio* and *D. nigrofasciatus* did not have significantly fewer primary melanophores than control hybrids between *wt D. rerio* and *D. nigrofasciatus* (Tukey-Kramer comparisons, all  $P>0.05$ ). **C** Mean secondary melanophore number was significantly greater in *D. rerio* than *D. nigrofasciatus* ( $F_{1,18}=136.03$ ,  $P<0.0001$ ) and control hybrids exhibited an intermediate number of secondary melanophores that differed significantly from both parental species ( $F_{2,26}=77.21$ ,  $P<0.001$ ; Tukey-Kramer comparisons,  $P<0.05$ ). Only hybrids between *jag*<sup>c5</sup> *D. rerio* and *D. nigrofasciatus* exhibited secondary melanophore numbers significantly less than control hybrids (Tukey-Kramer comparison,  $P<0.05$ ; see text). Bars  $\pm 1$  SD

which are contained within the *jaguar*<sup>c7</sup> deficiency (N. Gosse, S.L. Johnson, unpublished data). All 17 hybrids tested amplified z4396, indicating the presence of a *D. rerio jaguar*<sup>+</sup> or *jaguar*<sup>c7</sup> chromosome; 7 of these hybrids failed to amplify both z13822 and z7381, indicating that they carried the *jaguar*<sup>c7</sup> deficiency chromosome. Although 3 of the *jaguar*<sup>c7</sup> carriers exhibited moderately broken stripes, none had fewer stripes as compared to siblings carrying the wild-type *jaguar* allele. These analyses tend to exclude *jaguar* from having a major role in promoting the difference in stripe number between wild-type *D. rerio* and *D. kerri*, though conceivably other genes in the pathway in which *jaguar* acts could be involved in this interspecific difference. This possibility is also suggested by the close similarity of the *jaguar D. rerio* × *D. kerri* hybrid non-complementation phenotype to *D. kerri*, as well as the *jaguar D. rerio* × *D. albolineatus* non-complementation phenotype to *D. albolineatus*, as compared to the broken stripe phenotype of heterozygous *jaguar*<sup>c5</sup> or *jaguar*<sup>b230</sup> *D. rerio*.

#### *D. nigrofasciatus* candidate genes

Pigment patterns of hybrids between mutant *D. rerio* and *D. nigrofasciatus* typically did not differ from control hybrids (e.g. Figs. 6F, 7), with the exception of hybrids between *jaguar*<sup>c5</sup> *D. rerio* and *D. nigrofasciatus*, which exhibited fewer melanophore stripes and secondary melanophores than controls (Fig. 7A, C), likely due to a dominant effect of the *jaguar*<sup>c5</sup> mutation (above). Although the *leopard* locus has been suggested to be responsible for the difference between wild-type *D. rerio* and *D. nigrofasciatus* (Frankel 1979), hybrids between *leopard D. rerio* and *D. nigrofasciatus* did not have fewer melanophores or fewer stripes than control hybrids (Fig. 7). Finally, despite the similarity of *ednrb1* and *primrose* mutant *D. rerio* to *D. nigrofasciatus*, hybrids carrying either of these mutations did not differ from controls. These analyses tend to exclude these loci from having major effect roles in contributing to the difference between wild-type *D. rerio* and *D. nigrofasciatus* pigment patterns.

## Discussion

Pigment patterns are a tractable system for identifying molecular and cellular mechanisms underlying adult form in vertebrates, and how changes in these mechanisms have resulted in phylogenetic transformations of adult traits. The results of this study are a first step in assessing the genetic bases for pigment pattern diversity in the genus *Danio*. Our data provide insights into phylogenetic relationships among danios, the genetics of pigment pattern differences across *Danio* species, and potential roles for candidate genes in contributing to this phenotypic variation.

The genus *Danio* comprises as many as 45–50 species that exhibit considerable variation in pigment pattern,

vertebral count, body size, and other characters (Goodrich et al. 1954; Meyer et al. 1995; Fang 1997a, b, 1998; McClure 1999). Small- and large-bodied danios were traditionally grouped in different genera (*Brachydanio* and *Danio*, respectively), and phylogenetic reconstructions based on mitochondrial and nuclear genes have revealed separate clades of large and small species (Meyer et al. 1995; Zardoya et al. 1996). Our analyses were not intended to definitively resolve the phylogenetic relationships within this speciose genus, but rather to elucidate relationships among taxa we used for examining hybrid pigment patterns. Nevertheless, our taxonomically limited analyses support the grouping of large and small danios within a single genus: the large bodied *D. dangila* is predicted to be more closely related to small danios (e.g. *D. rerio*) than other large danios (e.g. *D. pathirana*), based on both mitochondrial rDNA sequences and viability of hybrids. More recent analyses also have placed a small-bodied danio within a clade of otherwise large-bodied species (D.M. Parichy, unpublished data). Thus, the traditional genus *Danio* is likely to be paraphyletic relative to *Brachydanio*, supporting the view that all of these species should be considered provisionally within *Danio*. We caution, however, that inclusion of additional species into phylogenetic analyses could conceivably alter these reconstructed relationships, highlighting the need for a thorough molecular and morphological revision of the genus (e.g. Fang 1998). Finally, our analysis of rDNA and *fms* sequences, as well as patterns of hybrid viability and fertility, identified *D. nigrofasciatus* as a sister species to *D. rerio* among the taxa we examined. As both *D. rerio* and *D. nigrofasciatus* exhibit particularly narrow and well-defined melanophore stripes, this result parsimoniously suggests a qualitative change in stripe-forming mechanisms within this lineage as compared to other danios. Additional developmental and phylogenetic analyses will be required to determine if an ancestor of the clade including *D. rerio* and *D. nigrofasciatus* possibly exhibited a pigment pattern resembling one or the other of these species, or an intermediate pattern.

The mechanisms underlying pigment pattern diversification in *Danio* are unknown. Nevertheless, our analyses of interspecific hybrids provide a first insight into the genetic bases of species differences in this genus. Hybrids between *D. rerio* and either *D. dangila*, *D. albolineatus*, or *D. kerri* exhibited pigment patterns that were similar to *D. rerio*, indicating that alleles of pigment pattern genes in these other species are recessive to *D. rerio* alleles. Since allelic dominance and recessivity often reflects underlying levels of gene activity (e.g. Kacser and Burns 1981; Besmer et al. 1993), the present results are consistent with a model in which the *D. rerio* pigment pattern results principally from gain-of-function alleles relative to these other species, or pigment patterns of other species result from loss-of-function alleles relative to *D. rerio*. The development of stripes in hybrids between *D. albolineatus* (which lacks stripes) and both *D. nigrofasciatus* and *D. kerri* (which exhibit stripes),

further supports a model in which the loss of a striped pigment pattern in *D. albolineatus* has resulted from the evolution of loss-of-function alleles in this species relative to striped danios.

In contrast to other species, hybrids between *D. rerio* and *D. nigrofasciatus* exhibited pigment patterns that were largely intermediate between the two parental species. Whereas numbers of melanophores in primary melanophore stripes of hybrids were not significantly different from *D. rerio*, numbers of melanophores in secondary melanophore stripes, and total stripe numbers, were intermediate between species and more similar to *D. nigrofasciatus*. These findings suggest the existence of different genes contributing to the development of primary stripe melanophores as compared to secondary stripe melanophores and total stripe number, or a common set of loci having antagonistic, pleiotropic effects on these characters. These analyses also are consistent with a model in which greater numbers of primary stripe melanophores in *D. rerio* result from gain-of-function alleles in this species relative to *D. nigrofasciatus* (or loss-of-function alleles in *D. nigrofasciatus* relative to *D. rerio*).

Our finding that alleles of pigment pattern genes in other species often are recessive to *D. rerio* alleles allowed us to use interspecific complementation tests to ask whether the same genes affected in *D. rerio* pigment pattern mutants also might contribute to pigment pattern differences across species. Complementation phenotypes exhibited by most hybrids between *D. rerio* pigment pattern mutants and other danios suggest that most of the loci tested are not likely to have major effect roles in determining pigment pattern differences across species. For example, *leopard* was an a priori candidate for contributing to the uniform pigment pattern in *D. albolineatus* (e.g. Haffter et al. 1996; Asai et al. 1999), and has been suggested to be the locus responsible for the pigment pattern differences between *D. nigrofasciatus* and *D. rerio* (Frankel 1979). Nevertheless, hybrids between *leopard* *D. rerio* and either of these species did not differ discernibly from control hybrids with wild-type *D. rerio*, suggesting that *leopard* probably is not causally related to the pigment pattern differences between these species and *D. rerio*. Nevertheless, we do not rule out the possibility that loci tested here might have minor effect roles in contributing to interspecific pigment pattern differences. Finally, phenotypes of interspecific hybrids identified one locus as a potential contributor to a pigment pattern difference between species: hybrids between *fms* mutant *D. rerio* and *D. albolineatus* failed to develop stripes, though this effect depended on the *fms* allele or genetic background tested. The finding of unique amino acid substitutions in *D. albolineatus fms* is consistent with a difference in the activity of this locus, though functional consequences for these substitutions await experimental evaluation. Further molecular, genetic, and cellular analyses are now underway to test roles for *fms* and *fms*-dependent cell populations during stripe loss in *D. albolineatus*.

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