

Genetic divergence among sympatric colour morphs of the Dalmatian wall lizard (*Podarcis melisellensis*)

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Abstract If alternative phenotypes in polymorphic populations do not mate randomly, they can be used as model systems to study adaptive diversification and possibly the early stages of sympatric speciation. In this case, non random mating is expected to support genetic divergence among the different phenotypes. In the present study, we use population genetic analyses to test putatively neutral genetic divergence (of microsatellite loci) among three colour morphs of the lizard *Podarcis melisellensis*, which is associated with differences in male morphology, performance and behaviour. We found weak evidence of

genetic divergence, indicating that gene flow is somewhat restricted among morphs and suggesting possible adaptive diversification.

Keywords Polymorphism · F_{ST} · F_{IS} · Non-random mating · Population divergence · Microsatellites

Introduction

Phenotypic polymorphisms are a prominent form of diversification in many animal taxa, and a fascinating aspect of biological diversity. They are often used as model systems in studies central to evolutionary biology. The long term maintenance of alternative morphs in a population implies that all morphs have equivalent fitnesses, and often the different morphs show differences in other phenotypic traits as well. When alternative morphs coexist in a population, correlational (or epistatic) selection may favor certain trait combinations at the expense of other combinations, which may result in modifications of the genetic architecture and developmental pathways and lead to genetic divergence among morphs (Lande and Arnold 1983; Forsman and Appelqvist 1998; Forsman et al. 2008). In resource based or trophic polymorphisms for example, discrete morphs can show differential morphological adaptations and resource use (see review in Smith and Skúlason 1996). In other cases, phenotypic polymorphisms have been linked to alternative reproductive strategies (Oliveira et al. 2008), with accompanying differences in size, shape, and/or performance (Sinervo et al. 2000; Meyers et al. 2006; Pryke and Griffith 2006; Huyghe et al. 2007).

Phenotypic morphs associated with alternative reproductive strategies are usually found to be adaptive

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(Brockmann and Taborsky 2008). Polymorphisms may be the outcome of phenotypic plasticity (condition-dependence), may be highly genetically determined, or may be the result of a combination of the two. Further differentiation could then be promoted in the presence of non-random mating barriers such as positive assortative mating among individuals of the same morphotype or selection against hybrids. This could theoretically lead to reproductive isolation between morphotypes, even in the absence of temporal or spatial segregation. Genetic divergence between such sympatric morphs provides an outstanding opportunity to study the early stages of adaptive diversification and sympatric speciation (Smith and Skúlason 1996; Hendry et al. 2009).

Whether and how often sympatric speciation occurs in natural populations are much debated topics, but different speciation models have demonstrated the theoretical feasibility and some empirical examples are available. Theoretical models have been described for sympatric speciation through natural and sexual selection (Lande and Kirkpatrick 1986; Van Doorn and Weissing 2001; Gavrilov and Waxman 2002; Härdling and Bergsten 2006), but these models also have identified many possible constraints on diversification in sympatry (Coyne and Orr 2004; Bolnick and Fitzpatrick 2007). Empirical evidence of reproductive isolation and incipient sympatric speciation through natural selection (ecological speciation) has been found in several studies. For instance, in the marine periwinkle (*Littorina saxatilis*), males prefer females of their own ecotype (Johannessen et al. 2008), and in Darwin's finches (*Geospiza fortis*) there is strong assortative pairing within small and large beak morphs, resulting in restricted gene flow between these morphs (Huber et al. 2007). Other examples of sympatric speciation have been found in stickleback fish (McKinnon and Rundle 2002), *Rhagoletis* flies (Linn et al. 2004), and other herbivorous insects (e.g. Funk et al. 2002; Matsubayashi et al. 2009). Sexual selection as a sole mechanism driving sympatric speciation is unlikely in the absence of any ecological diversification (Coyne and Orr 2004; Van Doorn et al. 2004; Bolnick and Fitzpatrick 2007; Ritchie 2007), but sexual selection could be responsible for mating polymorphisms, possible cradles for additional (ecological) sources of disruptive selection.

To test genetic divergence among alternative sympatric morphs, one must identify genetically diverged subpopulations (morphs) and assign individuals (probabilistically) to these subpopulations. The highest resolution to estimate relatedness among individuals of unknown pedigree can be achieved by using data from highly polymorphic loci. In this study we estimated the putatively neutral divergence at microsatellite loci between colour morphs of a population of the lizard *Podarcis melisellensis*, occurring on the island of Lastovo, Croatia. In this population, males and females

occur in three different phenotypes (morphs): they show white, yellow or orange ventral coloration (Huyghe et al. 2007). Male morphs show differences in size, head shape, jaw muscle mass, bite force capacity and fighting ability (Huyghe et al. 2007, 2009a), while female morphs do not differ in any of the aforementioned traits (Huyghe et al. unpublished data). We therefore assume that these male morphs have evolved as alternative “tactics” to deal with different selective pressures partly shaped by sexual selection, while the mechanisms through which female morphs originated remain unknown. The relative frequencies of the morphs within a population of *P. melisellensis* and individual coloration seem to remain stable across years (Huyghe et al. unpublished data). Polymorphisms in coloration or colour patterns are widespread in natural populations, and have contributed significantly to the understanding of the conditions that promote the evolution and maintenance of polymorphisms (Sinervo et al. 2007; Forsman et al. 2008; Svensson et al. 2009). As the colour morphs in *P. melisellensis* are associated with morph-specific suites of characters, positive assortative mating might promote increased phenotypic and genetic divergence, and eventually complete reproductive isolation. Hence, this polymorphic population provides an ideal study system to test genetic divergence by non-random (assortative) mating.

The random mating hypothesis for permanent polymorphism states that different morphs of a polymorphic population can successfully interbreed so that random mating occurs (Ford 1945). In this context, we tested the null hypothesis of random mating, i.e. lack of genetic divergence, among colour morphs in the polymorphic lizard *Podarcis melisellensis*.

Methods

Field methods

Podarcis melisellensis is a medium-sized lacertid lizard occurring along the Adriatic coast and on islands in the Adriatic Sea. Populations of *P. melisellensis* seem to vary in their degree of polymorphism; some populations (islands) include one, two, or three colour (Huyghe et al. pers. obs.). On the island of Lastovo (Croatia, 42°16'N, 16°54'E, 53 km²), lizards have a bright white, yellow or orange ventral colour. These three morphs are distributed randomly and share the same microhabitat (Huyghe et al. 2007). During fieldtrips in 2006 and 2007, we collected a small piece of tail tissue (<3 mm) from 271 individuals of this population, comprising 140 males (78 white, 32 yellow and 30 orange) and 131 females (98 white, 25 yellow and 8 orange). Tail tissue was preserved in 95% ethanol for

transport and storage until DNA analysis (Huyghe et al. 2009b).

Laboratory processing

Microsatellite genotypes from 13 loci were used to assess gene flow and genetic subdivision among the colour morphs, thus indirectly testing for assortative mating.

DNA was extracted using a modified Chelex extraction protocol (adapted from R. J. Nelson, Institute of Ocean Sciences, personal comm., Small et al. 1998). An approximately 2 mm³ piece of tissue was incubated in chelex extraction buffer (10% Chelex (Bio-Rad), 0.1% SDS and 0.1% of 20 mg/mL Proteinase K) for 60 min at 65°C and 15 min at 95°C, then refrigerated (long-term storage in freezer).

Forward microsatellite primers were fluorescently labeled (6fam dye: Sigma and vic, pet, ned dye: Applied Biosystems). Multiplex Polymerase Chain Reaction (PCR) amplifications were performed in a 12.5 µL reaction volume containing 1.5 µL of template DNA, 100× primer mix containing each primer at 0.02 µM, 4× Qiagen multiplex PCR master mix (Qiagen), and 3× RNase free H₂O. Amplification entailed an initial denaturation step of 15 min at 95°C, followed by 30 cycles of 30 s denaturation at 95°C, 90 s of annealing at locus-specific temperatures (Huyghe et al. 2009b), and 60 s of extension at 72°C. After the last cycle, a final extension was done at 60°C for 30 min. Negative controls containing only PCR reagents were included in each PCR run.

For fluorescent detection on an AB 3130 XL Genetic Analyzer, PCR products were mixed with formamide and an internal size standard (500Liz, GeneScan). The size of the PCR products was determined using the Genemapper v 4.0 software program (Applied Biosystems).

Statistical analyses

Individual lizards were unambiguously assigned to one of the colour morph classes by visual inspection (Huyghe et al. 2007). Measures of genetic variation within morph classes, including allelic diversity, allelic frequency and heterozygosity, were calculated using Genepop v.3.3 (Raymond and Rousset 1995) and Fstat v.2.9.3.1 (Goudet 1995). To assess whether collections were random samples from a panmictic population, we tested for deviations from Hardy-Weinberg equilibrium (HWE) using the Markov chain method implemented in Genepop v.3.3 (dememorization = 1000, batches = 100, iterations per batch = 1000). We then compared deviations from HWE across and within colour morphs. Loci not in HWE were checked with Microchecker v.2.2.3 (Chakraborty et al. 1992; Brookfield 1996) to detect possible interpretation errors due to the presence of null

alleles, stuttering and/or differential amplification efficiency of large vs small alleles (large allele dropout). We tested for linkage disequilibrium between pairs of loci using Genepop v.3.3. across and within colour morph classes.

Genetic heterogeneity among colour morphs was analysed in several ways. First, we tested for differences in allele frequencies by using the ‘genic differentiation’ option in Genepop v.3.3. Second, we estimated genetic differentiation using *F*-statistics (F_{ST}) and *R*-statistics (R_{ST}) (Weir and Cockerham 1984; Queller and Goodnight 1989) using Fstat v.2.9.3.1. To further assess population structuring, we used a factorial correspondence analysis (FCA) in Genetix v.4.05 (Belkhir et al. 1996) to determine the similarity of allelic composition among the colour morph classes. Genetix performs a FCA in which composite axes are generated that maximize differences among individuals of the different morphs based upon allele frequencies, and then plots individuals in three dimensions according to their genotype. We used a multivariate analysis of variance to test for differences in the FCA factors for the three colour morph classes, using SPSS v.16. We also used the Bayesian approach implemented in Structure v.2.2 (Pritchard et al. 2000) to identify ancestry of the three colour morph classes. We ran five simulations for each putative number of clusters ($K = 1-5$). In each case, we used the admixture model with burn-in length of 100000 and Monte Carlo Markov chain iteration value 500000. Finally, we assessed possible family structure within the colour groups by calculating mean relatedness (*R*) and the variance of relatedness for each colour morph class, using 1000 permutations with Identix v.1.1.5 (Belkhir et al. 2002) and pairwise relatedness (percentage of half- and full sibs) among the individuals of each group.

Results

Testing overall genetic diversity (Table 1) yielded very similar results to those in a previous study (Huyghe et al. 2009b). The only exception was locus Pmeli19, which showed a significant deviation in genotype frequency from HWE in Huyghe et al. (2009b), but not so in the present work, probably because of the larger sample size. The number of alleles per locus in the present analysis was only slightly higher (maximal increase of 5 alleles per locus) than in Huyghe et al. (2009b), suggesting that our sample reflects the overall population diversity fairly well.

Two loci deviated from HWE in each colour morph class: Pmeli02 and Pmeli18 (Table 2). Microchecker suggested possible null alleles, provoking a general excess of homozygotes for most allele size classes at these loci. When null alleles are present, observed genotypes represent one of several possible true genotypes, and depending

Table 1 Overall genetic diversity of the entire populations (all morph classes pooled)

Locus	<i>N</i>	<i>N_A</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P</i>
Pmeli-02	263	42	0.657	0.910	0.278	<i><0.001</i>
Pmeli-04	254	29	0.795	0.885	0.102	<i><0.001</i>
Pmeli-05	270	19	0.803	0.805	0.002	0.201
Pmeli-07	254	14	0.795	0.885	0.102	<i><0.001</i>
Pmeli-08	267	27	0.920	0.929	0.010	0.289
Pmeli-10	269	13	0.792	0.804	0.015	0.086
Pmeli-11	271	15	0.755	0.781	0.033	0.034
Pmeli-13	270	20	0.803	0.839	0.043	0.677
Pmeli-14	271	27	0.920	0.942	0.023	0.594
Pmeli-15	271	18	0.803	0.811	0.010	0.108
Pmeli-16	268	26	0.852	0.936	0.090	0.006
Pmeli-18	231	30	0.460	0.832	0.447	<i><0.001</i>
Pmeli-19	270	45	0.961	0.954	-0.007	0.176

Significant *P*-values (<0.05) for deviation from HWE that remain significant after sequential Bonferroni correction are in italic

N number of individuals, *N_A* number of alleles, *H_O* observed heterozygosity, *H_E* expected heterozygosity

Table 2 *F_{IS}* values and deviations from HWE for each of the colour morph classes

Locus	White		Orange		Yellow	
	<i>F_{IS}</i>	<i>P</i>	<i>F_{IS}</i>	<i>P</i>	<i>F_{IS}</i>	<i>P</i>
Pmeli-02	0.262	<i><0.001</i>	0.374	<i><0.001</i>	0.264	<i><0.001</i>
Pmeli-04	0.111	<i><0.001</i>	0.082	0.552	0.142	0.025
Pmeli-05	-0.001	0.451	0.006	0.212	0.026	0.379
Pmeli-07	0.111	<i><0.001</i>	0.082	0.594	0.142	0.016
Pmeli-08	0.011	0.191	0.006	0.800	0.027	0.127
Pmeli-10	0.016	0.426	0.023	0.151	0.024	0.227
Pmeli-11	0.036	0.117	0.127	0.071	-0.031	0.597
Pmeli-13	-0.003	0.937	0.058	0.008	0.069	0.941
Pmeli-14	0.043	0.146	-0.001	0.454	-0.012	0.613
Pmeli-15	0.005	0.599	0.086	0.231	-0.015	0.784
Pmeli-16	0.065	0.047	0.122	0.031	0.066	0.055
Pmeli-18	0.481	<i><0.001</i>	0.416	<i><0.001</i>	0.482	<i><0.001</i>
Pmeli-19	-0.002	0.603	0.014	0.089	-0.015	0.398
Overall	0.124	<i><0.001</i>	0.125	<i><0.001</i>	0.111	<i><0.001</i>
Overall ^a	0.062	<i><0.001</i>	0.059	0.014	0.049	0.007

Significant *P*-values (<0.05) for deviation from HWE that remain significant after sequential Bonferroni correction are in italic

^a Without loci Pmeli18 and Pmeli02

on their frequency, analyses need to adjust for their presence (Dakin and Avise 2004; Wagner et al. 2006). To test the impact of removing these loci, we ran all analyses again without these loci (see Table 2 for comparison of overall *F_{IS}* values with and without these two loci). None of the

significance levels of the results were altered. The estimated null allele frequencies of Pmeli02 (frequency *P* = 0.14) and Pmeli 18 (frequency *P* = 0.32) were substantial, but since they apparently did not affect significance levels of the analyses, we included the loci with possible null alleles (Wagner et al. 2006), and report only the results for analyses including all loci.

We found some evidence for genetic divergence among the colour morphs of *P. melisellensis*. First, more loci showed heterozygote deficits when data from the three colour morphs were pooled (Table 1), than when morphs were considered separately (Table 2). Within colour morph classes, *F_{IS}* values were a mix of positive and negative values, as expected with a random collection, and mainly the loci with possible null alleles were not in HWE. However, when the three groups are combined, almost all the *F_{IS}* values are positive, suggesting a Wahlund effect caused by mixing unrecognized genetically different subpopulations. Although allele frequencies of the individual loci did not differ significantly among colour morphs, and genetic divergence measures of these loci were not significantly different from zero among morphs, overall allele frequencies did differ significantly, Table 3). Also, pairs of loci did not show more or less significant levels of linkage disequilibria within vs across colour morphs (Table 4).

The factors, generated by the FCA, differed significantly among colour morphs ($F_{8,530} = 48.93$, *P* < 0.001). Subsequent univariate analyses revealed that the first two factors separated the three morphs, even though there was substantial overlap (Fig. 1). Bayesian cluster analysis (Structure) was unable to identify clusters in the dataset, but this is expected when groups are cryptically

Table 3 Genotypic differentiation among colour morph classes, overall *F_{ST}* values are significant (*P* = 0.001)

Locus	<i>F_{ST}</i>	<i>R_{ST}</i>	Genic differentiation (<i>P</i>)
Pmeli-02	-0.003	-0.004	0.431
Pmeli-04	-0.003	-0.005	0.736
Pmeli-05	0.009	0.016	0.301
Pmeli-07	-0.003	-0.005	0.723
Pmeli-08	0.002	0.004	0.241
Pmeli-10	-0.001	-0.003	0.258
Pmeli-11	-0.001	-0.001	0.379
Pmeli-13	-0.003	-0.005	0.644
Pmeli-14	-0.001	-0.001	0.468
Pmeli-15	-0.001	-0.001	0.426
Pmeli-16	0.001	0.001	0.063
Pmeli-18	-0.002	-0.003	0.067
Pmeli-19	0.001	0.001	0.678
Overall	-0.001	-0.001	0.328

Table 4 Pairs of loci that show significant linkage disequilibrium (P -values <0.05) within and/or across colour morph classes

Pairs of loci	White	Orange	Yellow	All
Pmeli04 & Pmeli07	0.03	0.01	<i><0.001</i>	<i><0.001</i>
Pmeli05 & Pmeli04	0.67	1.00	<i><0.001</i>	<i><0.001</i>
Pmeli08 & Pmeli10	<i><0.001</i>	1.00	0.63	<i><0.001</i>
Pmeli08 & Pmeli18	<i><0.001</i>	1.00	0.37	<i><0.001</i>
Pmeli11 & Pmeli19	0.13	0.05	<i><0.001</i>	<i><0.001</i>
Pmeli05 & Pmeli18	0.53	0.21	<i>0.01</i>	0.03
Pmeli15 & Pmeli02	0.02	0.38	0.19	0.04
Pmeli13 & Pmeli18	<i>0.01</i>	1.00	0.59	0.10
Pmeli15 & Pmeli18	0.04	0.78	0.57	0.24
Pmeli11 & Pmeli18	1.00	0.70	0.03	0.28
Pmeli13 & Pmeli02	0.03	1.00	1.00	0.32

Significant P -values (<0.05) that remain significant after sequential Bonferroni correction are in italic

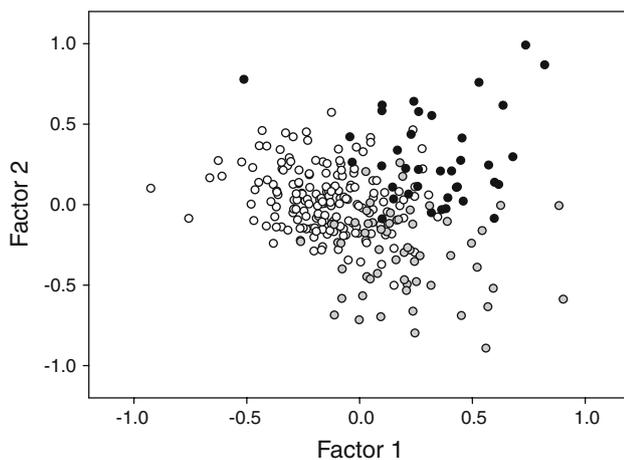


Fig. 1 Graph representing the relationship between Factor 1 (x -axis) and Factor 2 (y -axis) generated from a factorial correspondence analysis in Genetix 4.03 using the allelic frequencies to document differences between colour morph classes (white circles: white morph; grey circles: yellow morph; black circles: orange morph)

differentiated, as Structure is only appropriate to identify individuals from fairly well-defined populations (Pritchard et al. 2000). Moreover, the ability of Structure to correctly assign individuals to their subgroups increases with F_{ST} , and Structure discerns subpopulations correctly only at levels of genetic differentiation $\geq F_{ST} = 0.03$ (Latch et al. 2006). The overall F_{ST} value of -0.001 in our dataset is far below this critical threshold. Mean \ln values of the runs for each K ranging from 1 to 5 were, respectively -16743.7 ; -16658.2 ; -16718.3 ; -16974.3 and -17030.8 . Finally, within morph relatedness assessment (Identix) revealed a higher percentage of half and full sibs in the white morph, compared to the yellow and orange morphs (Table 5).

Table 5 Mean relatedness (Queller and Goodnight's R) and the variance of the relatedness for each colour morph class, the percentage of half sibs and the percentage full sibs within each group

	White	Orange	Yellow
Mean R	-0.0061	-0.0269	-0.0190
Variance R	0.0133	0.0113	0.0130
% Half sibs	2.46	0.95	2.08
% Full sibs	0.10	0.07	0.10

Discussion

Our microsatellite analysis suggests that the different colour morphs of *P. melisellensis* may represent weakly distinct gene pools. Genetic divergence among the morphs was not obvious in all population structure analyses: individuals of the same morph were clustered in a priori assignment tests (cfr FCA), but assigning the individuals back to each colour group was not possible (cfr Structure). Interestingly, if neutral genetic differentiation (weakly) parallels colour polymorphism, non-random mating may be occurring. We hypothesize that this weak genetic divergence could be due to some degree of assortative mate choice among partners of a similar colour morph. Especially within the white morph, there seemed to be a signal of assortative mating, as the proportion of half and full sibs was higher than within yellow or orange morphs. Such assortative mating could minimize the break-up of co-adapted gene complexes built by correlational selection (Sinervo and Svensson 2002). Positive assortative mating ensures that recombinational or segregational load is minimized and that adaptive genetic correlations can be maintained due to the strengthening of linkage disequilibrium in a sexually selected runaway process (Sinervo and Svensson 2002). In case of condition-dependent polymorphism, assortative mating might also lead to population divergence (West-Eberhard 2005). Theoretically, this may lead to reproductive isolation resulting in sympatric speciation, even though this is not likely to happen in the absence of additional ecological differentiation among the morphs. No significant ecological differences were found in microhabitat use, thermal behaviour, locomotor performance or diet in *P. melisellensis* (Huyghe et al. 2007). Morphs do differ in some morphological traits (body and head size), muscle mass and bite force capacity (Huyghe et al. 2007, 2009a), and in their fighting ability (Huyghe et al. unpublished data). Thus, morphs differ in traits involved in male-male competition for sexual partners, suggesting sexual selection.

In polymorphic systems such as the one observed for *P. melisellensis*, where morphs may represent co-adapted trait complexes, disruptive selection can also result in concomitant selection for assortative mate choice. However, as

morphs should, by definition, be able to fully interbreed (Ford 1945), mating barriers leading to reproductive isolation are expected to be absent in polymorphic populations. Therefore, the maintenance of genetic variation through assortative mating should be countered by condition-dependent mate choice, preventing reproductive isolation and ensuring the continued existence of the different morphs in the population. Whether these preference functions manifest themselves in a population depends on the fluctuating morph \times environmental interactions, i.e. the ability of females to bear the costs of choosiness in fluctuating selective environments. In the side-blotched lizard (*Uta stansburiana*) for instance, females show variation in preference for males, and use a multicondition preference function dependent upon the females' genotype (morph type) and her reproductive state (number of clutches laid). Females exhibited positive assortative mating prior to the first clutch, but orange females switched choice, preferring yellow males, prior to the second clutch (Bleay and Sinervo 2007). Among morph gene flow can also be sustained in populations where males use the so-called "sneaking" mating tactic, which may override assortative mating tactics. In the Gouldian finch (*Erythrura gouldiae*), study of male and female mate choice showed that males were particularly choosy, associating and pairing only with females of their own morph type (Pryke and Griffith 2007), whereas females showed a strong preference for mates with the most elaborate sexually dimorphic traits, without regard for morph type.

This study provides some evidence for weak genetic differentiation among colour morphs of *P. melisellensis* at neutral loci, which is in line with the findings of other studies on polymorphic vertebrate populations. Walter et al. (2009) found very limited genetic differentiation among two colour morphs in trimorphic fish populations (*Telmatherina antoniae*), despite assortative mating for similar colour. Low levels of genetic differentiation in the colour polymorphic cichlid *Pseudocrenilabrus philander* indicated a deviation from panmixis, suggesting that this population might have been in a stage of incipient sympatric divergence (Koblmüller et al. 2008). In a study on three colour polymorphic hamlet fish populations (*Hypoplectrus* sp.), highly significant genetic differences were found between the morphs, as well as strong assortative mating (Puebla et al. 2007). *Hypoplectrus* morphs seemed to be reproductively isolated and were therefore considered incipient species (Puebla et al. 2007). In a recent study of the polymorphic Gouldian finch (*Erythrura gouldiae*), limited gene flow between the morphs was attributed to inviability effects in offspring resulting from genetically mixed phenotypes (Pryke and Griffith 2009), revealing an underlying genetic incompatibility between different genotypes. These morphs were considered to represent

transient stages in the speciation process, rather than freely interbreeding morphs. These examples of color polymorphism in populations associated with varying levels of genetic differentiation show that the latter, indicative of recent divergence, can, but do not necessarily lead to reproductive isolation. Mating preferences such as positive assortative, condition-dependent, or a combination of these could explain divergence-hybridization patterns that are observed in polymorphic populations.

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