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Mate choice and the genetic basis for colour variation in a polymorphic dart frog: inferences from a wild pedigree

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Abstract

Understanding how reproductive barriers evolve during speciation remains an important question in evolution. Divergence in mating preferences may be a common first step in this process. The striking colour pattern diversity of strawberry dart frog (*Dendrobates pumilio*) populations has likely been shaped by sexual selection. Previous laboratory studies have shown that females attend to male coloration and prefer to court with males of their own colour, suggesting that divergent morphs may be reproductively isolated. To test this hypothesis, we used molecular data to estimate pedigree relationships from a polymorphic population. Whereas in the laboratory both red and yellow females preferred to court with males of their own phenotype, our pedigree shows a pattern of assortative mating only for red females. In the wild, yellow females appear to be less choosy about their mates, perhaps because they incur higher costs associated with searching than females of the more common red phenotype. We also used our pedigree to investigate the genetic basis for colour-pattern variation. The phenotype frequencies we observed were consistent with those expected if dorsal background coloration is controlled by a single locus, with complete dominance of red over yellow. Our results not only help clarify the role of sexual selection in reducing gene flow, but also shed light on the mechanisms underlying colour-pattern variation among sympatric colour morphs. The difference we observed between mating preferences measured under laboratory conditions and the pattern of mate choice observed in the wild highlight the importance of field studies for understanding behavioural reproductive isolation.

Keywords: heritability, mate choice, pedigree, polymorphism, sexual selection, speciation

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Introduction

The question of how reproductive isolating barriers evolve among incipient species is one of the central problems of speciation. In many cases, divergent populations show strong assortative mating in the absence of intrinsic post-zygotic incompatibilities, suggesting that divergence in mating signals and associated female preferences may be a common first step in the speciation process (McMillan *et al.* 1997; Seehausen *et al.* 1999; Jiggins & Mallet 2000; Jiggins *et al.* 2004). Whereas reproductive barriers can continue to accumulate and strengthen after new species have formed, the barriers that cause speciation are those that reduce gene flow before reproductive isolation is complete. For this reason, studies of recently or nearly diverged populations, where the action of these barriers can be more directly observed, provide the most accurate picture of the process (Coyne & Orr 2004; Rundle & Nosil 2005; Sobel *et al.* 2010).

The strawberry dart frog (*Dendrobates pumilio*, formerly *Oophaga pumilio*, see Santos *et al.* 2009) is amazingly variable in coloration on the islands of the Bocas del Toro archipelago and adjacent mainland of Panama (Daly & Myers 1967; Summers *et al.* 2003). This

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variation apparently arose with the formation of the archipelago over the past 6000 years (Summers *et al.* 1997; Anderson & Handley 2002). Although most colour and pattern variants (hereafter, colour morphs) are allopatric, there are a few areas where two or more distinctive colour morphs occur sympatrically. Despite their amazing diversity, all of the Panamanian colour morphs are considered to be conspecific based on the similarity of male mating calls (Daly & Myers 1967; Prohl *et al.* 2007) and the fact that all populations (with the exception of frogs from the much more distant island of Escudo de Veraguas) fall into one clade with minimal genetic divergence in rapidly evolving molecular markers (Summers *et al.* 1997; Hagemann & Prohl 2007; Wang & Shaffer 2008; Hauswaldt *et al.* 2010).

Laboratory experiments, in which females are simultaneously exposed to males of two or more phenotypes, suggest that female *D. pumilio* prefer to court with males of their own colour over males of other colours (Summers *et al.* 1999; Reynolds & Fitzpatrick 2007; Maan & Cummings 2008, 2009; Richards-Zawacki & Cummings 2011). Whereas most of these studies have involved only females from monomorphic populations, assortative mating preferences were also observed for females from a polymorphic population on the island of Bastimentos in Panama (Richards-Zawacki & Cummings 2011). Both red and yellow females from this population preferred to court with males of their own phenotype when given a choice between red or yellow. If the female preferences observed in these choice experiments are representative of the mate choice decisions that female *D. pumilio* make in the wild, some degree of behavioural reproductive isolation may already be present, even among sympatric colour morphs in polymorphic populations. This assumption, however, deserves explicit testing as in several frog species, mating preferences observed in the laboratory are not reflected in the pattern of mating in the field (Gerhardt 1992).

Preference vs. choice

Differences between patterns observed in laboratory choice experiments and field studies may be common because these two approaches are in fact measuring different aspects of female mating behaviour. Whereas choice experiments are appropriate for studying 'mating preferences' (sensory and behavioural properties that influence the propensity of females to mate with certain phenotypes, sensu Heisler *et al.* 1987), field studies provide estimates of 'mate choice' (the pattern of mating that arises in part as a result of mating preferences). Mate choice depends not only on mating preferences, but also on how females acquire and use information about potential mates. In natural populations, constraints on time, memory and mobility prevent females from visiting all possible mates (Janetos 1980). Therefore, the degree to which mate choice can be predicted from studies of mating preferences will depend upon the sampling strategies females use to select their mates.

If female *D. pumilio* mate with the first male they encounter (random mating), preferences observed in the laboratory would not be a good predictor of the pattern of mate choice that females exhibit in the wild. Even if females sample multiple males, the attractiveness of a given mate may depend upon the others with whom he is being compared (Bateson & Healy 2005). Hence, mating preference results from laboratory experiments may or may not predict patterns of mate choice, depending upon how many males are compared and how the experiments were designed. In either case, the previous experiments could have either underestimated the complexity or misrepresented the nature of sexual selection among *D. pumilio* morphs. Whereas studies of mating preference are important for understanding the sensory underpinnings of differences in mating behaviour among morphs, a clearer understanding of the pattern of mate choice exhibited in natural populations is needed to assess the extent to which mating behaviour leads to reproductive isolation. One way to obtain such information is by testing for a pattern of assortative mating using pedigree information from a polymorphic population.

The power of pedigrees

Divergence in mating preferences among populations, like any evolutionary change, requires a combination of two elements: (i) heritable genetic variation that underlies the distribution of phenotypes and (ii) a mechanism (such as selection or genetic drift) that alters that distribution from generation to generation. Pedigrees can be used to study both of these elements of evolutionary change: they can be used to quantify the phenotypic covariance of relatives, providing insight into the genetic basis of traits, and breeding patterns from pedigrees can be linked to these traits to estimate selection (Kruuk & Hill 2008). Although geneticists have recognized the power of pedigrees for more than a century, only recently have biologists begun to realize their potential in providing insight into the forces driving evolutionary dynamics in wild animal populations (Pemberton 2008). For example pedigrees have recently been used to study breeding patterns (e.g., Blackmore & Heinsohn 2008; Grant & Grant 2008; Ursprung *et al.* 2011a), heritability and the quantitative genetic architecture of traits (e.g., Charmantier *et al.*

2006) and gene flow (e.g., Zeyl *et al.* 2009) in wild animal populations. As direct observations of reproduction are usually unavailable, pedigrees for wild populations are usually estimated using molecular marker data. In these cases, DNA derived from tissue, scat, fur or feathers are used to infer relationships statistically. The resulting pedigree estimates are particularly useful in the study of wild animal mating systems, especially for taxa where direct observation of mating is impossible, prohibitively time-consuming or potentially misleading (e.g., Carpenter *et al.* 2005; Gottelli *et al.* 2007; reviewed in Jones & Wang 2010).

Study objectives

Using molecular information gathered in conjunction with a mark recapture study, we estimated pedigree relationships among *D. pumilio* individuals from a polymorphic population on the northwest tip of the island of Bastimentos. Using simulations, we tested the effect of our mating system assumptions on the confidence of our pedigree assignments. The phenotypes of mated pairs and their offspring were then used to test for a pattern of assortative mating by colour, consistent with results of a laboratory study (Richards-Zawacki & Cummings 2011). We also investigated the mode of inheritance of colour–pattern variation by asking whether the patterns we observed could be explained by a single locus of large effect showing simple Mendelian dominance. Our results not only contribute important new insight into the degree to which colour morphs are reproductively isolated, but also shed light on the mechanisms underlying colour–pattern variation and mating preferences among morphs. These findings help to improve our understanding of the role of sexual selection in the rapid evolution of colour–pattern polymorphism among Panamanian *D. pumilio*, and emphasize the importance of field studies for studies of mate choice.

Methods

Field sampling and measurements of phenotype

A total of 677 *Dendrobates pumilio* (312 male, 274 female, 91 juvenile) were captured by hand from a 0.75 ha area of secondary-growth forest on the northwest tip of the island of Bastimentos (9.3468°N, 82.2064°W). The study site was bounded on its northern and western sides by cleared land (a cemetery and residential areas) and on its eastern and southern sides by a stream. We have never encountered *D. pumilio* in the marshy habitat that lies on the other side of the stream; however, occasionally individuals can be found in and around large trees in the cleared areas. Frogs in this population differ in dorsal coloration and each colour morph appears more or less evenly distributed across the study area (Fig. S1, Supporting information). The frogs were captured between July 2007 and January 2009 and transported to the Smithsonian Tropical Research Institute's Bocas del Toro Research Station. There, digital photographs of the dorsal and ventral surfaces, as well as body size (snout-vent length, SVL) measurements of each frog were taken against a grey card with a flat spectral response across the visible spectrum (Digital Gray Card™, Robin Myers Imaging). Photographs were taken, within 6 h of capture, from a distance of 10 cm under uniform lighting, and after the frogs had acclimated for at least 30 min to room temperature (23 °C). Frogs were then weighed using a digital scale (Gempro-500, accurate to 0.002 g), classified by sex and the tip of the fourth toe of each hindlimb was removed. Like some other poison frogs (e.g., *Allobates femoralis*, Ursprung *et al.* 2011b), *D. pumilio* regenerates its digits after toe-clipping and no adverse effects of this tissue sampling method were seen over the course of our study. Sex was determined based on SVL and the presence (male) or absence (female) of a dark throat patch. Males in this population usually had dark throat patches by 18.0 mm SVL; therefore, any frog 18.0 mm or larger with no dark throat patch was treated as female. Frogs smaller than 18.0 mm SVL were treated as juveniles. Because frogs in this population breed year-round, other than distinguishing juveniles from adults, we were unable to distinguish cohorts of similar age within our sample. Of the 169 adult frogs recaptured during this study, six (3.6%) had been classified as the wrong sex upon first capture. All of these were males whose dark throat patch was missed or was not present at first capture. Frogs were returned to their point of capture within 48 h.

Colour analysis

To quantify colour variation, colour and contrast values among a subset of digital photographs were standardized by adjusting midtones to a target value using the 'auto colour' command in Adobe Photoshop CS 8.0. Colours were characterized by averaging the red, green, blue (RGB) colour values from three random points on each of the dorsal and ventral surfaces (excluding the dark throat patch of males). Variation among individuals was summarized using a principal components analysis. A two-step cluster analysis using the Bayesian Information Criterion was used to estimate the number and composition of natural colour groups in the population. All statistical analyses were performed in SPSS 18.0. Results of the cluster analysis were compared

to assignments of the same frogs to colour groupings made by a human observer (CRZ) to determine whether visual inspection of photographs was a reliable way of assigning frogs to colour groups.

Molecular genetic methods

Toe clip tissues were preserved in a salt-saturated dimethyl sulfoxide (DMSO) and ethylenediaminetetraacetic acid (EDTA) solution at room temperature for up to 20 months prior to extraction. Genomic DNA was isolated using a QIAGEN DNeasy kit according to the standard protocol for animal tissue. Extracted samples were diluted to 2.5 ng/μL prior to amplification by polymerase chain reaction. Tissue samples from 677 individuals were amplified at 15 microsatellite loci using primers previously developed by Wang & Summers (2009; Dpum12, Dpum13, Dpum14, Dpum24, Dpum44, Dpum92 and Dpum110) and Hauswaldt *et al.* (2009; OopB8, OopB9, OopC3, OopC11, OopD4, Oop E3, OopF1 and OopH5). Forward primers were labelled with a 5′-fluorescent tag (6-FAM, NED, VIC or PET). polymerase chain reactions were carried out in 10 μL volumes containing 1× buffer (Applied Biosystems), 0.2 mM each dNTP, 0.5 μM forward and reverse primers, 0.25 U Ampli*Taq* Gold *Taq* DNA polymerase (Applied Biosystems) and 2.5 ng template DNA. For all of the Hauswaldt *et al.* (2009) primers, Dpum12 and Dpum13, the $MgCl_2$ concentration was 2.5 mM. For the rest of the Wang & Summers (2009) primers, the $MgCl_2$ concentration was 3.75 mM. For Dpum12 and Dpum13, 5% DMSO was also added to the polymerase chain reaction master mix. Polymerase chain reaction cycling conditions were as in Hauswaldt *et al.* (2009) and Wang & Summers (2009). Each locus was amplified individually and run on an ABI 3730 Genetic Analyser (Applied Biosystems). Eleven reference samples with known genotype for each locus were included in each run to ensure reliable scoring of genotypes across different gels. Fragments were sized with LIZ-500 size standard (Applied Biosystems) and collected with GeneScan version 3.1 (Applied Biosystems). Scoring was performed with GeneMarker (SoftGenetics, LLC).

Population genetic analysis

We tested for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using GenePop 4.1 (Raymond & Rousset 1995). Allele frequencies, observed (H_O) and expected (H_E) heterozygosity, number of alleles and frequency of null alleles for each locus were estimated using CERVUS 3.0 (Kalinowski *et al.* 2007). Genetic structure among colour groups was estimated using the infinite alleles model (F_{ST}) in ARLEQUIN 3.0 (Schneider *et al.* 2000).

Pedigree inference

As we could not distinguish cohorts (other than juveniles vs. adults) by body size or other metric, we pooled all adult males as candidate fathers, all adult females as candidate mothers and all frogs (male, female and juvenile) as candidate offspring for pedigree analysis. Realizing the challenges this poses for pedigree estimation (reviewed in Koch *et al.* 2008), we used a multifaceted approach involving three pedigree estimation programs to improve confidence in our inferred relationships and the conclusions that were drawn from them. Pedigree analyses were performed using CERVUS 3.0 (Kalinowski *et al.* 2007), COLONY 2.0 (Jones & Wang 2009) and MASTERBAYES 2.47 (Hadfield *et al.* 2006), as described below.

CERVUS is a likelihood based program that uses simulations to determine thresholds for parentage assignment. For our analysis, we used simulations of 10 000 offspring with a genotyping error rate of 1%. For simulation, CERVUS requires an estimate of the average number of candidate mothers and fathers per offspring (which should include all adults known or thought to be present at the time of breeding). To estimate these values, we used the POPAN open-population model in MARK 5.1 (White & Burnham 1999) on mark–recapture data recorded during five sampling sessions: (i) July 13–14, 2007; (ii) March 24–April 1, 2008; (iii) June 16–17, 2008; (iv) October 20–22, 2008 and (v) January 9–14, 2009. Photographs of the dorsal colour-pattern were used to identify individuals. This was possible because in this population, markings are both unique and fixed soon after metamorphosis. The reliability of this method is supported by the observation that of 679 frogs diagnosed by dorsal pattern as being distinct individuals, in only two cases (0.3%) did we later find identical genotypes. Photographs allowed us to diagnose both of these as recaptures where the colour-pattern match had initially been missed, rather than identical genotypes occurring in two individuals. The best-supported POPAN model resulted in estimates of $N = 965$ females and $N = 1044$ males, where N is an estimate of the total number of frogs that came into the population during the study period (the super population size). This resulted in estimates of 0.308 and 0.302 respectively, for the proportion of candidate mothers and fathers sampled.

Unlike CERVUS, which uses pairwise comparisons to assign parentage, COLONY considers the likelihood of the entire pedigree structure in assigning pedigree relationships. Our COLONY analysis was attempted in multi-core processor mode, using the full likelihood method and a medium run length. We assumed both male and female polygamy and a 1% error rate. This analysis proved to be prohibitively long for our full data set, likely as a

result of the large number of candidate offspring, and was terminated prior to completion after several months. However, we were able to use COLONY to provide a check of the parent–offspring trios identified by CERVUS. We ran COLONY ten times, each time with all individuals as potential parents and a different subset of individuals as potential offspring. Each offspring subset contained all offspring identified by CERVUS plus an equal number of randomly chosen individuals. Parent–offspring relationships supported at 95% confidence in at least one COLONY run were used in subsequent analyses.

Pedigree analyses in MASTERBAYES can be run using either maximum likelihood or Bayesian approaches. If only genotypic data are being used, confidence levels for assignments can be calculated analytically using maximum likelihood, removing the need for a Bayesian approach (Hadfield *et al*. 2006). Nevertheless, we ran both maximum likelihood and Bayesian parentage analyses in MASTERBAYES. The offspring pool for these analyses was limited to all juvenile individuals plus adult individuals identified as offspring by CERVUS because preliminary trials with MASTERBAYES indicated poor performance when there is overlap between parent and offspring pools (individuals were often assigned as their own parent). The parent pool consisted of all adult individuals except those identified as trio offspring by CERVUS. For the maximum likelihood analysis, results from a run where MASTERBAYES was allowed to estimate the number of unsampled males and females were compared with those of a run where estimates from the mark–recapture analysis were provided. For the Bayesian analysis, we ran the Markov chains for 250 000 iterations with a burn-in of 50 000 iterations and a thinning interval of 100.

Effects of multiple matings on confidence in pedigree assignments

Our CERVUS analysis assumed no inbreeding and that no close relatives were present among the candidate parents. The critical delta scores for 90% and 95% confidence obtained from this analysis were used to identify mother–father–offspring trios. To examine how the presence of relatives with a coefficient of relationship (r, Wright 1922) of 0.25 or more would affect confidence in our parent–offspring assignments, we ran a series of simulations in CERVUS with different numbers (1–10) and types of relatives. We then used field estimates of clutch size and survival to adulthood to estimate the true confidence, and the number of full siblings likely to be present in our data set, as a function of the number of repeat matings between parents. Details of these calculations can be found in the supporting information.

Mode of inheritance of dorsal coloration

Using the mother–father-offspring trios identified by our pedigree analysis, and the frequency of the yellow phenotype in this population, we tested the hypothesis that dorsal coloration could be largely controlled by a single locus with two alleles and complete dominance of red over yellow (i.e. simple Mendelian dominance: X = red, x = yellow; Fig. 1). This is the scenario that best explains limited observations from captive breeding of these two morphs: yellow pairs have only ever produced yellow offspring, whereas pairs where at least one parent is red (red + red and red + yellow) produce a mix of red and yellow offspring (CRZ, unpublished data). The frequency of yellow dorsal coloration in the Bastimentos population (q^2) was estimated from photographs of all individuals captured during the 18-month mark–recapture study. From this value, the frequency of the recessive (q) and dominant (p) alleles as well as the expected frequency of homozygous (p^2) and heterozygous (2pq) red frogs were derived, assuming HWE. The observed and expected frequencies of yellow offspring for different combinations of parent phenotypes were compared to test their fit to Hardy–Weinberg expectations.

Results

Colour variation

The principal components analysis of averaged R, G and B values extracted a single significant component (PC) for each of dorsal (n = 217) and ventral (n = 68) coloration. For dorsal coloration, the first PC (eigenvalue = 1.905) explained 63.5% of the variance in coloration and was positively correlated with variation in G and B (coefficient scores: R = −0.89, G = 0.505, B = 0.512). The second PC explained 33.2% of the variance, was marginally non-significant (eigenvalue = 0.997) and was strongly positively correlated with variation in R (coefficient scores: R = 0.988, G = 0.164, B = 0.011). For ventral coloration, the first PC (eigenvalue = 2.481) explained 82.7% of the variance in coloration and was approximately equally influenced by R, G and B (coefficient scores: R = 0.330, G = 0.396, B = 0.371). Frogs classified by eye as belonging to red and yellow dorsal colour groups are clearly distinguishable in a plot of the first two dorsal PCs (Fig. 2a). However, these two groups are not differentiated in terms of ventral coloration (Fig. 2b). A two-step clustering analysis suggested that dorsal coloration falls into two natural clusters, which almost perfectly match the assignments made by eye (2 of 217, or <1% of assignments differed). This supports the idea that colour groupings made by visual inspection of photographs accurately represent natural

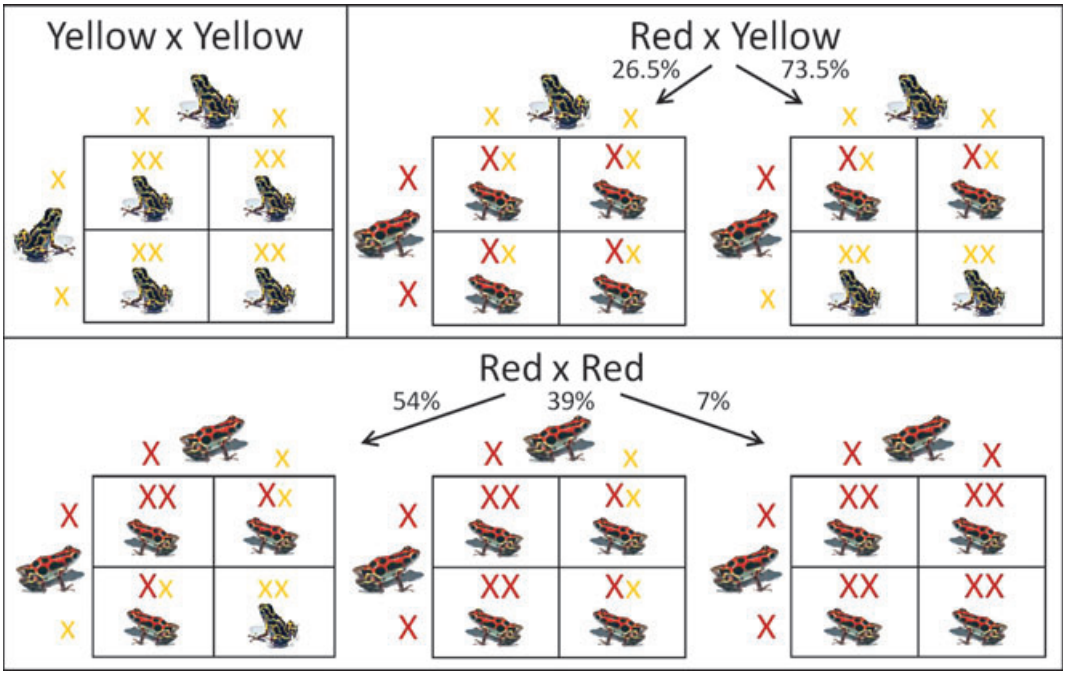

Fig. 1 Expectations for offspring phenotypes if red is completely dominant over yellow. The frequency of yellow dorsal coloration in the population (q^2) was found to be 0.35. From this value, assuming Hardy–Weinberg equilibrium, the frequency of the recessive ($q = 0.592$) and dominant ($p = 0.408$) alleles, as well as the expected frequency of homozygous ($p^2 = 0.166$) and heterozygous ($2pq = 0.484$) red frogs were derived. The proportion of red frogs carrying a recessive allele was calculated as $2pq/(2pq + p^2) = 0.735$. From this value, the expected frequencies of each genotype combination for red + yellow and red + matings were calculated.

groupings in this population. Through visual inspection of photographs, the frequency of yellow dorsal coloration was found to be 0.35 for both adult males (110 of 312) and females (96 of 274).

Population genetic analysis

Linkage disequilibrium was detected between OopD4 and Dpum110. Because linkage between these two loci was not found in other *Dendrobates pumilio* populations (CRZ unpublished data) and because linkage between one pair of loci is unlikely to bias confidence in parentage assignments (Marshall *et al.* 1998), these two loci were used in the pedigree analyses. Four loci (Dpum12, Dpum13, Dpum14 and Dpum24) were found to be out of HWE after Bonferroni correction. Comparisons among individuals that were genotyped multiple times indicate that two of these loci were unreliable because they produce non-repeatable results (Dpum12 and Dpum13). The other two loci (Dpum 14 and Dpum 24) produced repeatable results but harboured null alleles at high frequencies. Only the 11 loci in HWE were used in the pedigree analyses to assign mother–father–offspring relationships. However, alleles at Dpum14 and Dpum24 were later used to check the accuracy of those assignments (see pedigree analysis, below). Across the 11 microsatellite loci in HWE and 677 frogs, we found an average of 18.27 alleles per locus and an average H_O of 0.785 (Table 1). Frogs from the two dorsal colour groups (red and yellow) were not differentiated genetically ($F_{ST} = 0.0003$, $P = 0.17$).

Pedigree inference

From among the 677 genotyped frogs, our CERVUS pedigree analysis identified 32 mother–father–offspring trios with 90% confidence, of which 24 were also identified with 95% confidence. For each of these trios, a second round of screening was performed by comparing mother, father and offspring alleles at two additional loci (Dpum14 and Dpum24). As both these loci are known to harbour null alleles, incidences where a mismatch could be attributed to the presence of a null allele were ignored. However, there were two cases at the 90% confidence level where alleles at these two loci were incompatible with the proposed parent–offspring relationship. These trios were considered false positives and not used in subsequent analyses, bringing the total to 30 trios with 90% confidence and 24 with 95% confidence (Fig. S3, Supporting information). Eleven (36.7%) of the 90% confidence trios had juvenile frogs at the offspring position. The proportion of trios with juvenile offspring was similar (8 of 24, or 33.3%) for the 95% confidence trios. Considering that we only sampled 91

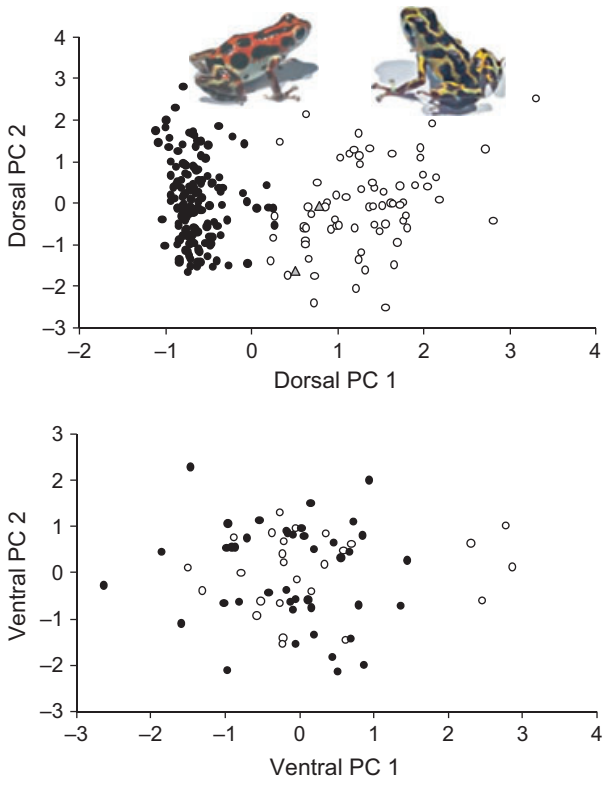

Fig. 2 Plots of dorsal (a) and ventral (b) colour variation among individual *Dendrobates pumilio*. Symbols indicate dorsal colour cluster: black circles = frogs classified as red by eye and by cluster analysis; open circles = frogs classified as yellow by eye and by cluster analysis; grey triangles = frogs classified as red by eye but yellow by cluster analysis.

juveniles out of a total of 677 individuals, the probability of parents being assigned was higher for juveniles (12.1%) than for adults (3.2%). This pattern is expected as juvenile offspring are more likely to have parents still alive and available for sampling within the population than offspring that have already reached sexual maturity and may be anywhere between 10 months and several years old themselves.

Parent–offspring relationships for 25 of the 30 trios supported by CERVUS were also supported by COLONY and/or MASTERBAYES with 95% confidence (Fig. S3, Supporting information). For MASTERBAYES, the same relationships were supported regardless of whether the number of unsampled parents was estimated directly from the data or whether estimates of these numbers from the POPAN mark–recapture analysis were used. The same relationships were also supported regardless of whether the Bayesian or maximum likelihood module of MASTERBAYES was used.

The frequency of yellow dorsal coloration among adult male frogs in this population is estimated to be 0.35. Therefore, under the null hypothesis of random mating,

Table 1 Genetic diversity and results of test for departure from HWE for 677 in individuals of *Dendrobates pumilio*

Locus	Number of alleles	H_O	H_E	Null allele frequency	Uncorrected *P*-value
		Used in pedigree estimation			
OopH5	19	0.874	0.878	0.002	0.1912
OopB9	14	0.869	0.876	0.004	0.6465
OopC3	14	0.871	0.897	0.014	0.2806
OopF1	16	0.870	0.875	0.002	0.3985
OopE3	25	0.920	0.923	0.001	0.7778
OopC11	25	0.867	0.888	0.013	0.1002
OopD4	18	0.706	0.718	0.009	0.0675
OopB8	21	0.924	0.919	−0.003	0.1820
Dpum110	17	0.850	0.867	0.010	0.0087
Dpum92	2	0.009	0.009	−0.001	0.9999
Dpum44	30	0.878	0.884	0.003	0.1558
Mean	***18.27***	***0.785***	***0.794***		
		Not used in pedigree estimation			
Dpum14*	8	0.063	0.079	0.107	<0.0001
Dpum24*	15	0.649	0.908	0.166	<0.0001
Dpum12**	6	0.811	0.721	−0.077	<0.0001
Dpum13**	9	0.767	0.774	0.020	<0.0001

Loci denoted by (*) were used only as a secondary check of parent–offspring assignments. Loci denoted by (**) were not used at all. Abbreviations: H_O = observed heterozygosity, H_E = Expected heterozygosity. Critical *P*-value for HWE test ($\alpha = 0.05$), after Bonferroni correction = 0.0045.

female frogs of either color should share parenthood with yellow males 35% of the time. Yellow females appear to follow this pattern. In the set of trios supported by CERVUS at 90% confidence, 38% of yellow females mated with yellow males (Table 2). Alternative analyses including more conservative subsets of these trios (i.e. those supported by CERVUS at 95% confidence, those containing juveniles as offspring and those also supported by COLONY and/or MASTERBAYES) produced similar results (Table S1, Supporting information). The frequency of yellow–yellow pairings does not differ from the expectations for random mating in any of these analyses (Binomial Exact Test, $P \geq 0.437$), although for some analyses the power to detect such a difference would be low because of the small sample size of yellow females.

Unlike yellow females, red females show a statistically significant pattern of assortative mating (Binomial Exact Test $P \leq 0.049$). This is true whether we consider the CERVUS trios (Table 2), where 91% of red females mated with red males, or any of the more conservative alternative analyses (Table S1, Supporting information), where the frequency of red–red pairings was ≥88%. For the CERVUS analyses with all trios considered, our results are robust to potential error in our estimation of the frequency of the yellow phenotype ($q^2 = 0.35 \pm 0.05$ for 95% confidence and 0.35 ± 0.1 for 90% confidence).

Table 2 Frequencies of assortative/disassortative mating for mated pairs supported by CERVUS with 90% confidence

	Male phenotype: observed (expected)		Binomial exact test
	Own	Other	*P*-value (1-tailed)
Red female (N = 22)	0.91 (0.65)	0.09 (0.35)	0.006
Yellow female (N = 8)	0.38 (0.35)	0.63 (0.65)	0.572

The error rate for parentage assignments made by CERVUS tends to be higher than that in COLONY and MASTERBAYES (Walling *et al.* 2010). Although we screened our initial CERVUS trios for matching genotypes at an additional two loci, it is still possible that some of the trios supported by CERVUS but not by other programs reflect this larger error rate. However, in our study, the presence of erroneous parentage assignments among trios would tend to obscure a pattern of assortative mating rather than strengthen it. For this reason, and because our results were unchanged when only the subset of relationships supported by two or more pedigree analyses (CERVUS and COLONY or MASTERBAYES) was considered, we feel confident that the significant pattern of assortative mating seen among red females in CERVUS is an accurate reflection of the mating behaviour of this population.

Effects of multiple matings on confidence in pedigree assignments

Our CERVUS analysis was conducted using the assumption that no close relatives of the candidate parents and offspring are present in the sample. However, six individuals that were captured as adults during our first sampling session were recaptured 18 months later, which indicates that frogs in this population are capable of living at least 2 years, and perhaps much longer. In this case, our sample likely comprises more than two generations of frogs, and potentially several types of close relatives. If close relatives are present in the data set, using the critical values from the CERVUS simulation assuming no relatives will lead to differences between assumed (90% or 95%) and true confidence in pedigree relationships. To estimate the impact that close relatives would have on confidence levels for our analysis, we conducted a series of simulations with different numbers and types of close relatives of candidate parents and offspring. The results suggest that our confidence estimates are robust to the presence of relatives with r = 0.25 (half siblings, grandparents or grandchildren) among the candidate parents and offspring (Fig. 3a–c) and to the presence of full siblings (r = 0.5) among the candidate parents so long as the candidate parent and

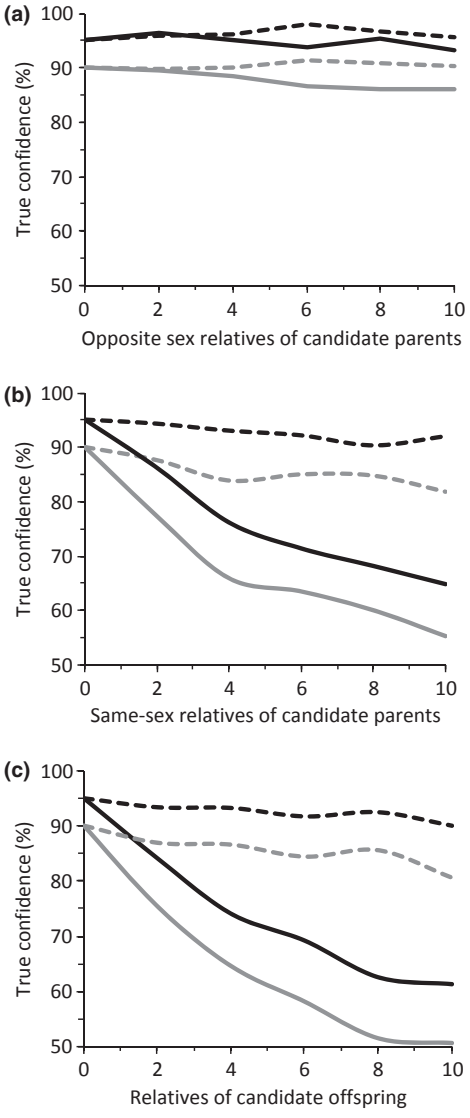

Fig. 3 Effect of close relatives of parents and offspring on true confidence in pedigree analysis. If relatives are present in the data set, using the critical values from the CERVUS simulation assuming no relatives will lead to differences between intended and true confidence. True confidence is plotted as a function of the number and type of relative present. Line colour indicates which confidence level from the no-relatives simulation was used for comparison: Black = 95%, grey = 90%. Line type indicates whether the coefficient of relationship (r) is 0.5 (solid) or 0.25 (dashed). The presence of half siblings and/or grandparent/grandchild relationships (r = 0.25) has little effect on the confidence of pedigree assignments (a–c). However, the presence of same-sex full siblings of parents (b) and full siblings of offspring (c) can lead to confidence levels that are much lower than intended.

its close relatives are of the opposite sex (e.g. father's sister or mother's brother) (Fig. 3a). However, if several same-sex full siblings of the candidate parents are present (e.g. mother's sister or father's brother), the true confidence in our pedigree assignments may be much lower than 90% or 95% (Fig. 3b). The presence of full siblings among the candidate offspring has a similarly dramatic effect on confidence (Fig. 3c).

Mode of inheritance of dorsal coloration

Observations from captive breeding of these two morphs (CRZ, unpublished data) suggest that red dorsal coloration shows complete dominance of red over yellow. However, that experiment has not yet yielded sufficient data to merit statistical testing. Using the mother–father–offspring trios identified by our pedigree analysis, we tested the null hypothesis that dorsal coloration could be controlled by a single locus with two alleles and complete dominance of red over yellow (Fig. 1). Table 3 shows the observed and expected frequencies of yellow offspring from different combinations of parent phenotypes using the results from CERVUS at 90% confidence. The observed frequencies of offspring phenotypes were not significantly different from expected, regardless of which confidence level (90% or 95%) was used, or whether trios were limited to those with juvenile offspring or those also supported by COLONY and/or MASTERBAYES (Table S2, Supporting information). This suggests that dorsal coloration in this population could be controlled in large part by a single locus with complete dominance of red over yellow. However, for some parent combinations (e.g. red + yellow) and data sets (e.g. juvenile offspring), our sample size is small and thus the power to detect a departure from Hardy–Weinberg expectations would be quite low. The observed frequencies of offspring phenotypes were also not significantly different from expectations based on our mark–recapture study (i.e. 35% yellow), regardless of which confidence level or subset of trios was used (Tables 3 and S2, Supporting information). This suggests that our pedigree contains a representative subset of mother–father–offspring relationships and that the patterns of mate choice and inheritance seen among these trios can reproduce the morph frequencies we observed in a larger sample of the population.

Discussion

One of the central problems of speciation is the origin of isolating barriers that actually or potentially prevent gene flow in sympatry (Mayr 1942; Coyne & Orr 2004). Reproductive barriers can take many forms (e.g. prezygotic, post-zygotic), each of which can prevent gene flow in different ways. Because speciation is usually a long process during which many barriers can arise, several will likely be present by the time reproductive isolation is nearly complete (reviewed in Coyne & Orr 2004). The most important are those that reduce gene flow to the greatest extent prior to complete speciation. A number of species show strong assortative mating in the absence of post-zygotic isolation, suggesting that divergence in mating preferences may be a common first step in the speciation process (McMillan *et al.* 1997; Seehausen *et al.* 1999; Jiggins & Mallet 2000; Jiggins *et al.* 2004).

The amazing colour variation found among strawberry dart frog populations in Panama appears to have evolved recently and rapidly, suggesting the action of strong selection (Brown *et al.* 2010). Studies of recently diverged populations, like the colour morphs of *Dendrobates pumilio*, provide direct insight into which barriers play a major role in reducing gene flow, leading to reproductive isolation. In this study, we used a wild pedigree, estimated using molecular markers, to investigate whether females of this species mate assortatively by colour to clarify the extent to which sympatric morphs are reproductively isolated from one another as a result of mating behaviour. Using simulations, we investigated the impact of our assumption of no close relatives on true confidence in pedigree assignments. We also used our pedigree to investigate the genetic basis for colour-pattern variation in this population, providing the first glimpse into the heritability of a trait on which mate choice appears to be based.

Table 3 Comparison of offspring phenotypes to expectations if dorsal coloration is controlled by a single locus with the red allele being dominant over yellow

Parents		Offspring phenotypes		Frequency of yellow offspring		Binomial exact test
Phenotypes	Genotypes	Red	Yellow	Observed	Expected	*P*-value (2-tailed)
Yellow × yellow	xx + xx	0	3	1.00	1.00	
Red × yellow	xx + X_	4	3	0.43	0.37	0.99
Red × red	X_ + X_	16	4	0.20	0.14	0.61
	Total	20	10	0.33	0.35	0.99

Effect of multiple matings on confidence in pedigree assignments

The effect of the 'no-relatives' assumption on confidence appears to depend very much on the type of relationship. Our simulations suggest that the presence of half siblings, grandparents or grandchildren ($r = 0.25$) of candidate parents or offspring would have very little effect on confidence (Fig. 3). Other potential relationships within our study population (e.g. cousins, great-grand parents or great-grand children) would likely have even less effect as their coefficient of relationship is lower ($r \leq 0.125$). The presence of full siblings ($r = 0.5$), however, could cause true confidence to be much lower than the assumed cut-off levels for assignment of pedigree relationships. This would be the case if full siblings of the offspring or same-sex full siblings of the candidate parents were present in the data set. However, the presence of opposite-sex full siblings of the candidate parents (e.g. sister of father or brother of mother) had little effect on confidence. Taken together, our simulations suggest that the presence of any full siblings of the candidate offspring, and approximately one half of any full siblings of candidate parents (same-sex siblings) present could cause true confidence in pedigree assignments to be much lower than assumed. But how likely is it that these types of relatives are present in our data set?

Because *D. pumilio* has a small clutch size (avg. 4.2 eggs, Prohl 2005) and both males and females are known to mate with multiple partners (Prohl & Hodl 1999), it is unlikely that large numbers of full siblings of offspring or parents would be present within our sample. The high mortality of this species in early development (only one out of six eggs are likely to survive to metamorphosis, Prohl & Hodl 1999; Maple 2002) and the fact that we only sampled about 30% of the population mean that a female would have to mate with the same male many times before we would likely sample two of their full sibling offspring (Fig. S2, Supporting information). Under this scenario, even if a female were to mate with the same male 10 times, we would only have sampled, on average, two of the resulting offspring. Our simulations suggest that this would lead to a true confidence of 89% instead of the intended 95% for assignment of a parent–offspring relationship, which is still higher than the assignment cut-off of 80% used in many parentage studies (e.g., Krutzen *et al.* 2004; Delgado *et al.* 2008). Given that this high number of matings between the same male and female is likely to be rare for *D. pumilio*, and the fact that it only slightly lowered our confidence, we feel justified in trusting our pedigree assignments.

Preference vs. choice

Laboratory studies, which compare the courtship behaviour of females towards males of different phenotypes, have shown that female *D. pumilio* attend to male coloration and prefer to court with males of their own colour (Summers *et al.* 1999; Reynolds & Fitzpatrick 2007; Maan & Cummings 2008, 2009; Richards-Zawacki & Cummings 2011). This suggests the potential for behavioural pre-zygotic isolation, caused by divergent female preferences, to limit gene flow among morphs. However, for some species (including several frogs, see Gerhardt 1992), mating preferences observed in the laboratory are not reflected in the pattern of mating in wild populations. This appears also to be the case for at least one colour morph of *D. pumilio*.

A previous laboratory experiment found evidence of assortative mating preferences for both red and yellow females from the polymorphic population on the northwest corner of the island of Bastimentos (Richards-Zawacki & Cummings 2011). This suggests that females prefer males of their own colour over males of other phenotypes they would come in contact with in the wild. The patterns of mate choice we observed in this study, however, tell a slightly different story. Whereas red females showed a significant tendency to mate assortatively in the wild, yellow females did not. In fact, the pattern of mate choice for yellow females did not differ from expectations for random mating.

There are a number of potential explanations for the discrepancy between the laboratory study and this field study. For example it is possible that yellow females do mate assortatively in the field and that this study's small sample size ($n = 8$ yellow females) is not accurately representing the behaviour of yellow females. It is also possible that the contrasting results for yellow females are attributable to differences in lighting between the two studies and its effects on females' perceptions of colour. We investigated this possibility by plotting red and yellow dorsal colour phenotypes and calculating colour contrasts under both forest lighting and the lighting conditions used in the laboratory study (filtered incandescent light) using a *D. pumilio*-specific visual model (details in Supporting Information). Whereas laboratory lighting did generate a slightly greater colour contrast than forest light, this shift is small in comparison to the natural variation in colour found among individuals of each colour morph (Fig. S4, Supporting information). It therefore seems unlikely that lighting differences can explain the difference in yellow female behaviour between the two studies.

Another possible explanation for the apparent inconsistency could stem from the fundamental difference

between 'mating preferences' and 'mate choice'. Although yellow females showed a significant preference for males of their own phenotype in the laboratory, the process by which females sample and assess the quality of alternative males under natural conditions could prevent that preference from being expressed in the form of assortative mate choice in the field. In the laboratory experiment, females were presented with one male of each phenotype. However, in the wild, the frequency of the yellow phenotype for both males and females in this population is 0.35, meaning that on average, two out of every three males that yellow females encounter will be red. A yellow female would, therefore, have to sample more males than a red female to find a suitable mate of her own colour. If sampling incurs a cost, (e.g. increased risk of predation, expenditure of time and energy because of added searching, directed aggression from other searching females; Real 1990) mating assortatively would be more costly for yellow females than for red. This may have led to yellow females being less choosy than red ones, despite the fact that both inherently prefer males of their own colour (Real 1990).

Differences in mate preference between females of the two *D. pumilio* morphs could be a result of phenotypic differences in visual sensitivity, as has been demonstrated for sister species of African cichlids (Seehausen *et al.* 2008). Another possibility is that females choose their mates by comparing prospective partners to a reference template, which differs for red and yellow females (Widemo & Saether 1999). This template could be the female herself, or another individual she has encountered (i.e. sexual imprinting). Sexual imprinting has not been reported in frogs, although it is widespread in birds (Price 1998; ten Cate & Vos 1999) and mammals Kendrick *et al.* 1998; Penn & Potts 1998). The unusual form of parental care exhibited by *D. pumilio*, which is qualitatively similar to that of many sexually imprinting species, makes them a potential candidate for mating preferences to be learned via sexual imprinting on their mothers' phenotypes. Female *D. pumilio* transport their tadpoles to the water-filled axils of bromeliads (one tadpole per axil) and return periodically to lay unfertilized eggs for their offspring to eat (Weygoldt 1980; Brust 1993). Tadpoles do not survive to metamorphosis without their mother's eggs (Brust 1993), and they solicit eggs from their mothers by rapidly vibrating their tails in response to her approach (Weygoldt 1980). Studies of captive *D. pumilio* suggest that they are capable of individual recognition as highly territorial males immediately recognize and challenge strangers (Weygoldt 1987). Perhaps tadpoles of this species recognize and imprint on their mothers as they are fed. Future studies comparing the visual systems of females and behavioural studies to test for sexual imprinting will help to understand how variation in preference may have evolved among colour morphs.

Insights into the genetic basis of polymorphism

Despite growing interest in the evolution and behaviour of *D. pumilio* populations, little is known about the genetic basis of their highly variable pigmentation patterns. When bred in captivity, frogs from monomorphic populations breed true, suggesting that coloration is largely heritable (Summers *et al.* 2004; CRZ unpublished data). Studies of other frog species suggest that colour and melanistic pattern inheritance is typically under simple genetic control (e.g., Fogelman *et al.* 1980), although polygenic control has been proposed in some taxa (e.g., Matthews & Pettus 1966). The results of cross-breeding among several other colour morphs of *D. pumilio* by Summers *et al.* (2004) suggest that dorsal melanistic patterning is controlled by a single locus with the presence of such a pattern being dominant over its absence. This is consistent with what is known about the genetic control of melanistic patterning in most other anurans in which it has been investigated (Hoffman & Blouin 2000). However, cross-bred offspring in the Summers *et al.* (2004) study were often intermediate in dorsal background coloration between their two parents, suggesting that this aspect of pigmentation is either controlled by multiple loci, or caused by a single gene with incomplete dominance. In our study population, however, we do not see this pattern.

In our study population, the presence of melanistic patterning on the dorsum appears to be fixed, precluding us from investigating its inheritance. However, dorsal background coloration is polymorphic. Prior to this study, limited captive breeding of frogs from the northwest corner of Bastimentos led us to suspect that in this population, background coloration might be controlled by a single locus with red being completely dominant over yellow. Captive pairs of red frogs produced both red and yellow offspring, whereas pairs of yellow frogs only ever produced yellow offspring. New metamorphs were clearly either red or yellow, never an intermediate colour (CRZ, unpublished data). We used the pedigree relationships inferred in this study to test the hypothesis that dorsal coloration in this population is controlled by a single gene, with red being dominant to yellow. The observed phenotype frequencies were similar to those expected and we were unable to reject this hypothesis for the control of dorsal background coloration. However, this result should be interpreted with caution as tests for departure from Hardy–Weinberg, especially with small sample sizes, are fairly weak. Our

results are further supported by the presence of two, distinct groupings in our colour analysis, rather than a continuum from red to yellow. However, further pedigree analyses, pigment characterization and/or mapping of the genes controlling coloration in this species will go a long way towards clarifying the genetic basis underlying the spectacular variation seen within and among populations.

Conclusion

The spectacular variation in colour-pattern among populations of *D. pumilio* in Panama has long fascinated biologists, especially given the recency and rapidity with which it is thought to have evolved. Several recent studies have made great strides in understanding the geographic, behavioural and ecological factors involved in shaping patterns of diversity among these populations (e.g., Saporito *et al.* 2007; Maan & Cummings 2008, 2009; Wang & Shaffer 2008; Brown *et al.* 2010; Hauswaldt *et al.* 2010; Wang & Summers 2010). However, more studies of polymorphic populations, where the action of current reproductive barriers among morphs can be more directly observed, are needed to clarify selection's role in driving diversification. Pedigrees from wild populations can be powerful tools for addressing these and other fundamental questions in evolutionary biology. Our pedigree analysis contributes new insight into the degree to which colour morphs of *D. pumilio* are reproductively isolated, and also sheds light on the mechanisms underlying colour-pattern variation. These findings bring us one step closer to understanding of the role of sexual selection in the rapid evolution of colour-pattern polymorphism among Panamanian *D. pumilio* and highlight the importance of quantifying patterns of female mate choice (in addition to mating preference) in studies of behavioural reproductive isolation.

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Data accessibility

Microsatellite genotypes and colour analyses: DRYAD entry doi: 10.5061/dryad.qv6hr4mq.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Map of study area showing its situation with respect to surrounding non-forested areas and the distribution of *D. pumilio* color morphs (red = red circle, yellow = yellow circle) during a survey conducted on May 18, 2009.

Fig. S2 True confidence in pedigree assignments (solid lines) and the number of full siblings of candidate offspring in the dataset (dashed lines) as a function of the number of matings between parents.

Fig. S3 Parent-offspring trios supported by one or more pedigree analyses.

Fig. S4 Dorsal coloration of yellow (n = 9) and red (n = 10) males from the Richards-Zawacki & Cummings (2011) study plotted in *D. pumilio* color space under two lighting conditions.

Table S1 Frequencies of assortative/disassortative mating for mated pairs supported by (a) full CERVUS analysis at 95% confidence, (b) CERVUS relationships with juveniles in the offspring position, (c) CERVUS relationships also supported by COLONY and/or MASTERBAYES.

Table S2 Comparison of offspring phenotypes to expectations if dorsal coloration is controlled by a single locus (with red being dominant over yellow) for offspring from: (a) full CERVUS analysis at 95% confidence, (b) CERVUS relationships with juveniles in the offspring position, (c) CERVUS relationships also supported by COLONY and/or MASTERBAYES.

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