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Non-gradual variation in colour morphs of the strawberry poison frog *Dendrobates pumilio*: genetic and geographical isolation suggest a role for selection in maintaining polymorphism

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Abstract

The relative roles that geographical isolation and selection play in driving population divergence remain one of the central questions in evolutionary biology. We approached this question by investigating genetic and morphological variation among populations of the strawberry poison frog, *Dendrobates pumilio*, in the Bocas del Toro archipelago, Panama. We found significant population genetic structure and isolation by distance based on amplified fragment length polymorphism markers. Snout vent length (SVL), coloration and the extent and size of dorsal black spots showed large variation among the studied populations. Differences in SVL correlated with genetic distance, whereas black spot patterns and other coloration parameters did not. Indeed, the latter characters were observed to be dramatically different between contiguous populations located on the same island. These results imply that neutral divergence among populations may account for the genetic patterns based on amplified fragment length polymorphism markers and SVL. However, selective pressures need to be invoked in order to explain the extraordinary variation in spot size and coverage, and coloration. We discuss the possibility that the observed variation in colour morphs is a consequence of a combination of local variation in both natural selection on an aposematic signal towards visual predators and sexual selection generated by colour morph-specific mate preferences.

Keywords: genetic diversity, morphological variation, neutral divergence, Poison dart frogs, selection, speciation

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Introduction

Populations belonging to the same species often exhibit geographical variation in quantitative characters such as size and coloration (Haldane 1932; Mayr 1963; Endler 1977; Gray & McKinnon 2006). The main evolutionary forces behind such divergence are drift and selection. Drift is the neutral part of divergence and only small amounts of gene flow are sufficient to genetically homogenize geographically separated populations. Thus, under this scenario, divergence is predicted to be highly dependent on the degree of isolation between populations (Wright 1931; Wang 2004). Divergence mediated by selection is less dependent on isolation and may occur among populations where gene flow is present, the magnitude of divergence being dependent on the existing genetic variation and the strength of selection (Dobzhansky 1937; Endler 1977). However, drift and selection may interact in creating population divergences (Wright 1931, 1977).

A wealth of studies have attempted to link geographical variation in quantitative characters such as size and coloration with speciation processes (reviewed by Gray & McKinnon 2006). It has been argued that intraspecific variation, especially when there is a strong geographical component, may be seen as a starting point of a speciation

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process (Coyne & Orr 2004). However, intraspecific variation in quantitative characters may arise for a range of reasons and because of different scenarios of migration and drift. A central issue is whether such differences among populations are accidental, owing to random or deterministic differences within semi-isolated populations or if the differences among populations are caused by selection against hybridization at secondary contact zones (so called reinforcement).

Regardless of the reason for divergence in quantitative characters among populations, it is necessary in any study of geographical variation to set the divergence in quantitative characters against a background of neutral expectation. Therefore, it is important to compare the results of quantitative characters which may be under natural or sexual selection to those from genetic markers which can be assumed to evolve in a neutral way (Kremer *et al.* 1997; Butlin & Tregenza 1998; McKay & Latta 2002). Furthermore, it is crucial to use several approaches when inferring population structure from neutral genetic markers. Several such approaches that do not rely on a priori assumptions of population structure have recently been suggested (e.g. Pritchard *et al.* 2000).

The strawberry poison frog, *Dendrobates pumilio*, shows extreme colour polymorphism in parts of its range (Daly & Myers 1967; Myers & Daly 1976; Summers *et al.* 2003). Other closely related species living in sympatry with *D. pumilio* do not show the same extent of colour polymorphism (Summers *et al.* 1997). The bright coloration in *D. pumilio* is presumably an aposematic signal that has co-evolved with toxicity, as in many other species of poison frogs in the family Dendrobatidae (Summers & Clough 2001). The colour pattern in *D. pumilio* is visible as early as the tadpole stage and is consistent throughout life (Summers *et al.* 2004). Additionally, many of the colour morphs are maintained under common garden conditions suggesting genetic control of the colour pattern (Summers *et al.* 2004). Despite the extensive colour variation, analyses of call variation (Myers & Daly 1976) and mitochondrial DNA (Summers *et al.* 1997) suggest that the different colour morphs used in this study should be considered a single species. The colour morphs of *D. pumilio* are likely to be distinguished by conspecifics (Siddiqi *et al.* 2004) and it has been shown that females from different island populations differing in coloration show significant mating preferences for their own colour morph under controlled conditions (Summers *et al.* 1999). This indicates that coloration may be an important reproductive barrier in *D. pumilio* and a potential basis for future speciation.

Previously, no sufficiently fine-scale molecular method has been used to investigate the relationships among the morphologically diverged populations of *D. pumilio* at a locality/population level. In this study, we use amplified fragment length polymorphism (AFLP) to investigate genetic structure among populations. AFLP has the advantage of providing a sufficiently large number of markers to pick up even fine-scale genetic variation. The aims of this study were to investigate the morphological [snout vent length (SVL), spot patterns and coloration] and genetic structure among populations of *D. pumilio* in an area of its range where morphological variation is extensive. We discuss three, not mutually exclusive, hypotheses (neutral divergence; aposematic natural selection; and sexual selection) that may explain the observed variation.

Materials and methods

Study species

The strawberry poison frog, *Dendrobates pumilio*, is a diurnal neotropical frog in the family Dendrobatidae with a distribution ranging from the Atlantic versant, humid lowlands and premontane slopes of eastern Nicaragua (0–940 m above sea level, asl), through to the lowlands of Costa Rica and northwestern Panama (0–495 asl) (Savage 2002; Fig. 1). It is locally very abundant and classified as a Least Concern species (IUCN, www.iucnredlist.org). Geographical variation in coloration occurs throughout its range but is most pronounced in the Panamanian archipelago of Bocas del Toro (see below, Daly & Myers 1967; Savage 2002). The species primarily inhabits leaf litter and low vegetation (Pröhl 2003) but we noticed that some populations were more arboreal than others.

Study area

We visited 14 locations with *D. pumilio* from 23 March to 10 April 2005 in the Bocas del Toro archipelago in northwest Panama (9°20′N, 82°15′W, Fig. 1, Table 1). We aimed at 20 individuals per locality, with the exception of the Bastimentos town (BBT) locality where a higher number of individuals were sampled because of the presence of more than one distinct colour morph. In total, 300 individuals were sampled without destruction (see below, Table 1). We studied localities on different islands as well as within one of the islands. Thereby, we included populations that were isolated because of physical barriers such as seawater, and populations without any obvious physical barriers which presumably have present gene flow. Research and collection permits were obtained from the Panamanian authorities (ANAM) via the Smithsonian Tropical Research Institute.

Sex was determined in adult frogs by investigating throat coloration (Walls 1994). Male throats appear darker when uninflated as their calling behaviour stretches the skin. Each individual was digitally photographed at a standardized distance using a custom-made camera stand equipped with a Minolta DiMAGE 7i camera with a Hoya Skylight filter and flash to normalize light conditions. Individuals were placed in a plastic Petri dish equipped with

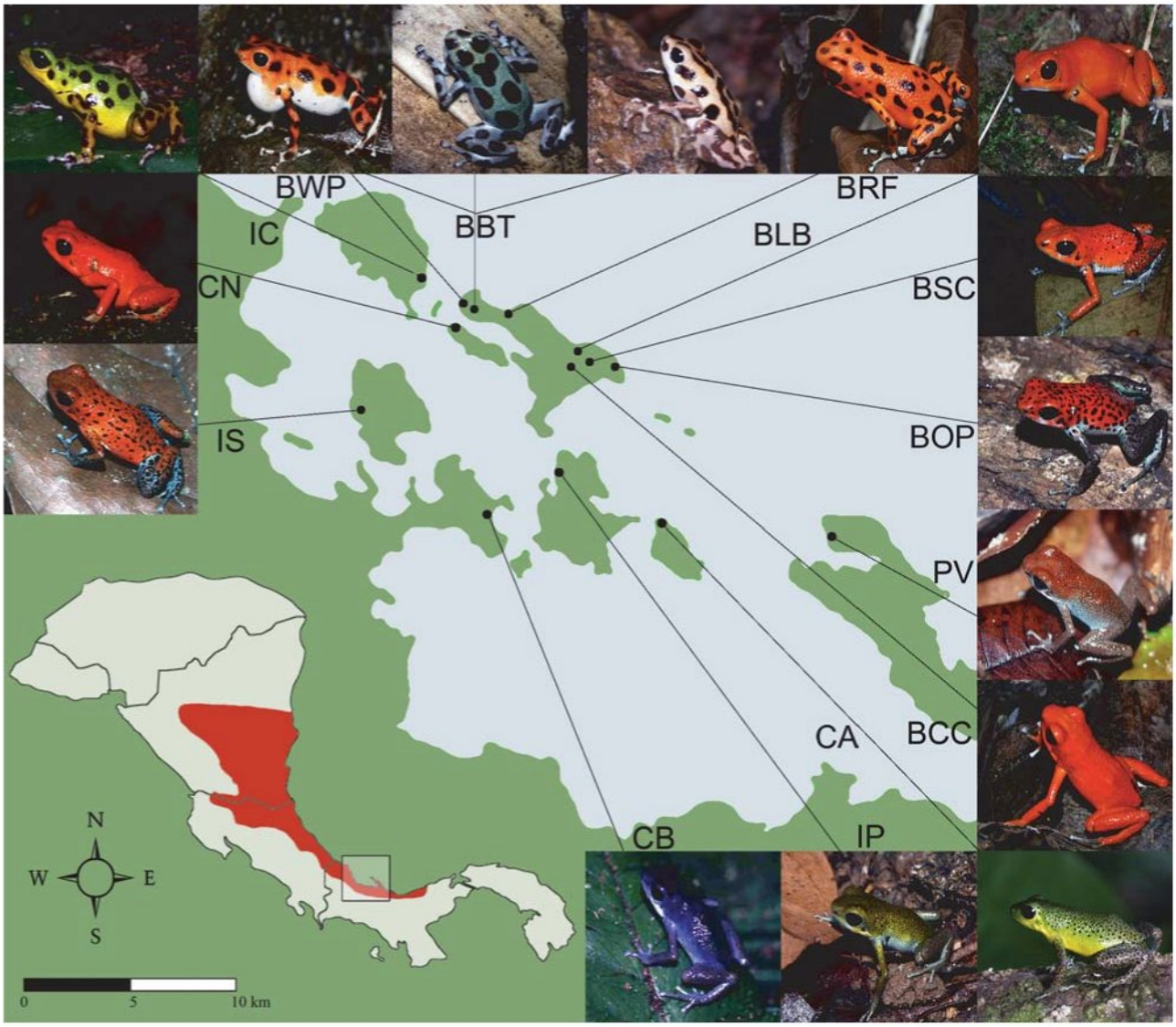

Fig. 1 Distribution and local variation of *Dendrobates pumilio*. The map to the left show Central America with the distribution in Nicaragua, Costa Rica and Panama. The insert indicate the sample locations in Bocas del Toro, Panama, and examples of the sampled morphs.

millimetre grid paper. A transparent lid was placed on top of the individual in order to restrain the frogs while being handled and allow for accurate size measurements to be taken. We later determined SVL, coloration and spot pattern from the digital photographs. Before release, we took a tissue sample from each individual by removing the toe-pad of digit number III on one of the hind legs according to standard operating procedure guidelines (www.nwhc.usgs.gov) using disinfected scissors. The tissue samples were stored in 95% ethanol.

Image analyses

We defined an area for the colour and pattern measurements. This was carried out by drawing lines from the vent to the rear side of the eyes and forward connecting at the point of the snout. This area was uniform and easily comparable between individuals and thus appropriate for image analysis. We used ADOBE PHOTOSHOP vs. 8.0 to measure SVL and to estimate the background colour of the dorsum. SVL was determined with the aid of the millimetre grid paper. Colour was measured by using the colour picker tool set to a five by five pixels average. Four points were measured and the mean calculated. We estimated colour by hue, saturation and brightness. Hue is the colour reflected from or transmitted through an object. It is measured as a location on the standard colour wheel, expressed as a degree between 0° and 360°. In common use, hue is identified by the name of the colour such as red, orange, or green. Saturation, sometimes called chroma, is

Table 1 Study localities of *Dendrobates pumilio* in Bocas del Toro area with abbreviations, sample sizes and number of sexed individuals

Locality	Abb.	Island/Mainland	Sample size	Males	Females	Not sexed
Salt Creek	BSC	Bastimentos	21	9	8	4
Long Beach	BLB	Bastimentos	20	8	10	2
Old Point	BOP	Bastimentos	20	9	9	2
Cedar Creek	BCC	Bastimentos	20	12	8	0
Bastimentos Town	BBT	Bastimentos	36	14	21	1
West Point	BWP	Bastimentos	21	13	7	1
Red Frog Beach	BRF	Bastimentos	20	6	11	3
Isla Colón	IC	Isla Colón	22	14	7	1
Isla San Cristobal	IS	Isla San Cristobal	20	8	11	1
Cerro Brujo	CB	Mainland	19	14	5	0
Isla Popa	IP	Isla Popa	20	6	12	2
Cayo Nancy	CN	Cayo Nancy	20	14	6	0
Punta Valiente	PV	Mainland	20	9	7	4
Cayo Agua	CA	Cayo Agua	21	7	13	1
Total			300	143	135	22

the strength or purity of the colour. Saturation represents the amount of grey in proportion to the hue, measured as a percentage from 0% (grey) to 100% (fully saturated). On the standard colour wheel, saturation increases from the centre to the edge. Brightness is the relative lightness or darkness of the colour, usually measured as a percentage from 0% (black) to 100% (white) (ADOBE PHOTOSHOP version: 8.0 Adobe Systems Incorporated).

The use of a compact digital camera was chosen for logistic reasons as many of the sampled localities were remote. We are aware of that this method is suboptimal for estimating colour (Montgomerie 2006; Stevens *et al.* 2007), and although spectral reflectance data would be preferred, we argue that our method suffices as we are interested in relative and not absolute colour values. One possible limitation is that there is colour variation detectable with tetrachromatic visibility which is undetectable using our red, green, and blue (RGB) system. Earlier spectral reflectance measurements of 15 populations of *D. pumilio*, including six localities in the vicinity of those in this study, revealed no more than one reflectance peak for dorsal coloration (Summers *et al.* 2003). Thus, we are confident that the available hue variation will be detected using a trichromatic colour system (Vorobyev *et al.* 1998).

We controlled for variance in light conditions by including a reference surface measurement in each picture. Pictures from different populations were chosen, and thus include different light conditions due to factors such as forest foliation, cloud coverage, and time of the day. The standard error was 0.38°, 0.55% and 0.51% ($N = 20$) for hue (population means 8.3°–156°), saturation (population means 9.9–80.3%) and brightness (population means 31.9–84.6%), respectively, indicating a slight effect of differing light condition and exposure inconsistency on our data.

The spot pattern was analysed with IMAGEJ software (Abramoff *et al.* 2004). This software produces a binary image from a digital photo, and calculates the number and size of the black and white areas. We took two measurements of the black spot pattern: mean spot size (mm^2); and the percentage of black in the measured dorsal area (%).

DNA extraction

We extracted all samples with high salt purification and ethanol precipitation as follows. Each sample was placed in 350 μL SET buffer (0.15 M NaCl, 0.05 M Tris, 1 mM EDTA pH 8.0), 12.5 μL proteinase K (10 mg/mL) and 15.5 μL SDS (25%) in an Eppendorf tube and was incubated at 55 °C for approximately 2 h until the tissue was totally dissolved. We then added 300 μL NaCl 6 M, and vortexed the samples vigorously for 10–20 s, centrifuged for 10 min at 14 000 g and transferred 600 μL of the supernatant to a new tube. We then added 150 μL Tris 0.001 M, pH 8.0, mixed, added 750 μL freezer-cold 99.5% ethanol, mixed again and let the sample precipitate over night at –20 °C. Samples were then centrifuged for 15 min at 14 000 g, the supernatant discarded, the pellet washed with 1 mL freezer-cold 70% ethanol and again centrifuged 10 min at 14 000 g. Finally, the pellet was dried and dissolved in 40 μL TE buffer.

AFLP

We used a protocol based on Bensch *et al.* (2002) modified from Vos *et al.* (1995) with some further modifications. Ten microlitres of the extraction was cut by a mix of 6.9 μL ddH_2O, 2 μL TA-buffer 10× (100 mM Tris-Ac pH 7.9, 100 mM MgAC, 500 mM KAC, 10 mM DTT), 1 μL BSA 1 μg/μL, 0.05 μL *Eco*RI 50 μ/μL and 0.05 μL Tru1 50 μ/μL for 1 hour at 37 °C.

To ligate adaptors, we added 5 μL of the mix of the 4.15 μL ddH_2O, 0.5 μL T4 ligase buffer 10×, 0.025 μL E-adaptor 100 μM, 0.25 μL M-adaptor 100 μM and 0.1 μL T4 ligase 5 μ/μL to cut the DNA and incubated for 3 hours at 37 °C. The E-adaptor and M-adaptor were built by primers 5′-CTCGTAGACTGCGTACC-3′, 5′-AATTGGTACGCAGTC-TAC-3′ and 5′-GACGATGAGTCCTGAG-3′, 5′-TACTCAG-GACTCAT-3′.

Ligated samples were diluted 10 times with ddH_2O and amplified in a nonselective polymerase chain reaction (PCR). The reactions consisted of 1.0 μL ddH_2O, 2.0 μL $MgCl_2$ 25 mM, 2.0 μL Fermentas *Taq* buffer 10×, 4.0 μL dNTP 1 mM, 0.06 μL E-primer 100 μM, 0.06 μL M-primer 100 μM, 0.08 μL Fermentas *Taq* 5 μ/μL, 0.8 μL BSA 1 μg/μL and 10 μL of the diluted ligated samples. E-primer and M-primer were 5′-GACTGCGTACCAATTCN-3′ and 5′-GATGAGT-CCTGAGTAAN-3′, where 'N' is an arbitrarily selected base. The reactions were incubated as follows: (94 °C 2 min); (94 °C 30 s, 56 °C 30 s, 72 °C 60 s) × 20 cycles; (72 °C 10 min). The products were diluted 10 times with ddH_2O.

The diluted product was selectively amplified in a touchdown PCR. The product (2.5 μL) was added to the mix of 2.9 μL ddH_2O, 1.0 μL $MgCl_2$ 25 mM, 1.0 μL Fermentas *Taq* buffer 10×, 2.0 μL dNTP 1 mM, 0.06 μL flouresin marked E-primer 100 μM, 0.06 μL M-primer 100 μM, 0.08 μL 5 μ/μL and 0.4 μL BSA 1 μg/μL. E-primer and M-primer were 5′-GACTGCGTACCAATTCNNN-3′ and 5′-GATGAGTC-CTGAGTAANNN-3′, where 'N' is an arbitrarily selected base with the first 'N' the same as in the nonselective amplification. The reactions were incubated as follows: (94 °C 2 min); [94 °C 30 s (65–0.7 °C/cycle) 30 s, 72 °C 60 s] × 12 cycles; (94 °C 30 s, 56 °C 30 s, 72 °C 60 s) × 23 cycles; (72 °C 10 min).

Finally, 2 μL of the products were added to a mix of 9.75 μL ddH_2O and 0.25 μL ET-ROX 400 size standard and run on a MegaBACE 1000 (Amersham Biosciences). Runs were analysed using the MegaBACE FRAGMENT PROFILER version 1.2 (Amersham Biosciences). All individuals were scored together for fragments between 50 bp and 400 bp for presence (1) or absence (0).

Eight primer combinations (M-primer: E-primer; -CGT: -TAG; -CTA: -TAG; -CGT: TCT; -CAG: TCT; -CAG: TAG; -CAC: TAG; -CTA: TCT; -CTA: TGC) yielded a total of 139 polymorphic markers. DNA extractions from 12 randomly picked individuals from 11 locations were repeated using all primer combinations to test for repeatability. Five markers amplified in an uncertain matter (less than 83% repeatability) and were thus excluded. The remaining 134 markers used in analysis showed an overall repeatability of 97.3%.

Statistical analyses

We used R (www.r-project.org) to perform standard statistical tests and Mantel tests. We used two-way ANOVAS with type III sums of squares to test for differences in morphological traits among populations and between the sexes. The *P* values for ANOVAS were Bonferroni corrected to account for each type of multiple tests. Mantel tests based on Pearson's product-moment correlation between morphological traits, genetic distance and geographical distance, including the test for isolation by distance, were performed with the aid of the R-package VEGAN. Ten thousand permutations of the rows and columns of the first dissimilarity matrix were used to assess the level of significance. The *P* values were Bonferroni corrected for each type of multiple tests.

To estimate the number of independent genetic clusters, *K*, we used our AFLP data without prior population information on the individuals with STRUCTURE 2.0 (Pritchard *et al.* 2000). The simulation runs were set for a burn in period of 10 000 and 100 000 Markov chain Monte Carlo with the 'no admixture' model. We performed five repeats for each simulation of *K* clusters. The output is given by Ln *P*(*D*), an estimate of the posterior probability of the data for a given *K*. Further, we used the measure of Δ*K* which was proposed by Evanno *et al.* (2005) to be a more accurate way to detect the true number of clusters of individuals.

To calculate the global F_{ST} (Weir & Cockerham 1984) and Nei's measure of genetic distance (Nei 1978), we used TFPGA (www.marksgeneticsoftware.net). For the estimate of F_{ST}, the confidence level was set to 95% and 10 000 bootstrap iterations were used to generate the confidence intervals. To calculate the F_{ST} analogue Φ_{PT} for pairwise population comparisons, we used GENALEX 6 (Peakall & Smouse 2006). Φ_{PT} is calculated as $V_{AP}/(V_{AP} + V_{WP})$ where V_{AP} is the variance among populations and V_{WP} is the variance within populations. Probability values were based on 999 permutations.

For the scenario of drift–migration equilibrium among populations, we estimated the selection needed to sustain the populations distinct in dorsal background coloration. We used a model of migration–selection balance (Falconer 1989) as described in (Hoekstra *et al.* 2004) where the frequency change of a foreign allele in a population is given by:

$$\Delta q = -\frac{spq[q + h(p - q)]}{1 - sq(2hp + q)} + mQ - Mq$$

where *s* is the selection coefficient against the foreign allele, *q* is the frequency of the foreign allele, *p* is the frequency of the local allele, *h* is the dominance coefficient, *m* is the immigration rate, *Q* is the frequency of the foreign allele among immigrant and *M* is the emigration rate. We assumed no dominance in the determination of this trait in accordance with the observed mixed coloration of colour-morph hybrids (Summers *et al.* 2004). Under the assumption of drift–migration equilibrium, the migration rate was

Table 2 ANOVA table of the studied *Dendrobates pumilio* morphological traits in the Bocas del Toro archipelago and the effects of the factors locality, sex and their interaction. B indicates significance after Bonferroni correction

	SumSq	d.f.	*F*	*P*	B
SVL					
Sex	0.2337	1	0.2844	0.59432	
Locality	569	13	53.2285	< 0.00001	***
Sex:Locality	22	13	2.0145	0.02015	
Residuals	205	250			
Hue					
Sex	34	1	0.2900	0.590740	
Locality	358255	13	231.8575	< 0.00001	***
Sex:Locality	3930	13	2.5437	0.002661	*
Residuals	29001	244			
Saturation					
Sex	16	1	0.1912	0.662316	
Locality	111424	13	104.4244	< 0.00001	***
Sex:Locality	2565	13	2.4037	0.004624	*
Residuals	20027	244			
Brightness					
Sex	16	1	0.5894	0.4434	
Locality	73099	13	205.5251	< 0.00001	***
Sex:Locality	362	13	1.0190	0.4334	
Residuals	6676	244			
Coverage					
Sex	34	1	0.7082	0.4009	
Locality	82568	13	133.8068	< 0.00001	***
Sex:Locality	211	13	0.3424	0.9844	
Residuals	10917	230			
Spot size					
Sex	0.067	1	0.0968	0.756	
Locality	314.492	13	35.1384	< 0.00001	***
Sex:Locality	0.495	13	0.0553	1.000	
Residuals	158.348	230			

estimated using the infinite island model (Wright 1931) where $F_{ST} \approx 1/(4Nm + 1)$. By setting delta q to zero and solve for s, we obtain the lowest limit of selection needed to lower the frequency of a foreign allele in the population.

For a visual comparison between the STRUCTURE inferred clusters and the genetic distance between sampled localities, we reconstructed a dendrogram based on Nei's measure of genetic distance. A UPGMA option was used in PHYLIP version 3.5 with 10 000 bootstrap resamplings and the tree was visualized with TREEVIEW version 1.6.6 (Page 1996).

Results

We found highly significant variation among localities in all morphological traits (Table 2). The effect of sex was small and nonsignificant in all characters (Table 2). However, we found a significant interaction between the effects of sex and population on hue and saturation, indicating variation in the extent of sexual dimorphism among populations in these characters.

Using STRUCTURE, we got the highest Ln $P(D)$ for nine genetic clusters, also supported by the measure of ΔK. The groupings according to the STRUCTURE algorithm were concordant with the UPGMA clustering (Fig. 2). One cluster only contained small proportions of assigned individuals from a few localities and is not shown in the figure. It is not unlikely that population size, and thereby drift, varies among populations. Thus, the dendrogram reflects a common evolutionary past confounded by demographic differences and should not be interpreted as a strict phylogenetic reconstruction.

The global F_{ST} was 0.232 ± 0.015 suggesting distinct population differentiation. All pairwise Φ_{PT} values were significantly different from zero (Table 3).

In our selection–migration model, we considered the morphologic and genetic structure obtained from our data. The only populations polymorphic for dorsal background coloration were BBT and West Point (BWP). These populations also showed high variation in the neutral genetic markers (results not shown) and clustered together with Red Frog Beach (BRF) and Isla Colón (IC). This pattern would be expected if there was recent migration between IC, BWP, BBT and BRF, or migration from IC to the latter three. Unlike the rest of the investigated populations, these are situated in a densely inhabited area with extensive tourism and translocation of frogs by humans is thus not

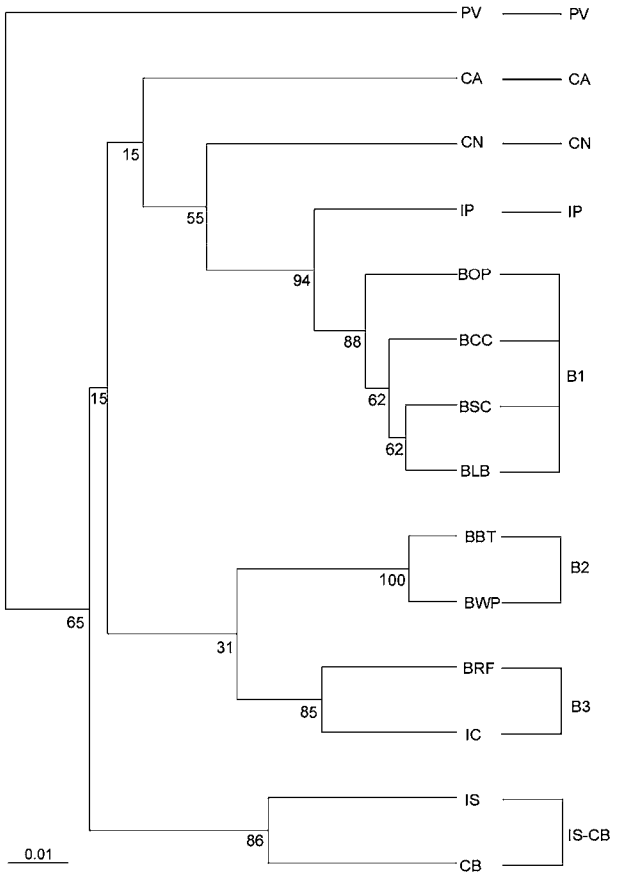

Fig. 2 UPGMA dendrogram based on Nei's Genetic distance to the left (bootstrap values below branches). The eight clusters given by STRUCTURE 2.0 are shown to the right.

unlikely. Therefore, the BBT and BWP populations were excluded from the model, and the five remaining populations on Bastimentos Island were pooled as they show similar dorsal background coloration. Mean F_{ST} was then estimated to 0.2049. The frogs appear in high densities and by assuming an effective population size (*Ne*) of between 100 and 1000 individuals, we included an interval that should be representative for the considered populations. This yields a migration rate of between 9.7e-3 and 9.7e-4. We did not observe any foreign colour morph in any of the populations included here. Hence, q was set to 0.01, as any higher q would certainly have resulted in the observation of such an aberrant morph. The minimum selection needed to decrease q at the assumed population sizes was then 0.19 and 1, respectively.

We found significant isolation by distance in AFLP genetic variation (Mantel's $r = 0.71$, $P < 0.001$, Fig. 3). Among populations, differences in SVL correlated with increasing genetic distance (Mantel's $r = 0.45$, $P = 0.0372$, Fig. 3) and, although not significant, showed a trend to correlate with geographical distance (Mantel's $r = 0.476$, $P = 0.0552$). The only measure of population differences in coloration that correlated with either geographical or genetic distance was brightness (geographical distance: Mantel's $r = 0.3865$, $P = 0.0396$; genetic distance: Mantel's $r = 0.140$, $P = 0.91$, Fig. 3). None of the other measures of coloration (hue, saturation, spot size and coverage) correlated with either geographical distance (hue: Mantel's $r = 0.121$, $P = 1.00$; saturation: Mantel's $r = 0.303$, $P = 0.48$; spot size: Mantel's $r = 0.039$, $P = 1.00$; coverage: Mantel's $r = 0.067$, $P = 1.00$) or genetic distance (hue: Mantel's $r = -0.063$, $P = 1.00$; saturation: Mantel's $r = 0.011$, $P = 1.00$; spot size: Mantel's $r = 0.190$, $P = 1.00$; coverage: Mantel's $r = 0.268$, $P = 0.52$, Fig. 3).

Discussion

The morphological variation in size and coloration in *Dendrobates pumilio* is more pronounced in the Bocas del Toro archipelago and adjacent mainland localities than elsewhere within its distribution (Daly & Myers 1967; Savage 2002; H. Pröhl, personal communication). The dramatic colour and spot pattern variation found in this study is contrary to that which would be expected if the system was under common stabilizing selection. Such stabilizing selection is assumed if the predator system shares similar prey avoidance where an aberrant coloration would not be recognized by naïve predators. If the distinct differences in coloration mainly result from its aposematic signal content, this would indicate that there must have been a change in predator preference. The area considered in this study is more fragmented, in terms of suitable habitat, than other parts of the species distribution. This implies that subpopulations are more likely to evolve as independent units rather than as a unified population. The extraordinary colour variation observed in Bocas del Toro and adjacent mainland areas may be explained by a model of 'shifting balance' (Wright 1932, 1977), also discussed in the similar case of *Heliconius* butterflies (Mallet & Singer 1987; Turner & Mallet 1996). In theory, increased drift due to low population size, and possibly a relaxation of predation pressure, may cause periods of relaxed selection. Such a scenario would allow a novel morph to increase to a high frequency. Presumably, if a novel colour morph reaches a high enough frequency through drift, it may eventually become fixed due to selection.

According to the selection model used, the minimum selection needed to decrease the frequency of a foreign allele was between 0.19 and 1. This estimation is based on several assumptions and the result is within a large interval. Nevertheless, this would imply that if the neutral genetic structure reflects actual drift–migration equilibrium, the selection would need to be relatively high to prevent migrated colour morphs to increase to detectable levels. If, however, the neutral genetic structure reflects isolated diverging populations without drift–migration equilibrium,

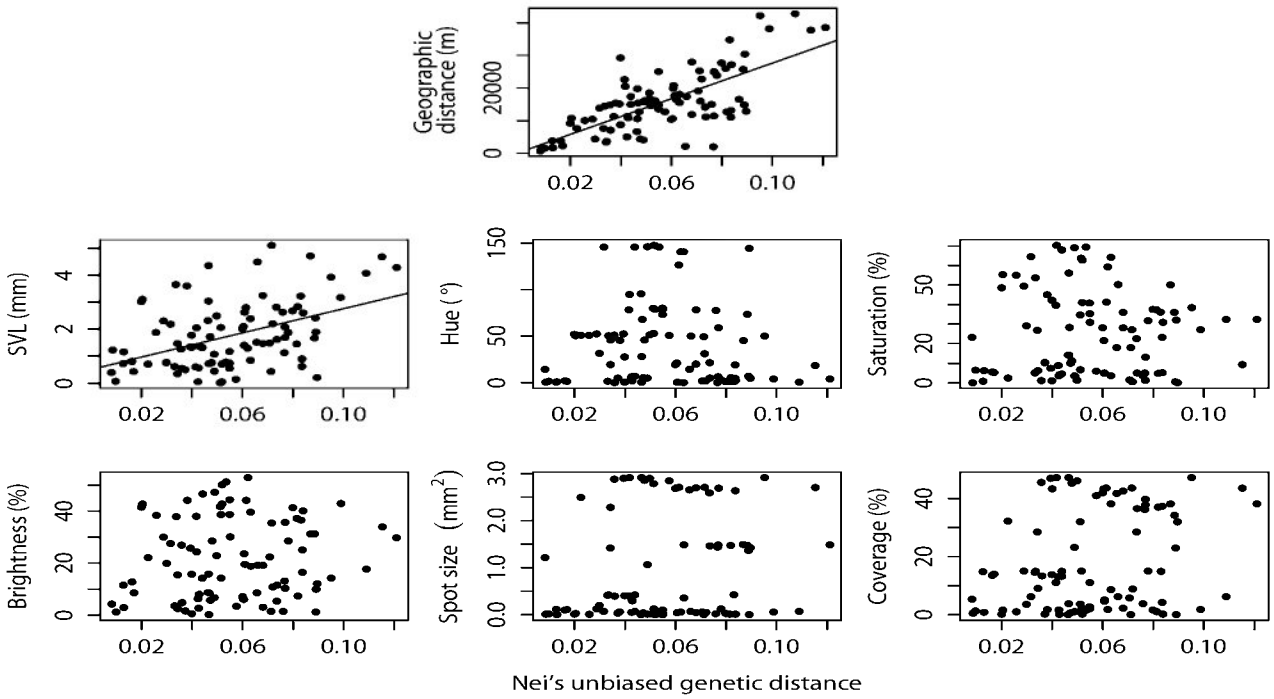

Fig. 3 Isolation by distance and relationships between genetic distance and morphological differences in population means. Lines through figures of significant correlations are best fit linear regressions.

Table 3 Pairwise Φ_{PT} values among the localities of *Dendrobates pumilio* below diagonal and probability values based on 999 permutations above diagonal

	BSC	BLB	BOP	BCC	BBT	BWP	BRF	IC	IS	CB	IP	CN	PV	CA
BSC		0.035	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
BLB	0.016		0.001	0.047	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
BOP	0.064	0.058		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
BCC	0.044	0.016	0.054		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
BBT	0.296	0.263	0.339	0.269		0.011	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
BWP	0.356	0.320	0.401	0.329	0.028		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
BRF	0.178	0.152	0.232	0.151	0.198	0.266		0.001	0.001	0.001	0.001	0.001	0.001	0.001
IC	0.193	0.152	0.228	0.165	0.152	0.184	0.111		0.001	0.001	0.001	0.001	0.001	0.001
IS	0.332	0.327	0.379	0.348	0.389	0.454	0.357	0.316		0.001	0.001	0.001	0.001	0.001
CB	0.232	0.229	0.264	0.218	0.294	0.348	0.228	0.198	0.164		0.001	0.001	0.001	0.001
IP	0.114	0.113	0.149	0.107	0.321	0.373	0.181	0.199	0.344	0.212		0.001	0.001	0.001
CN	0.206	0.180	0.250	0.187	0.323	0.373	0.254	0.237	0.453	0.325	0.166		0.001	0.001
PV	0.347	0.335	0.363	0.341	0.413	0.446	0.373	0.356	0.474	0.381	0.317	0.415		0.001
CA	0.248	0.229	0.256	0.229	0.352	0.408	0.261	0.222	0.374	0.237	0.207	0.274	0.387	

and considerably lower present migration, this model is not applicable. Selection on coloration could then be much lower. This last view is more intuitive since the migration over open salt water is highly unlikely due to poor swimming abilities, and the osmoregulatory properties (Shoemaker & Nagy 1977) of these land-living amphibians. This would also explain the genetic and morphological properties of the polymorphic populations discussed above. Even if these populations show distinct genetic differences, the possible human induced migration, if still low, would be sufficient to explain colour polymorphism in BBT and BWP. However, we should also consider the alternative explanation for the observed polymorphic populations where human disturbance could have locally altered the predation pressure.

Several aspects of morphological variation, with the exception of SVL and brightness, were extensive and differences among localities were nongradual. This is

contrary to what is expected if these characters had evolved solely because of genetic drift between semi-isolated populations. In this case, we would expect isolation by distance such as we found for neutral genetic AFLP markers. The significant population genetic structure among the studied populations of *D. pumilio* suggests limits to gene flow among populations and colour morphs. All of the pairwise comparisons between populations were significant. Furthermore, significant among-site divergence between populations on the same island was detected. The results from our two approaches of analysing relationships between populations (STRUCTURE and UPGMA) were congruent.

The populations in this study have become isolated quite recently, within the last 6000–10 000 years as a consequence of post Pleistocene sea level rise, forming an archipelago from a previously continuous land mass (Summers *et al.* 1997). Even on the same island, populations may have become separated because of the formation of unsuitable swamp habitats that were not previously present. The patterns observed in the AFLP genetic data, SVL and brightness are in accordance with a gradual divergence of populations as these traits all display increased differences by increased distance. However, the genetic drift hypothesis cannot account for the divergence among populations in saturation, hue and spot patterns. For these traits, there is no relationship between genetic distance nor geographical distance and similarity. Given that colour is genetically determined (Summers *et al.* 2004), maintenance of the rapid changes seen in coloration is in response to strong natural or sexual selection rather than a result of environmentally induced phenotypic plasticity.

Variation in local predator faunas has been suggested to be responsible for local variation in predation pressure that may drive divergence among aposematic prey (Turner & Mallet 1996). In Heliconiid butterflies, predation by visual predators has been suggested to drive the evolution of mimicry rings. The predation pressure causes different prey species to converge on a similar colour pattern within areas but drive divergence within species among different geographical locations. Could a similar explanation apply to *D. pumilio*? We think not, as Summers *et al.* (1997) contrasted the patterns observed in *D. pumilio* with two other Dendrobatid species also inhabiting Bocas del Toro: *Phyllobates lugubris* and *Minyobates* sp. If there was a general change in predation pressure, this would affect all three species. Nevertheless, only *D. pumilio* shows extensive colour variation among island populations. If the predation pressure fluctuates more in the archipelago as compared to the main distributional area, temporary release from the selective constraint may explain why the island frogs are variable. However, the finding that *P. lugubris* and *Minyobates* sp. both lack colour variation (Summers *et al.* 1997) provides evidence against the hypothesis that relaxed predation is the only reason why colour variation has evolved in *D. pumilio.*

We suggest that both changed natural selection and increased sexual selection may be involved in explaining the shift from a uniform aposematic signal in most of the mainland distribution to morphological variation in the archipelago and adjacent mainland. *Dendrobates pumilio* in contrast to the other two species has evolved predominantly female parental care (Clough & Summers 2000). Summers *et al.* (1997) suggested that this increase in female reproductive costs may have lead to female choosiness and sexual selection on male coloration. Summers *et al.* (1999) attempted to test this hypothesis experimentally and found that females of *D. pumilio* preferred to mate with a male of their own colour morph. This provides tentative evidence that sexual selection through female choice may be involved in explaining the extent of phenotypic variation among populations. However, sexual dimorphism is slight. Females also display a colorful signal in all studied populations, which suggests that the signal is not only used in sexual signalling but also aimed at other receivers, presumably visual predators. Furthermore, even though morph-specific mate preferences have been shown to occur, more studies are needed in order to investigate why such distinct mate preferences have evolved and are maintained. Cross-breeding studies between distinct colour morphs of *D. pumilio* suggest that at least some combinations produce viable offspring suggesting low developmental costs of morph crossings. However, possible costs of cross breeding after the egg stage may be considerable (Summers *et al.* 2004) as the hybrids show an intermediate and dull coloration that probably lowers the strength of the aposematic signal. Neither female mate choice nor low fitness of hybrids can explain morph divergence but they may be involved in maintaining existing population differences.

We believe that all three of the hypotheses discussed above may be involved in the colour morph variation in *D. pumilio.* Neutral divergence is sufficient to explain divergence in AFLP and SVL. However, both natural selection and sexual selection probably need to be invoked to explain the extraordinary morphological variation in skin coloration and black spot pattern among populations in the Bocas del Toro archipelago. It is interesting that such pronounced divergence has been possible in such a short time frame (< 10 000 years). However, complete post–zygotic barriers to gene flow have not yet evolved as some morphs seem capable of interbreeding (Summers *et al.* 2004).

In Lake Malawi and other east African lakes, cichlids have diverged and speciated within the last million years (Meyer *et al.* 1990; Albertsson *et al.* 1999). It has been suggested that the radiation within lakes 'involved two mechanisms of speciation: an initial differentiation involving natural selection for feeding efficiency, followed by more recent differentiation involving sexual selection' (Coyne & Orr 2004). We propose that even though *D. pumilio* is not yet differentiated into different species, the divergence

among populations can be attributed to a similar scenario. It may be that differences among populations in natural selection mediated by visual predators caused initial differences in spot pattern and coloration. At a later stage, morph-specific mate preferences may be needed to maintain and enhance differences among populations.

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References

Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image Processing with ImageJ. *Biophotonics International*, **11** (7), 36–42.

Albertsson RC, Markert JA, Danley PD, Kocher TD (1999) Phylogeny of a rapidly evolving clade: the cichlid fishes of Lake Malawi, East Africa. *Proceedings of the National Academy of Sciences, USA*, **96**, 5107–5110.

Bensch S, Helbig AJ, Salomon M, Seibold I (2002) Amplified fragment length polymorphism analysis identifies hybrids between two subspecies of warblers. *Molecular Ecology*, **11**, 473–481.

Butlin RK, Tregenza T (1998) Levels of genetic polymorphism: marker loci versus quantitative traits. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **353**, 187–198.

Clough M, Summers K (2000) Phylogenetic and biogeography of the poison frogs: evidence from mitochondrial DNA sequences. *Biological Journal of the Linnean Society*, **70**, 515–540.

Coyne JA, Orr HA (2004) *Speciation*. Sinauer & Associates, Sunderland, Massachusetts.

Daly JW, Myers CW (1967) Toxicity of panamanian poison frogs (Dendrobates): some biological and chemical aspects. *Science*, **156**, 970–973.

Dobzhansky TG (1937) *Genetics and the Origin of Species*. Colombia University Press, New York.

Endler JA (1977) *Geographic Variation, Speciation and Clines*. Princeton University Press, Princeton, New Jersey.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, **14**, 2611–2620.

Falconer DS (1989) *Introduction to Quantitative Genetics*, 3rd edn. Longman Scientific & Technical, Harlow, UK.

Gray SM, McKinnon JS (2006) Linking color polymorphism maintenance and speciation. *Trends in Ecology & Evolution*, **22**, 71–79.

Haldane JBS (1932) *The Causes of Evolution*. Longmans, Green, London.

Hoekstra HE, Drumm KE, Nachman MW (2004) Ecological genetics of adaptive color polymorphism in pocket mice: geographic variation in selected and neutral genes. *Evolution*, **58**, 1329–1341.

Kremer A, Zanetto A, Ducousso A (1997) Multilocus and multitrait measures of differentiation for gene markers and phenotypic traits. *Genetics*, **145**, 1229–1241.

Mallet J, Singer MC (1987) Individual selection, kin selection, and the shifting balance in the evolution of warning colours: the evidence from butterflies. *Biological Journal of the Linnean Society*, **32**, 337–350.

Mayr E (1963) *Populations, Species and Evolution — an Abridgment of Animal Species and Evolution*. Belknap Press, Cambridge, Massachusetts.

McKay JK, Latta RG (2002) Adaptive population divergence: markers, QTL and traits. *Trends in Ecology & Evolution*, **17**, 285–291.

Meyer A, Kocher TD, Basasibwaki P, Wilson AC (1990) Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. *Nature*, **347**, 550–553.

Montgomerie R (2006) In: *Analysing colours in Bird Colouration, Vol. 1, mechanisms and measurements* (eds Hill GE, McGraw KJ). Harvard University Press, London, UK.

Myers CW, Daly JW (1976) Preliminary evaluation of skin toxins and vocalizations in taxonomic and evolutionary studies of poison-dart frogs (Dendrobatidae). *Bulletin of the American Museum of Natural History*, **157**, 173–262.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590.

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*, **12**, 357–358.

Peakall R, Smouse PE (2006) GENALEX6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.

Pritchard JK, Stephens M, Donnelly P (2000) Interference of population structure from multilocus genotype data. *Genetics*, **155**, 945–959.

Pröhl H (2003) Variation in male calling behaviour an relation to male mating success in the Strawberry poison frog (*Dendrobates pumilio*). *Ethology*, **109**, 273–290.

Savage JM (2002) *The Amphibians and Reptiles of Costa Rica: a Herpetofauna Between Two Continents, Between Two Seas*. University of Chicago Press, Chicago, Illinois.

Shoemaker VH, Nagy KA (1977) Osmoregulation in Amphibians and Reptiles. *Annual Review of Physiology*, **39**, 449–471.

Siddiqi A, Cronin TW, Loew ER, Vorobyev M, Summers K (2004) Interspecific and intraspecific views of colour signals in the strawberry poison frog *Dendrobates pumilio*. *Journal of Experimental Biology*, **207**, 2471–2485.

Stevens M, Párraga CA, Cuthill IC, Partridge JC, Troscianko TS (2007) Using digital photography to study animal coloration. *Biological Journal of the Linnean Society*, **90**, 211–237.

Summers K, Clough ME (2001) The evolution of coloration and toxicity in the poison frog family (Dendrobatidae). *Proceedings of the National Academy of Sciences, USA*, **98**, 6227–6232.

Summers K, Bermingham E, Weight L, McCafferty S, Dahlstrom L (1997) Phenotypic and genetic divergence in three species of dart-poison frogs with contrasting parental behaviour. *Journal of Heredity*, **88**, 8–13.

Summers K, Symula R, Clough M, Cronin T (1999) Visual mate choice in poison frogs. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **266**, 2141–2145.

Summers K, Cronin TW, Kennedy T (2003) Variation in spectral reflectance among populations of *Dendrobates pumilio*, the strawberry poison frog in the Bocas del Toro Archipelago, Panama. *Journal of Biogeography*, **30**, 35–53.

Summers K, Cronin TW, Kennedy T (2004) Cross-breeding of distinct colour morphs of the strawberry poison frog (*Dendrobates*

pumilio) from the Bocas del Toro Archipelago, Panama. *Journal of Herpetology*, **38**, 1–8.

Turner JRG, Mallet JLB (1996) Did forest islands drive the diversity of warningly coloured butterflies? Biotic drift and the shifting balance. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **351**, 835–845.

Vorobyev M, Osorio D, Benett ATD, Marshall NJ, Cuthill IC (1998) Tetrachromacy, oil droplets and bird plumage colours. *Journal of Comparative Physiology a — Neuroethology Sensory Neural and Behavioral Physiology*, **183**, 621–633.

Vos P, Hogers R, Bleeker M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.

Walls JG (1994) *Jewels of the Rainforest: Poison Frogs of the Rainforest*. TFH Publications, Neptune City, New Jersey.

Wang J (2004) Application of the one-migrant-per-generation rule to conservation and management. *Conservation Biology*, **18**, 332–343.

Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

Wright S (1931) Evolution in Mendelian populations. *Genetics*, **16**, 97–259.

Wright S (1977) *Evolution and the Genetics of Population, Volume 3. Experimental Results and Evolutionary Deductions*. University Chicago press, Chicago and London.

This work is part of Andreas Rudh's PhD research on diverging processes in natural populations of frogs. Björn Rogell is a PhD student mainly studying inbreeding depression and loss of adaptive potential in amphibian island populations. Professor Jacob Höglund's research group focuses on geographic distribution of genetic diversity in natural animal populations.
