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Geographic variation in sexual signals and behaviour in two species of poison frogs

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ABSTRACT

Background: Geographic variation in sexual signals is involved in the evolution of reproductive isolation among populations. In poison frogs, acoustic as well as visual signals vary geographically and are involved in mate choice. Here we compare geographic variation in sexual signals, behavioural strategies, and population structure among two closely related species. We end by discussing the causes and consequences of this variation for the speciation process.

Question: Are differences in advertisement calls and colour pattern associated with genetic population structure or with differences in anti-predator and reproductive behaviour in two species of poison frogs.

Organisms: Strawberry poison-dart frog (*Oophaga pumilio*) and granular poison-dart frog (*Oophaga granulifera*) from Central America.

Data: Morphological measurements, advertisement calls, distribution of colour morphs, mitochondrial (*Cytb*) and microsatellite markers from multiple frog populations across the species range. Behavioural observations and visual contrasts (as indicators of conspicuousness) from two colour morphs in each species.

Methods: Assess genetic population structure for both species. Analyse variation of (1) advertisement call parameters, (2) size, and (3) weight in relation to different levels of genetic population structure and colour morphs. Use visual modelling to assess conspicuousness of colour morphs. Compare behaviour between colour morphs (conspicuous vs. cryptic) and species.

Conclusions: The genetic-geographic population structure, variation in advertisement calls, and coupled divergence in colour pattern and behaviour were strikingly similar in the two species. Advertisement calls varied in accordance with the genetic population structure. In contrast, most colour patterns were restricted to one derived genetic lineage and unrelated to genetic structure. Colour patterns were correlated with differences in anti-predator behaviour and it was possible to distinguish strategies between more aposematic and more cryptic morphs. Colour patterns and advertisement calls are sexual signals and involved in pre-zygotic isolation in amphibians but in *Oophaga* they operate independently in different geographic-genetic settings.

Keywords: acoustic signals, dendrobatid frogs, genetic divergence, natural selection, *Oophaga*, sexual selection, visual signals.

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INTRODUCTION

A central aim of evolutionary biology is to understand the role of phenotypic divergence in speciation. Phenotypic divergence between populations may lead to speciation when the divergent traits are associated with reproductive isolation. Such traits include signals targeted to conspecifics and potential predators. These signals can vary between genetically divergent populations (e.g. Boughman, 2001; MacDougall-Shackleton and MacDougall-Shackleton, 2001; Rosenblum, 2006), suggesting that signal divergence may cause speciation. Geographic variation in signals is the result of natural or sexual selection, genetic drift, environmental effects or plasticity, but current evidence suggests that most species appear to arise by selection (Schluter, 2009). Signals may vary geographically within species or between closely related species as a consequence of divergent selection for ecological adaptation that may also drive mating preferences (i.e. ecological speciation) (reviewed in Rundle and Nosil, 2005). However, the mechanisms of interaction between sexual and natural selection during speciation remain poorly understood (Maan and Seehausen, 2011).

A small Neotropical fish, the Trinidadian guppy (*Poecilia reticulata*), provides one of the best examples for the interplay of natural and sexual selection on signal divergence. Streams differ in predator communities and low predation risk is associated with more elaborate and conspicuous coloration in male guppies coupled with female preferences for colourful males, while in streams with high predation risk males are duller and female preferences for colourful males is weaker or absent (Houde and Endler, 1990). Such mating preference in the choosing sex (mostly females) reduces the probability of mating between members of two groups with diverged sexual signals, thus resulting in pre-zygotic isolation, one essential component in the speciation process (Coyne and Orr, 2004). Therefore, geographic variation in sexual signals involved in courtship and mate assessment can promote speciation by behaviourally isolating populations (e.g. Gray and Cade, 2000; Boul *et al.*, 2007).

On the other hand, divergent selection across heterogeneous habitats, predator regimes, and mimicry rings entail geographic variation in signals targeted to potential predators (e.g. Jiggins *et al.*, 2001; Nosil *et al.*, 2005; Rosenblum, 2006; Chamberlain *et al.*, 2009; Mochida, 2011). This variation may lead to speciation, particularly if sexual preferences evolve for locally adapted traits (van Doorn *et al.*, 2009), or if hybrids of intermediate phenotypes are poorly adapted to either parental environment (Servedio, 2004). An extreme case occurs when divergence spans the whole spectrum of conspicuousness to potential predators, from aposematism to crypsis, and involves morphological as well as behavioural traits (Pröhl and Ostrowski, 2011; Rudh *et al.*, 2011; Willink *et al.*, 2013). Aposematism is the combination of unpalatability or toxicity with conspicuous coloration and relies on predator avoidance learning, while cryptic animals depend on being camouflaged against the background to avoid predation (reviewed in Ruxton *et al.*, 2004). Despite the increasing awareness of the interaction between behaviour and coloration in predator avoidance strategies (e.g. Ioannou and Krause, 2009; Speed *et al.*, 2010; Pröhl and Ostroswski, 2011), most studies to date aiming to link phenotypic and genetic divergence have not considered coloration and behavioural traits simultaneously.

In frogs and toads (Anura), variation in conspicuous signals directed towards conspecifics and predators is widespread. The advertisement calls act as their prime sexual signals and their significance for pre-zygotic isolation among lineages has been demonstrated in multiple mate-choice experiments (e.g. Boul *et al.*, 2007; for reviews, see Gerhardt and Huber, 2002; Wilkins *et al.*, 2013). While pronounced population genetic structure has been found in numerous species of anurans (e.g. Noonan and Gaucher, 2006; Pröhl *et al.*, 2006; Klymus *et al.*, 2010), the relationship between bioacoustic and genetic divergence remains unclear. In addition to variation in call traits, variation in colour pattern is prevalent in Neotropical poison frogs of the family Dendrobatidae. Colour diversity is associated with anti-predation strategies: numerous species are cryptic and non-toxic while others are toxic and possess highly conspicuous warning coloration (Santos *et al.*, 2003). Both strategies also occur within a species and this phenomenon is linked to colour polytypy (Pröhl and Ostrowski, 2011; Maan and Cummings, 2012; Willink *et al.*, 2013).

In the present study, we compared the patterns of phenotypic divergence in acoustic and visual signals with genetic divergence in two species of poison frogs that occupy similar habitats in Central America, aiming to shed light on the underlying mechanisms that link phenotypic divergence and speciation.

The study systems and past work

Two poison frog species from Central America are particularly suitable for the study of geographic variation of phenotypic traits and genetic divergence. The strawberry-poison frog (*Oophaga pumilio*) ranges from Northeast Nicaragua, crossing Costa Rica to Northwest Panama. In the lowland forests of the Caribbean slope, *O. pumilio* is an abundant species that occurs in altered as well as pristine forest habitats. The granular poison frog (*O. granulifera*) is restricted to the lowlands of the Pacific slope in Costa Rica and is less abundant; its occurrence is restricted to natural forests close to creeks.

In each species, two divergent mitochondrial (*Cytb*) lineages have been delineated (Hagemann and Pröhl, 2007; Wang and Shaffer, 2008; Hauswaldt *et al.*, 2011; Wang, 2011; Brusa *et al.*, 2013) that might be involved in the development of reproductive barriers. Further analyses with nuclear markers (microsatellites) revealed two genetic clusters (Hauswaldt *et al.*, 2011; Brusa *et al.*, 2013) that reflect more recent divergence and do not entirely coincide with the genetic lineages (Fig. 1). Individuals from different populations vary in size and weight as well as in advertisement calls, which, when used by males, attract mating partners and convey territorial defence (Pröhl *et al.*, 2007; Brusa *et al.*, 2013). In addition, gradation in aposematic and more cryptic coloration can be detected among populations of both species and some populations are polymorphic regarding colour morphs (Savage, 2002; Siddiqi *et al.*, 2004; Wang and Shaffer, 2008; Wang, 2011; Brusa *et al.*, 2013). The divergence into aposematic and cryptic coloration coincides with more conspicuous and more cryptic feeding and anti-predator strategies in both species (Pröhl and Ostrowski, 2011; Willink *et al.*, 2013). Similarities between both species in terms of their patterns of phenotypic and genetic divergence have yet to be explored.

This study had two goals. First, we compared the patterns of phenotypic divergence in *O. pumilio* and *O. granulifera*. To do this we described the geographic variation in acoustic signals, dorsal coloration, and size, and we investigated the behavioural and coloration conspicuousness of two colour morphs with divergent anti-predator strategies in each species. We compared the patterns of phenotypic divergence with genetic divergence and found striking similarities between the two species but in opposite latitudinal directions: (1) genetic divergence into two genetic lineages, roughly a northern and a southern clade; (2) variation in advertisement calls correlated to genetic differentiation; and (3) diversification into conspicuous and cryptic colour morphs and anti-predator strategies among populations lacking strong genetic structure. We discuss the selection pressures that might have played a role in the parallel geographic pattern of genetic, morphological, and behavioural divergence.

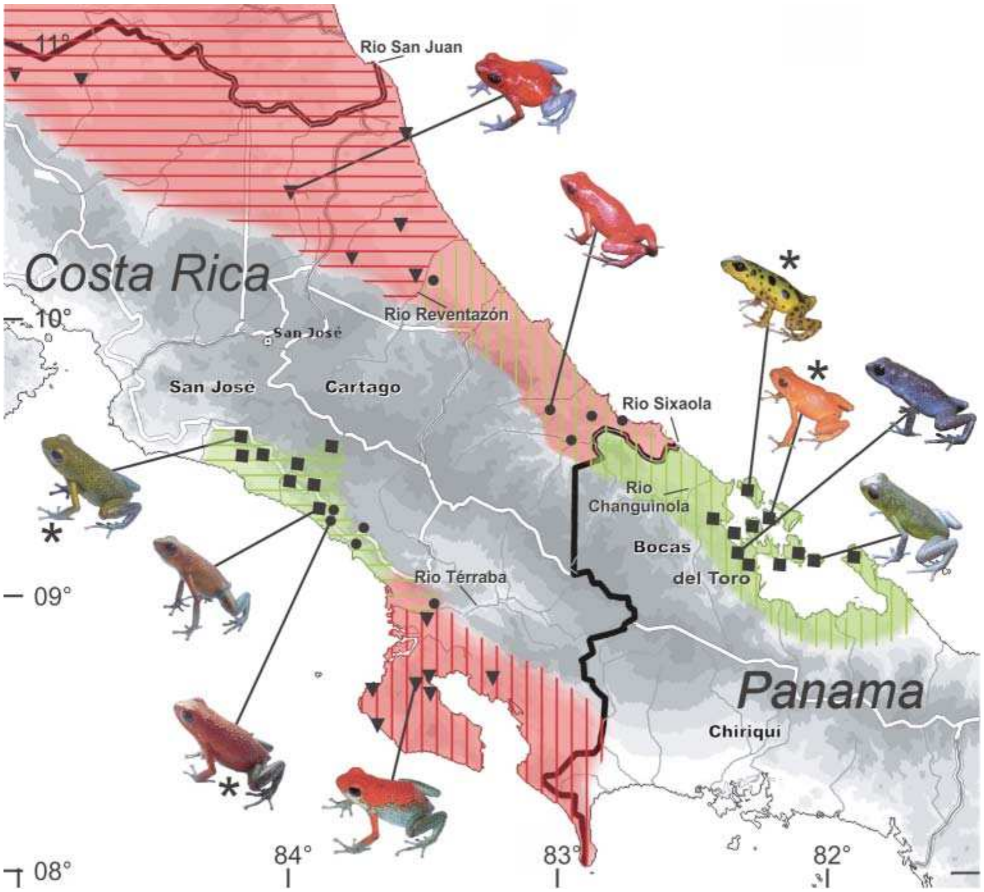

Fig. 1. Distribution of colour morphs of *O. pumilio* on the Caribbean slope and *O. granulifera* on the Pacific slope of Costa Rica and Panama. ▼, populations that belong to the red-blue group; ●, populations that belong to the red central group; ■, populations of the colour diverse group. Genetic clusters (based on microsatellites) are indicated as green and red areas whereby the red areas contain mainly red-blue and red frogs and the green areas contain mainly green and a variety of other colour morphs. Horizontal and vertical hatching indicates the northern and southern *Cytb* lineage, respectively (Hauswaldt *et al.*, 2011; Brusa *et al.*, 2013). Note that genetic clusters and genetic lineages overlap in the central distribution area of both species. We carried out behavioural observations in populations indicated by a star: Isla Solarte (red phenotype) and Isla Colón (green) for *O. pumilio*; Baru (red) and San Rafael (green) for *O. granulifera*.

MATERIALS AND METHODS

For this study we combined and re-analysed the data available on genetic population structure (Hauswaldt *et al.*, 2011; Brusa *et al.*, 2013), variation in size, weight, parameters of advertisement calls (Pröhl *et al.*, 2007; Brusa *et al.*, 2013), and distribution of colour morphs (Summers *et al.*, 2003; Hagemann and Pröhl, 2007; Wang, 2011; Brusa *et al.*, 2013) (Fig. 1). In addition, we evaluated differences in conspicuousness and behaviour for two colour morphs (Pröhl and Ostrowski, 2011; Willink *et al.*, 2013) to compare the pattern of genetic, morphological, and behavioural variation between two *Oophaga* species: *O. pumilio* and *O. granulifera*. Both species contain populations with red frogs with blue or blue-green legs, populations where frogs are completely or mainly red, and populations with a variety of colour morphs including cryptic ones. Therefore, we split both species into three phenotypic groups based on coloration: a red-blue group, a red central group, and a colour diverse group (Fig. 1). We then related the distribution of colour phenotypes and differences in calls (call parameters considered: call duration, call rate, duty cycle, pulse rate, dominant frequency) to the genetic population structure (*Cytb* lineages and genetic microsatellite cluster) and also compared size, weight, and call parameters among the phenotypic groups.

Behavioural observations and visual modelling

We compared the conspicuousness of anti-predator and reproductive behaviour between red and green populations (Fig. 1) from the colour diverse group of both species and linked behavioural and morphological conspicuousness assessed by spectrometric measurements and visual modelling. Behavioural observations and spectrometric measurements were described in Pröhl and Ostrowski (2011) and Willink *et al.* (2013) and are summarized briefly in the following text. Calculations of contrasts (visual modelling) were recalculated for this study.

In *O. pumilio*, we observed orange-red males on Isla Solarte and green males with black spots on Isla Colón. For *O. granulifera*, we selected a red population from Baru and a green population from San Rafael (Fig. 1). Behavioural observations were performed using focal animal sampling and included calling activity, movements, foraging, courtships with females, aggressive interactions between males, inactivity, escape distance, distance to next territorial neighbour, and the substrates (e.g. tree trunk, leaves) on which we found the focal males. Only the recording of the escape distance differed between the two species; it was the distance from a potential predator, represented by (a) the observer in the case of *O. pumilio* and (b) a bird dummy in the case of *O. granulifera,* at which the frog escaped from its current position to hide in the litter or vegetation.

We measured dorsal and ventral spectral reflectance (R) of both colour morphs of both species as well as from the natural substrates and ambient light (irradiance) with an Ocean Optics HR2000 Spectrometer and accessories (Pröhl and Ostrowski, 2011; Willink *et al.*, 2013). To compare visual conspicuousness between species, we calculated colour and brightness contrasts of red and green frogs against an average background of their habitat and their specific calling places. We calculated contrasts for the visual system of conspecific frogs and avian predators. The visual systems of some passerine birds (Hart *et al.*, 1998) and the strawberry poison frog (Siddiqi *et al.*, 2004) are sufficiently explored to allow sensitivity information of cone classes to be used in visual modelling. The visual systems of natural avian predators of poison dart frogs are not studied. Therefore, we used as a proximate the visual system of blue tits as in earlier calculations (Pröhl and Ostrowski, 2011; Willink *et al.*, 2013). Since the visual system of *O. granulifera* is unknown, we use *O. pumilio* sensitivity spectra for conspecific perception of this species as well.

One assumption of the model is that the photoreceptor noise and the relative number of cone classes determine the thresholds for colour discrimination. The photoreceptor noise of each cone class (ω) is given by the noise-to-signal ratio for each single cone (v), which is a constant for each taxa (here birds and frogs) and the relative number of cone types ($\acute{\eta}$) in the retina (Vorobyev *et al.*, 1998). Here we follow the suggestion of Maan and Cummings (2012) for noise-to-signal ratios and proportion of cone classes of the European starling (Table A, evolutionary-ecology.com/data/2839Appendix.pdf) to allow comparison with multiple *O. pumilio* phenotypes published in that study.

Statistical analyses

The data distribution of morphological and call parameters was checked with the Kolmogorov-Smirnov test and visual inspection of the data in histograms for deviation from normality. Despite some outliers in most variables, size, weight, and call parameters did not deviate significantly from a normal distribution (all $P > 0.2$), with the exception of call duration for *O. pumilio* ($P < 0.05$). Therefore, we applied different types of analyses of variance to determine whether most of the differences were due to different levels of divergence: between species, between lineages of *Cytb* (ancestral), genetic cluster (more recent divergence), and among phenotypic groups.

We calculated Euclidean distances based on standardized call parameters to explore variation in advertisement calls. To assess the effects of isolation by distance on variation in advertisement calls, we calculated correlations and partial correlation coefficients between bioacoustic distances (Euclidean distance), geographic distance (straight line pairwise distances in kilometres), and genetic distance (D_{est}), using Mantel tests in Arlequin 3.5.1.2 (Excoffier *et al.*, 2005). Pairwise and overall D_{est} values based on differences in microsatellite allele frequencies among populations were previously calculated with the R package DEMETICS (Jost, 2008; Gerlach *et al.*, 2010).

Visual contrasts were analysed with two-way analyses of variance (ANOVAs) testing the effects of frog species and phenotype on visual conspicuousness to conspecifics and potential predators. We used a Box-Cox transformation on contrast data to correct non-normality and heteroscedasticity (especially between species) in some of the contrasts. We performed separate analyses for the colour and brightness contrasts of the dorsal and ventral skin of frogs, for frog and bird visual systems, and for averaged contrasts against all backgrounds of each population and against the mean calling position of each population.

Behavioural rates of focal males (i.e. foraging, movement, calling activity, inactivity, aggression, and association with females) were analysed using generalized linear models (GLMs). A binomial error distribution was assumed, since the data are overdispersed proportions of a frog's time budget. We report association tests between the probability of engaging in these behaviours and the frog species and phenotype. We used a two-way ANOVA to evaluate the effects of the frog species and phenotypes on the distance to the nearest neighbour. Since different predator models were used to test the propensity to escape in *O. pumilio* and *O. granulifera*, we analysed the effects of frog phenotype on the escape distance separately with one-way ANOVAs. Distance data were log transformed to account for unequal variances between species and phenotypes.

RESULTS

Comparison of population structure and distribution of colour morphs between species

In *O. pumilio*, the northern lineage extends from northern Costa Rica (and possibly Nicaragua) to central Costa Rica on the Caribbean coast (Fig. 1). All frogs in this lineage are dorsally red and have blue legs. The southern lineage ranges from central Costa Rica to western Panama. Populations in Costa Rica are red with red or black legs, while a variety of colour morphs exist in Panama, especially in the area of Bocas del Toro (Fig. 1). In *O. granulifera*, a similar pattern is evident in coloration pattern and diversity but in the opposite geographic direction. The southern lineage extends from southwestern Costa Rica to south-central Costa Rica (Río Térraba) on the Pacific coast. Here frogs are dorsally red and the hindmost back and the legs are blue, similar to northern *O. pumilio*. In the northern *Cytb* lineage, which extends approximately 100 km to the north along the Pacific coast, the frogs are red with red legs or blue legs with red spots, and frogs in the northernmost population are olive-green with azure-green legs. A transition area (where frogs are neither red nor green) with intermediate colour morphs is located between the green and red phenotype. This area is 20–25 km wide, with phenotypes changing from red to orange, yellow, orange-green to green coloration (Brusa *et al.*, 2013; Willink *et al.*, 2013).

The genetic population structure inferred from microsatellites also revealed similarities between the two species: the largest amount of genetic variation across the species is captured by two genetic clusters (Fig. 1). However, the *Cytb* lineages and microsatellite based genetic cluster do not coincide exactly. For *O. pumilio*, the northern genetic cluster is shifted southwards to the border between Costa Rica and Panama and contains the red-blue and red morphs, while the southern cluster contains all the colour-diverse Panamanian populations. In *O. granulifera*, the southern cluster extends northwards past the border (Río Térraba) between the *Cytb* lineages and contains red frogs with blue lower back and legs and frogs with a completely red dorsum. The northern cluster contains red, intermediate, and green populations (Fig. 1).

Thus, in both species we find a geographic overlap between mitochondrial lineages and genetic clusters in the central distribution area: in *O. pumilio* the southern lineage overlaps widely with the northern cluster in South Costa Rica, while in *O. granulifera* the northern lineage overlaps with the southern cluster in a small area north of the Río Térraba (Fig. 1). Because of this particular distribution of genetic variation and phenotypes, we compared morphometric and bioacoustic signals among the three phenotypic groups of populations in each species.

Variation in size and weight and advertisement calls

The two species differed slightly in size and weight (ANOVA: size, $F = 5.52$, $P = 0.0197$; weight, $F = 6.48$, $P = 0.0116$). *Oophaga pumilio* was slightly smaller with a mean snout-to-vent length (SVL) and mean weight of 19.9 ± 1.9 mm and 0.67 ± 0.20 g respectively ($n = 152$). *Oophaga granulifera* was larger with a mean snout-to-vent length (SVL) and mean weight of 20.5 ± 0.69 mm and 0.73 ± 0.07 g respectively ($n = 68$) (Fig. 2). Intraspecific variation in size and weight was more pronounced in *O. pumilio* than in *O. granulifera* (Fig. 2). In *O. pumilio* most of the variation in the morphology was observed between the two microsatellite clusters and between the three phenotypic groups, while in *O. granulifera* most variation resided between the mitochondrial lineages (Table B, evolutionary-ecology.com/data/2839Appendix.pdf). In both species, the central red group was larger and heavier than the other two groups.

Four of five advertisement call traits differed between the two species (Wilks test: $F = 414.5$, $P < 0.01$). Most of the variation resided in call rate ($F = 962$, $P < 0.0001$) and call duration ($F = 1031$, $P < 0.0001$). Duty cycle ($F = 60.4$, $P < 0.0001$) and pulse rate ($F = 110.6$ $P < 0.0001$) also differed between species while the dominant frequency was similar

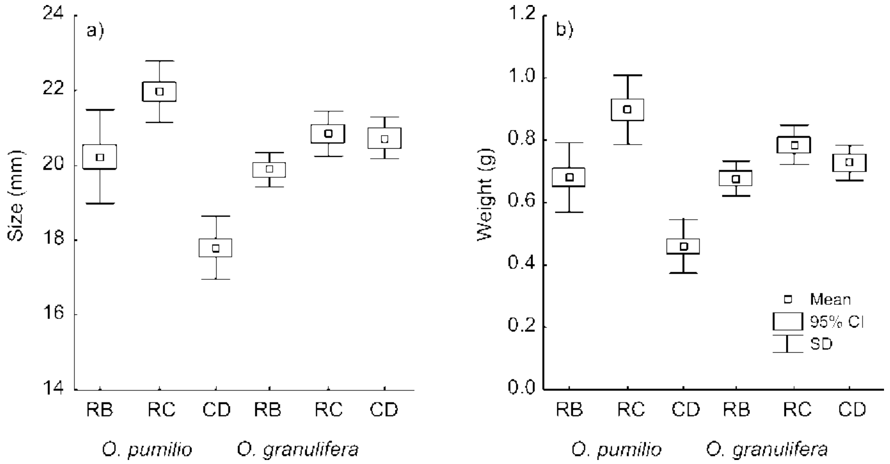

Fig. 2. Variation in size (a) and weight (b) across species and phenotypic groups: RB = red-blue group, RC = red central group, CD = colour diverse group.

($F = 0.12$, $P = 0.73$; Fig. 3). The calls of *O. pumilio* were shorter but were emitted at a much faster rate and with higher pulse rates than those of *O. granulifera* (Fig. 3a, b).

As with morphology, the species differed in their patterns of acoustic divergence. In *O. pumilio*, most call parameters showed particularly high differentiation between genetic clusters and phenotypic groups (Table B, 2839Appendix.pdf). Call rate and call duration varied more than the other call parameters at all levels (lineages, clusters, and groups), and showed particularly high differentiation between the two red groups and the colour diverse group (Fig. 3a, b). Only duty cycle diverged more between lineages than clusters or groups (Fig. 3c) (Table B, 2839Appendix.pdf).

In contrast, in *O. granulifera* morphological and call parameters varied the most between mitochondrial lineages. As in *O. pumilio* call rate and call duration varied more than pulse rate, duty cycle, and dominant frequency (Table B, 2839Appendix.pdf). Call duration decreased while call rate increased from south to north (Fig. 3a, b). Interestingly, pulse rate differed within the northern lineage between the red central group and colour diverse group (Fig. 3). Thus, in *O. pumilio* divergence in morphology and calls was most pronounced between microsatellite clusters, while in *O. granulifera* most of the variation was found between the *Cytb* lineages.

Correlation between variation in calls, genetic divergence, and geographic distance

In both species, the genetic distance (D_{est}) was correlated with geographic distance but the correlation was higher in *O. granulifera* (Mantel test; *O. pumilio*: $r = 0.23$, $P = 0.04$; *O. granulifera*: $r = 0.74$, $P = 0.003$). In *O. pumilio*, bioacoustic distance was correlated with geographic distance ($r = 0.59$, $P < 0.0001$), and to a lesser degree with genetic distance ($r = 0.32$, $P = 0.014$). In *O. granulifera*, the bioacoustic distance was similarly related to geographic distance ($r = 0.48$, $P = 0.013$) and to genetic distance ($r = 0.47$, $P = 0.013$).

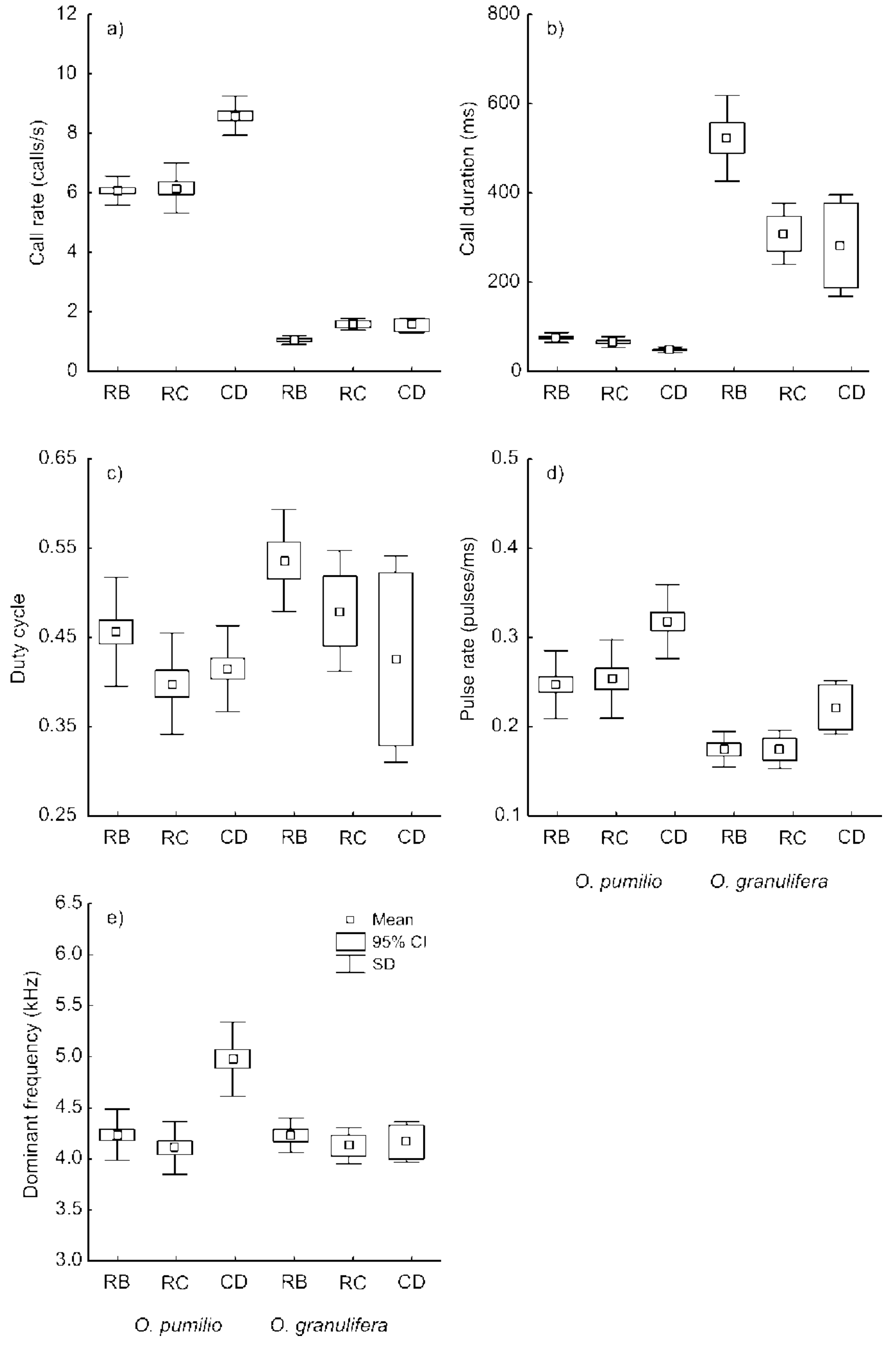

Fig. 3. Variation in parameters of the advertisement call across species and phenotypic groups. Phenotypic groups are shown on the horizontal axis: RB = red-blue group, RC = red central group, CD = colour diverse group.

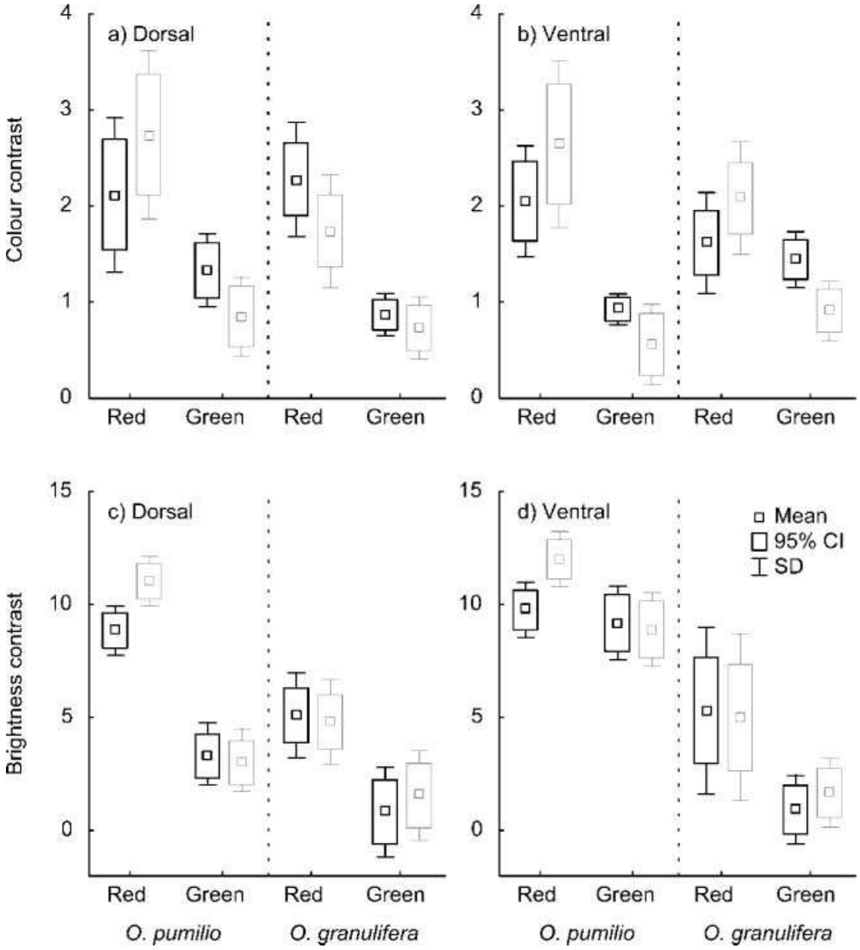

Fig. 4. Dorsal (a) and ventral (b) colour contrast and dorsal (c) and ventral (d) brightness contrast of *O. pumilio* and *O. granulifera* for bird vision. Black bars = mean contrast across different backgrounds; grey bars = mean contrast at the calling place of the males.

Frog conspicuousness: visual signals, anti-predator and sexual behaviour

In general, *O. pumilio* has brighter colours, particularly ventrally, and higher dorsal colour contrast than *O. granulifera*. However, for the eyes of potential predators, visual conspicuousness of frogs was more heavily influenced by the frog phenotype than by the species (Fig. 4; Table C, 2839Appendix.pdf). The red phenotypes are more conspicuous than the green phenotypes in terms of colour and brightness, and it matters whether they are seen dorsally or ventrally (Fig. 4). However, the strength of the phenotypic effect depended on the frog species and background substrate (Fig. A, 2839Appendix.pdf). Red males of *O. pumilio* used calling places that increased their conspicuousness in all types of visual contrasts, while green males of this species selected calling places that reduced their colour contrast compared with the average natural background (Fig. 4a, b). Red males of *O. granulifera* increased their ventral colour contrast by means of calling site selection

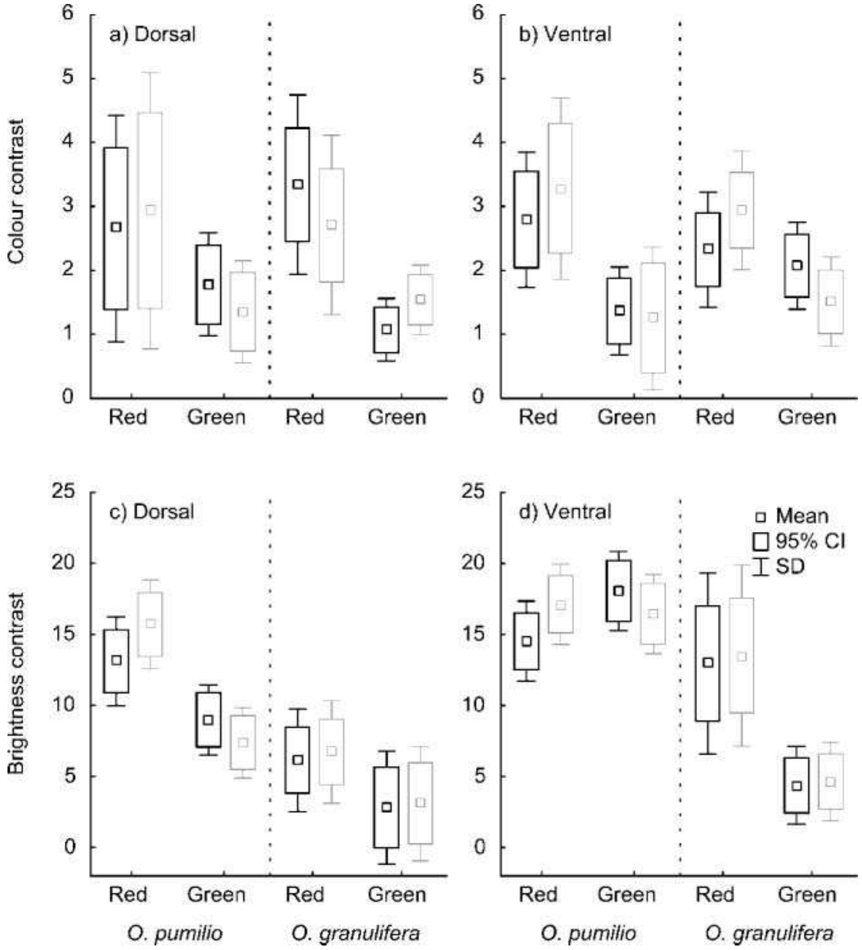

Fig. 5. Dorsal (a) and ventral (b) colour contrast and dorsal (c) and ventral (d) brightness contrast of *O. pumilio* and *O. granulifera* for frog (*O. pumilio*) vision. Black bars = mean contrast across different backgrounds; grey bars = mean contrast at the calling place of the males.

(Fig. 4b), but in this species the other types of visual conspicuousness were not enhanced at the preferred calling positions of males compared with other backgrounds available in the habitat.

For frog viewers, conspicuousness also differed between phenotypes in both species (Fig. 5). However, while the red phenotype remained more conspicuous in most visual contrasts, red and green morphs had similar ventral brightness contrasts in *O. pumilio*, and similar dorsal brightness contrasts in *O. granulifera* at calling positions (Fig. 5). The selection of calling positions only slightly increased the conspicuousness of red males of *O. pumilio* compared with other frogs (Fig. 5). For the vision of frogs, the magnitude of phenotypic effects also differed between species and depended on the background substrate (Fig. B, 2839Appendix.pdf).

Most behavioural traits of focal males differed between the red and green morphs in both species. In *O. pumilio* and *O. granulifera*, green males spent more time sitting inactive than

Table 1. Results of GLMs of differences in behavioural rates among species and phenotypes

		Foraging		Movement		Calling		Inactivity		Females	
	d.f.	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P
Species	1	1.88	0.170	7.96	**0.005**	14.85	**<0.001**	0.28	0.597	4.40	**0.036**
Phenotype	1	29.78	**<0.001**	3.10	*0.078*	0.61	0.434	11.30	**<0.001**	2.94	*0.086*
Species × Phenotype	1	0.01	0.936	1.12	0.289	4.97	**0.026**	1.12	0.290	0.92	0.337

did red males (Table 1, Fig. 6a). Also, in both species red males exhibited higher foraging rates and a statistical trend of higher movement rates (Table 1, Fig. 6b, c). However, the two *Oophaga* species differed in how males used their active time. Males of *O. pumilio*, regardless of the phenotype, spent more time moving and in association with females (Table 1, Fig. 6c, d), while males of *O. granulifera* spent more time engaged in aggressive behaviour towards neighbouring males (Fig. 6e). The time of aggressive calling was independent of the frog phenotype, both in *O. pumilio* ($\chi^2 = 1.81$, d.f. = 1, $P = 0.178$) and in *O. granulifera* ($\chi^2 = 0.33$, d.f. = 1, $P = 0.563$). In contrast, the effects of frog phenotype on advertisement calling activity differed between species (Table 1). In *O. granulifera*, red males spent more time engaged in advertisement calling, while both morphs of *O. pumilio* displayed similarly low calling activity (Fig. 6f).

The distance to the nearest neighbour did not differ between frog phenotypes for either species ($F_{1,42} = 0.59$, $P = 0.448$), but males of *O. granulifera* were more spaced ($F_{1,42} = 21.56$, $P < 0.001$, Fig. 6g). Propensity to escape was evaluated with different predator models, and in both species red males initiated an earlier escape response (*O. pumilio*: $F_{1,38} = 8.08$, $P = 0.007$; *O. granulifera*: $F_{1,23} = 4.82$, $P = 0.039$, Fig. 6h).

DISCUSSION

Phenotypic divergence in dendrobatid frogs

In both species of poison frogs, *O. pumilio* and *O. granulifera*, we observed substantial variation in morphology, especially in colour patterns, advertisement calls, sexual as well as anti-predator behaviour. It is possible that local varying selection pressures together with genetic drift play a role in diversifying coloration (e.g. Mochida, 2011; Noonan and Comeault, 2009). Divergence in sexual preferences for acoustic signals between evolving lineages or species, variation in body size and geographic clines are involved in differentiation of calls and songs and might entail behaviourally induced pre-zygotic isolation (Gerhardt and Huber, 2002; Noh and Henry, 2010; Wilkins *et al.*, 2013). Our results revealed striking similarities between the poison frogs *O. pumilio* and *O. granulifera* in geographic patterns of genetic and phenotypic divergence. We discuss our results in the context of natural and sexual selection and the importance of our findings for the speciation process.

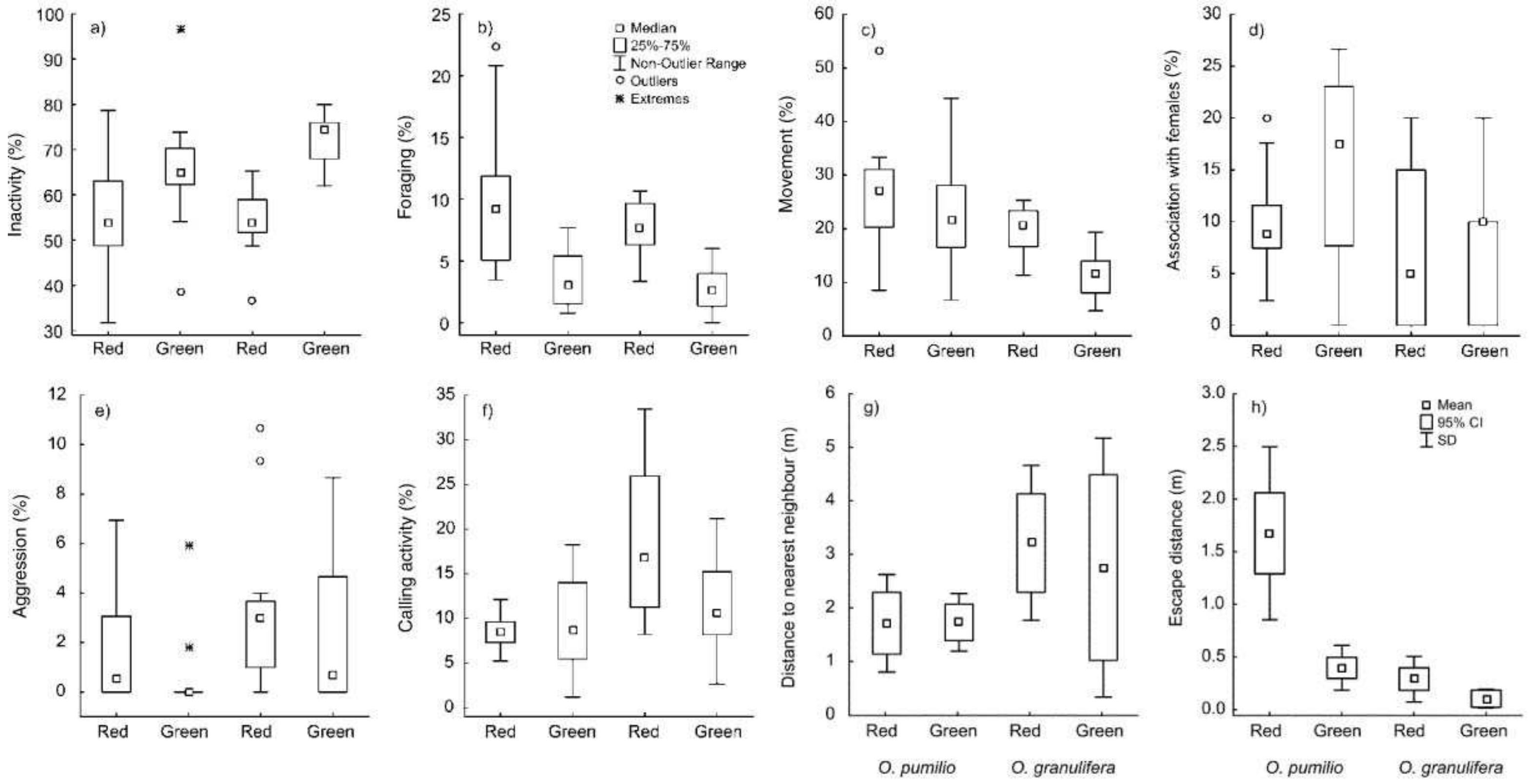

Fig. 6. Differences in eight behavioural elements of anti-predator and reproductive strategies that vary between species and colour morphs. For panels (a)–(f), data are percentage of observation time.

Parallel geographic patterns of genetic divergence in two poison frogs

Major genetic differentiation into two lineages in both species reflects separation into (1) a red-blue phenotype and (2) red and other phenotypes including polymorphic and cryptic coloured populations. The genetic lineages are old; *Cytb* divergence between the two lineages is 6% in *O. pumilio* and 4% in *O. granulifera* (Brusa *et al.*, 2013), thus divergence took place at approximately the same time in both species. Earlier studies indicated a pairwise rate of change of approximately 1.3–1.4% sequence divergence per million years in amphibian mitochondrial genes (for a review, see Pröhl *et al.*, 2010). This evolutionary rate indicates that the lineages of *Oophaga* diverged about 3 to 5 million years ago, i.e. roughly at the beginning of the Quaternary.

The role of rivers in the geographic and genetic divergence in Neotropical vertebrates is well documented (e.g. Maldonado-Coelho *et al.*, 2013). The formation of larger rivers seems to have contributed to the division into genetic lineages in our frog species: the Río Térraba (Fig. 1) geographically separates the red-blue *O. granulifera* from the northern populations, while the Río Reventazón separates the red-blue *O. pumilio* in the north from the red and colour diverse populations (Hagemann and Pröhl, 2007; Brusa *et al.*, 2013). However, the microsatellite data clearly show that there is recent gene flow across the boundaries between the *Cytb* lineages and therefore genetic clusters do not coincide exactly with genetic lineages. Gene flow across lineage boundaries and populations is more pronounced in *O. pumilio* than in *O. granulifera* (Fig. 1): in *O. pumilio* gene flow from the north across the Río Reventazón extends to the Panamanian border through the red populations, while it only extends through one population in the north of the Río Térraba in *O. granulifera* (Fig. 1).

The pair-wise genetic distance between populations was correlated with geographic distance in both species, although it was stronger in *O. granulifera*. High gene flow among populations and genetic clusters of *O. pumilio* might blur genetic structuring compared with *O. granulifera*. Local genetic drift is probably weaker in *O. pumilio* because populations are large, at least in the mainland of Costa Rica, and well connected through habitats strongly affected anthropogenically, while *O. granulifera* is restricted to natural forests along the Pacific slope (B. Willink and H. Pröhl, personal observations) and gene flow might be more limited among populations. On the other hand, geographic isolation by the sea in the Bocas del Torro region should also lower the isolation-by-distance effect in *O. pumilio* (see also Wang and Summers, 2010). Thus gene flow across populations of both species seems to be explained by a model of isolation by distance (Wright, 1943; Slatkin, 1993), maintained by higher or lower gene flow as found in other frog species (Pröhl *et al.*, 2006; Amézquita *et al.*, 2009; Ohmer *et al.*, 2009).

Divergence in acoustic signals related to genetic structure

Our results show that acoustic signals vary with genetic and geographic distance and differ among phenotypic and genetic groups, but in different ways in *O. pumilio* and *O. granulifera*. In both species, variation in advertisement calls is moderately correlated with geographic distance and to a lesser degree with genetic distance. Thus acoustic divergence can in part be explained by the genetic population structure and gene flow among populations. The remaining bioacoustic variation that cannot be explained by geographic/genetic divergence is probably the result of local selection pressures (Ohmer *et al.*, 2009; Kaefer *et al.*, 2012). One possibility is acoustic adaptation to the local habitat for enhancing signal transmission but there is little evidence for this effect in frogs (Kime *et al.*, 2000; Gerhardt and Huber, 2002). There is more evidence that sexual selection is involved in divergence of acoustic signals. Songs and calls are often species or lineage specific, detectable at longer distances than visual signals, and therefore suitable for discrimination. In particular, acoustic differences between genetic lineages together with divergence in sexual (female) preferences indicate a key role of acoustic sexual signals in the diversification and speciation process. In studies including vertebrates and invertebrates (Boul *et al.*, 2007; Noh and Henry, 2009; Grace and Shaw, 2012; for a review, see Wilkins *et al.*, 2013), acoustic signals are differentiated between closely related species or evolving lineages and the same applies to *Oophaga*.

Advertisement calls in both *Oophaga* species diverge at each of the different levels of genetic and phenotypic groupings (Table B, 2839Appendix.pdf). In *O. granulifera*, most variation in call parameters is associated with divergence in mitochondrial lineages, while in *O. pumilio* most call parameters differ between genetic clusters and call duration only is associated with old genetic divergence (lineages). Call rate and call duration differed more than other call parameters between species, lineages, clusters, and groups in both species and might be most important for sexual selection linked to pre-zygotic isolation. Call duration and call rate are associated with old genetic divergence (lineages) in *O. granulifera*; the same is true for differences in call duration in *O. pumilio*. In contrast, call rate and pulse rate were associated with recent divergence (clusters and groups) in *O. pumilio.* Our data suggest that pre-zygotic isolation based on differences in advertisement call might be strongest between the red central and colour diverse groups in *O. pumilio* (different clusters) and between the red-blue and red central group in *O. granulifera* (different lineages). Interestingly, pulse rate differs between the red central and colour diverse group in both species (same lineage). This observation points to the possibility that pulse rate contributes to the early stages of reproductive isolation potentially in combination with divergence in coloration. Multimodal signal divergence has rarely been investigated in the context of species formation but some studies indicate its relevance for pre-zygotic isolation (Uy *et al.*, 2008). Colour diverse *Oophaga* species offer the possibility to test for pre-zygotic isolation based on acoustic and visual signals and their interplay in different geographic settings (between lineages, clusters or groups).

Phenotypic divergence in anti-predator strategies and (lack of) correspondence with genetic divergence

Colour diversity in aposematic animals is the result of ecological adaptation to different habitats or predator pressure (Jiggins *et al.*, 2001; Arias *et al.*, 2008; Ng *et al.*, 2012). Unlike acoustic signals, there is no correspondence between the divergence in coloration and genetic structure, and different phenotypes can occur as well within or between species or evolving lineages (e.g. Jiggins *et al.*, 2001; Amézquita *et al.*, 2009; Twomey *et al.*, 2013). In *Oophaga* we find both scenarios. In both species, the aposematic red-blue lineage is ancestral (Wang and Shaffer, 2008; Brusa *et al.*, 2013) to the red and colour diverse lineage. In the derived lineage of *O. pumilio*, the formation of islands through rising sea-levels in the Bocas del Toro Archipelago several thousands years ago (Anderson and Hanley, 2002) probably contributed to the fast evolution of a large number of different colour morphs, through increased drift in small populations on islands and ecological diversification (Gehara *et al.*, 2013). A reduction in population size and genetic drift during population expansion in the diverged lineage could also explain the loss of the bright red coloration in both species, since conspicuousness becomes more costly in smaller populations (Speed and Ruxton, 2005). We suppose that genetic drift operated together with selection on frog conspicuousness and toxicity for the diversification of coloration patterns in these frogs (Pröhl and Ostrowski, 2011; Maan and Cummings, 2012; B. Willink *et al.*, submitted). Divergence in colour pattern as a consequence of ecological adaptation can facilitate reproductive isolation and speciation by assortative mating (Jiggins *et al.*, 2001; Schluter, 2009). Female preferences for the local colour morph seem to have developed in some but not all populations of colour diverse *O. pumilio* (Summers *et al.*, 1999; Maan and Cummings, 2008; Richards-Zawacki *et al.*, 2012). Whether and where these preferences contribute to pre-zygotic isolation between colour phenotypes is a matter of further research in dendrobatid frogs.

Conspicuousness and behaviour of species and colour divergent populations

For potential predators as well as for conspecifics, the difference in colour and brightness contrasts was more pronounced between the two phenotypes (red and green) than between the species. For both viewers and for most contrasts, the red morphs were more conspicuous than the green morphs, while visual conspicuousness was associated with behavioural conspicuousness. The divergence in conspicuous and cryptic strategies in both species is probably maintained by predator selection. Several authors have argued that birds are important predators shaping the evolution of colour divergence in poison frogs. This is because birds possess colour vision and have been observed to attack clay model frogs in predation experiments in both species (Hegna *et al.*, 2012; B. Willink *et al.*, submitted) and occasionally have been observed to attack live dendrobatid frogs (Master, 1998; Alvarado *et al.*, 2013). However, which colour signals are relevant to avian predators and the relative importance of birds versus other predator types may differ between species. In green *O. pumilio* (Isla Colón), overall predation of clay models was low, and birds preferred to attack the local cryptic morph over the red mainland morph (Hegna *et al.*, 2012). This has been attributed to honest signalling based on the correlation between conspicuousness (brightness contrasts) and toxicity in *O. pumilio* in the Bocas region in Panama (Hegna *et al.*, 2012; Maan and Cummings, 2012). In contrast, in *O. granulifera* birds avoided the local poison frog morphs in all studied populations independently of the colour morph, and lizard predation played an important role. Lizards avoided the green and intermediate phenotypes and preferentially attacked the red morphs (B. Willink *et al.*, submitted). In *O. granulifera*, the more cryptic green morph is more toxic than the red morph (Wang, 2011), thus there is an inverse association between toxicity and conspicuous coloration. The local cryptic morph is attacked by birds when it is only slightly toxic (*O. pumilio*), but attacked less by birds and lizards when it is highly toxic (*O. granulifera*). Also, in two South American poison frogs of the genus *Ranitomeya*, the local colour morph was better protected than a non-local aposematic morph against bird predators (Noonan and Comeault, 2009; Chouteau and Angers, 2012). Thus birds seem to be important as selective agents but the importance of other predator types needs to be explored in future research.

In terms of brightness contrast, *O. pumilio* was more conspicuous than *O. granulifera* in both phenotypes and for both viewers. Brightness contrast plays a role in sexual selection as well as predator selection (Osorio *et al.*, 1999). Red *O. pumilio* males from Solarte appear brighter to conspecifics and predators and are extremely toxic compared with other colour morphs (Maan and Cummings, 2012). In this population, females preferred brighter males (Maan and Cummings, 2009). Thus discouraging predators as well as attraction of females could have led to the extreme bright red coloration in Solarte males. Finally, the deviation in mating behaviour (Rudh *et al.*, 2011; this study) could contribute to assortative mating and reduce gene flow in contact zones between colour phenotypes.

CONCLUSION

Both *O. pumilio* and *O. granulifera* show similarities in their genetic population structure: they diverged into two mitochondrial lineages several million years ago. Secondary contact across genetic boundaries re-established gene flow between lineages and more recent events divided both species into two genetic clusters. The genetic structure affects morphology as well as advertisement calls. Variation in advertisement call traits seems to be the result of divergence into lineages, genetic clusters, and limited recent gene flow (isolation by distance) among populations. In contrast, most diversity in colour patterns is restricted to one lineage or one genetic cluster in both species, thus colour pattern diversity is affected to a lesser extent by genetic structure. The northern lineage in *O. pumilio* and the southern lineage in *O. granulifera* harbour red frogs with blue to green legs with longer calls emitted at a lower call rate and are ancestral to the derived lineages which contain different color morphs, including aposematic red and cryptic populations, which are linked by a transition zone in *O. granulifera* but mostly isolated on islands in *O. pumilio*. Divergence into a variety of colour morphs likely is related to a combination of genetic drift during the expansion of the diverged lineages into new habitats, a reduction in population size, and ecological adaptation to diverse local predator communities. Behavioural observation and assessment of coloration demonstrated that the more cryptic phenotype in both species also behaved in a more cryptic way, providing evidence that aposematic and cryptic strategies include coloration as well as behavioural aspects. Thus, the evolution of different anti-predator strategies represents similarities in the phenotypic divergence patterns in both species.

In summary, we found two levels of divergence in *Oophaga* species: (1) the call × genetic structure divergence, and (2) the coloration × behaviour divergence in different geographic scenarios. Acoustic and visual signals might be independently involved in ecological adaptation, pre-zygotic isolation, and speciation in poison frogs; whether and how they interact (e.g. at lineage boundaries) is a fascinating topic for future research.

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