A captive breeding experiment reveals no evidence of reproductive isolation among lineages of a polytypic poison frog

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Reproductive isolation is central to the generation of biodiversity, yet a clear understanding of the contributions of alternative reproductive barriers to this process remains elusive. Studies of young lineages that have diverged in ecologically important traits can offer insights into the chronology and relative importance of various isolating mechanisms during speciation. In poison frogs (Dendrobatidae), within-species lineages often differ dramatically in coloration, a trait subject to natural and sexual selection. Coloration in the strawberry poison frog (*Oophaga pumilio*) is particularly diverse and previous work suggests the potential for reproductive isolation. We used a captive breeding experiment to assess the extent of reproductive isolation among three allopatric, genetically distinct *O. pumilio* lineages that differ in coloration. We compared reproduction of within- and between-lineage pairs, predicting that if lineages are isolated, within-lineage pairs would be most successful. We also examined the fertility and productivity of F1 backcrosses of admixed offspring. We found no evidence suggesting behavioural pre-zygotic or post-zygotic reproductive isolation, indicating that isolation would not be maintained by intrinsic mechanisms in the event of secondary contact. Future work should address costs of between-lineage matings exerted by extrinsic natural and/or sexual selection against admixed offspring. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **00**, 000–000.

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INTRODUCTION

Reproductive isolation is essential to both the generation and maintenance of biodiversity. Gene flow can be limited by anything that influences the probability of individuals from different gene pools encountering one another, accepting each other as mates, or producing offspring that achieve fitness similar to individuals with genes from only one lineage (Coyne & Orr, 2004). Determining when these isolating mechanisms arise and how important each is early in speciation is challenging because any or all may continue to evolve after isolation is complete (Servedio & Kirkpatrick, 1997; Servedio, 2000; Rundle & Nosil, 2005; Sobel *et al.*, 2010). Therefore, studies assessing the extent and mechanisms of reproductive isolation among young lineages can be especially informative (e.g. McMillan, Jiggins & Mallet, 1997; Hatfield & Schluter, 1999; Jiggins *et al.*, 2001; Mendelson, 2003; Nosil, 2004; Mendelson, Imhoff & Venditti, 2007). Asking fundamental questions, such as whether some barriers are consistently more important or evolve earlier than others, requires studies of isolating barriers across diverse taxa. Despite their importance, comprehensive comparisons of potential isolating mechanisms are available in relatively few systems (e.g. Coyne & Orr, 1989, 1997; Mendelson, 2003; Moyle, Olson & Tiffin, 2004; Qvarnström, Rice & Ellegren, 2010), and are especially scarce for organisms with lengthy reproductive cycles that make them difficult to study. Only via the accumulation and synthesis of such examples is it possible to test the hypothesis that speciation is governed by general principles (e.g. Coyne & Orr, 1989, 1997; Sasa, Chippendale & Johnson, 1998; Russell, 2003; Moyle *et al.*, 2004; Price, 2007).

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The evolution of reproductive isolation can be driven by both adaptive and non-adaptive divergence among lineages (Coyne & Orr, 2004). The extent to which responses to selection will be accompanied by the evolution of isolating mechanisms is likely to depend on factors including the geographic distribution of populations and the mechanism(s) of selection driving divergence (Panhuis et al., 2001; Coyne & Orr, 2004; Rundle & Nosil, 2005). Taxa characterized by substantial within- or among-population variation in ecologically important traits provide tractable systems in which to examine the roles of adaptive and neutral processes in speciation (reviewed by Schluter, 2000; Coyne & Orr, 2004). For example, coloration varies both within and among populations in diverse taxonomic groups (Hoffman & Blouin, 2000; Gray & McKinnon, 2007). Such divergence may be especially likely to lead towards speciation because coloration is often subject to multiple mechanisms of both natural and sexual selection, offering numerous potential pathways to reproductive isolation (Endler, 1988; Boughman, 2002; Nosil, 2004; Gray & McKinnon, 2007).

Striking examples of colour and pattern divergence appear repeatedly in the poison frogs (Dendrobatidae: Hoffman & Blouin, 2000; Symula, Schulte & Summers, 2001; Roberts et al., 2007; Willink et al., 2014). In this group, colour is thought to play an aposematic function (Saporito et al., 2007; Noonan & Comeault, 2009) and to mediate female mate choice (e.g. Maan & Cummings, 2009). One remarkable example of colour diversification in this group comes from the strawberry poison frog (Oophaga pumilio) in the Bocas del Toro region of Panama. In and around the region's recently-formed archipelago (1–9 kya: Gehara, Summers & Brown, 2013), O. pumilio populations have diverged from a largely conserved ancestral phenotype (red body with blue legs) and now display colours spanning the visible spectrum (Hagemann & Pröhl, 2007; Wang & Shaffer, 2008; Hauswaldt et al., 2010; Fig. 1). Comparisons of genetic and phenotypic divergence in differently coloured Bocas del Toro O. pumilio suggest that drift alone cannot explain current diversity in coloration (Brown et al., 2010; Wang & Summers, 2010). The question of whether (and which) barriers to reproduction are evolving among these lineages remains unresolved.

The best evidence that O. pumilio morphs might be reproductively isolated comes from a polymorphic population, where the reconstruction of wild pedigrees indicates at least some isolation among colour morphs (Richards-Zawacki, Wang & Summers, 2012). In this same population, mate preference assays suggest reproductive character displacement, a pattern expected when females that preferentially mate with males of their own phenotype produce more or more fit offspring than females who mate randomly (Richards-Zawacki & Cummings, 2011). Studies like these, however, reveal little about the mechanisms underlying isolation, and reproductive barriers currently present in polymorphic populations would not necessarily be the same ones operating during initial contact between lineages (Richards-Zawacki & Cummings, 2011). So far, studies of reproductive isolation among O. pumilio morphs, allopatric or sympatric, have focused almost exclusively on a single pre-zygotic behavioural barrier to gene flow: divergent female preferences (a common early step in speciation: Panhuis et al., 2001; Coyne & Orr, 2004; Price, 2007). Tests of association preference in O. pumilio have revealed assortative female preferences in most, but not all, cases (Summers et al., 1999; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008; Richards-Zawacki & Cummings, 2011). Whether any other mechanisms of isolation are present remains untested. Post-zygotic barriers to gene flow often evolve more slowly than pre-zygotic isolating mechanisms, but can arise rapidly, especially in species where sexual selection is strong (Orr & Presgraves, 2000; Christianson, Swallow & Wilkinson, 2005; Stelkens, Young & Seehausen, 2010). Despite the recent formation of the Bocas del Toro archipelago, genetic distances between lineages (Wang & Summers, 2010) are at the level at which inviability and sterility (intrinsic post-zygotic isolation) often begin to evolve in frogs (see Supporting information, Fig. S1; Sasa et al., 1998).

As part of an effort to explore the full suite of potential isolating mechanisms in this system, we used a captive breeding experiment to assess the extent of reproductive isolation among one mainland and two island populations of O. pumilio from the Bocas del Toro region. To maximize the potential for detection of reproductive barriers, we studied lineages that differ dramatically in coloration, show significant genetic divergence (Hagemann & Pröhl, 2007; Rudh, Rogell & Hoglund, 2007; Wang & Shaffer, 2008; Hauswaldt et al., 2010), and show no evidence of ongoing gene flow (Wang & Summers, 2010). Captive breeding studies minimize confounding extrinsic effects when examining the tempo and mode by which reproductive isolation evolves, and are necessary when studying allopatric populations that can not be translocated. A small number of experimental crosses between O. pumilio lineages suggest a lack of complete isolation (Summers, Cronin & Kennedy, 2004). However, logistical constraints in that study (Summers et al., 2004) left open the question of whether matings between lineages are less productive than those within lineages and/or carry the cost of offspring that are sterile or suffer reduced
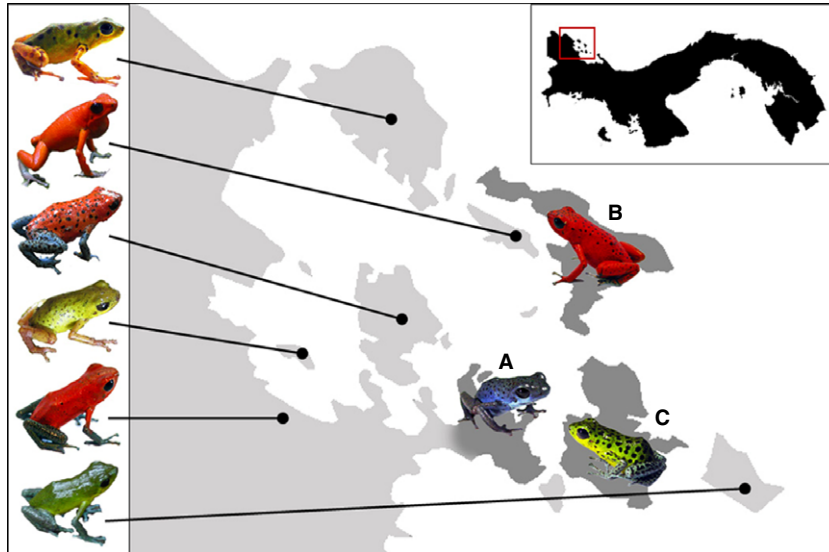
Figure 1. Map of the Bocas del Toro Archipelago of Panama showing the diversity of colour and pattern among *Oophaga pumilio* populations. Darker grey shading indicates the distributions of lineages in this study: (A) Aguacate peninsula, (B) Isla Bastimentos, and (C) Isla Popa.

fertility/fecundity, as is common early in speciation (Sasa *et al.*, 1998; Orr & Presgraves, 2000; Jiggins *et al.*, 2001). We began by comparing tadpole production of within- and between-morph pairs when held in captivity in Panama. If these lineages are reproductively isolated, either by behavioural mechanisms (as suggested by laboratory preference studies) or by intrinsic post-zygotic mechanisms (as suggested by genetic distances), within-morph pairs should produce more offspring than between-morph pairs. In a second phase of this study, we moved the colony to Tulane University (New Orleans, Louisiana) and used F1 backcrosses to ask whether admixed offspring were sterile, suffered reduced fertility/fecundity, or if their offspring suffered more developmental problems than offspring of other types of matings (by comparing ratios of juveniles/tadpoles). If admixed offspring are less successful, this previously untested mechanism could contribute to reproductive isolation, either directly or by driving the evolution of assortative mate preferences.

MATERIAL AND METHODS

STUDY SPECIES AND STUDY POPULATIONS

Oophaga pumilio is a small terrestrial frog that occurs in lowland forests and disturbed habitats (e.g. banana and cacao plantations) along the Caribbean side of Central America, from Nicaragua to Panama. Within populations, coloration of both sexes is similar. In the wild, males defend territories from which they court females, and behavioural observations have revealed no evidence of coercive mating (i.e. females can terminate courtship: Pröhl & Hödl, 1999). However, amplexus-like behaviour, otherwise absent in this species, has been reported in captivity at least once (Weygoldt, 1980). Following successful courtship, females lay a clutch of ~5 eggs in the leaf litter, where it is tended by males (Weygoldt, 1980; Pröhl & Hödl, 1999). Once eggs hatch, females transport tadpoles to water-filled leaf axils (but see Killius & Dugas, 2014) and regularly revisit these sites to provision tadpoles with unfertilized eggs (Weygoldt, 1980). A complete reproductive cycle takes at least 30 days (Pröhl & Hödl, 1999).

We studied reproductive isolation among three allopatric, differently coloured, lineages from the Bocas del Toro region of Panama (Fig. 1): red (Tranquilo Bay, Isla Bastimentos: 9°15′8.03″N, 82°8′43.30″W), green (Punta Laurel, Isla Popa: 9°8′25.98″N, 82°7′39.11″W) and blue (Shark Hole, Aguacate peninsula: 9°12′47.13″N, 82°12′49.29″W) morphs. Both mitochondrial and nuclear markers have revealed genetic structure (Wang & Shaffer, 2008; Wang & Summers, 2010), no gene flow was detectable among these lineages (Wang & Summers, 2010), and none is each other's closest relative (Hagemann & Pröhl, 2007; Rudh *et al.*, 2007; Wang & Shaffer, 2008; Brown *et al.*, 2010; Hauswaldt *et al.*, 2010). Females from all three populations attend to male coloration during association preference tests, and females from both lineages tested in earlier work (Isla Popa and Isla Bastimentos) prefer males with local coloration (Summers *et al.*, 1999; Maan & Cummings, 2008, 2009).

ANIMAL HOUSING AND MONITORING REPRODUCTION

In the first phase of this study, we compared the reproductive success of within- and between-lineage pairs. All animals were wild caught in February 2008 and housed at the Bocas del Toro Research Station of the Smithsonian Tropical Research Institute. We housed frogs in plastic enclosures (37 × 22 × 25 cm), each containing one male and one female. We maintained tanks in ambient light, temperature, and humidity conditions, and misted them daily; frogs consumed wild invertebrates (mostly *Drosophila* spp.) attracted to fruit placed in enclosures, and we supplemented this diet with vitamin-dusted termites. We lined each tank with leaf litter, and provided bromeliads and four water-filled PVC tubes as tadpole deposition sites. We quantified the total number of tadpoles produced by pairs with twice-weekly censuses, operationally defining 'tadpoles' as larvae present in rearing sites. While we presumably detected all tadpoles deposited in PVC tubes, this was apparently not the case in bromeliads as we occasionally found newly metamorphosed juveniles without previously detecting a tadpole. In such instances, we added a single tadpole to the pair's total; while this may have resulted in underestimation of tadpole production, it should not have done so in a systematic way. We moved any juvenile frogs (tadpoles that completed metamorphosis) to smaller rearing tanks, but did not analyse Panama juvenile production because some tadpoles were cross-fostered as part of another study, and this treatment led to reduced success of those tadpoles (unpubl. data). In Panama, all individuals participated in only one pairing.

In the second phase of the study, we were primarily interested in addressing the potential sterility or reduced fertility/fecundity of admixed offspring. In August 2009, we moved the breeding colony to Tulane University; while we collected some new frogs, roughly half of the individuals (37/67 females, 34/69 males) were included in both the Panama and Tulane phases of the experiment, although only one pair remained intact in both phases. We continued to house pairs in plastic enclosures, and maintained these in an environmental chamber held at 22–27 °C and ≥40% relative humidity under a 12L/12D light cycle (to mimic conditions in Bocas del Toro). We misted each tank twice daily, and fed frogs springtails and vitamin-dusted fruit flies (*D. melanogaster*) three times weekly. Immediately following the move to Tulane, very few tadpoles successfully completed metamorphosis. This problem was successfully addressed by changing the fruit fly rearing diet (Dugas, Yeager & Richards-Zawacki, 2013), and so we only included reproductive events that occurred on the improved diet in our Tulane tallies (February 2011–December 2014). At Tulane, we again provided pairs with four potential deposition sites (PVC tubes), but no bromeliads, allowing us to completely monitor tadpoles with twice-weekly censuses. We initially monitored clutch production, but could rarely assign new tadpoles to clutches, suggesting that we failed to detect most egg production, and so we did not consider this response variable. In early 2014, we sacrificed tadpoles from several pairs used in this study for unrelated projects. When >3 tadpoles were sacrificed, we ceased data collection for this pair because removal of dependent young might influence future reproductive output. Otherwise we removed any sacrificed tadpoles (2 ± 0.76 tadpoles sacrificed in each of eight pairs) from analyses of the ratio of juveniles to tadpoles for a pair. At Tulane, some individuals were included in more than one pairing, but none were ever paired with known genetic relatives (e.g. captive-bred offspring or siblings).

Because we defined 'tadpoles' as tadpoles that were transported to rearing sites (PVC tubes), this response variable integrates successful courtship, fertilization, egg development, male care (egg tending) and female care (tadpole transport). The same is true when we consider juvenile production, although we might expect this metric to reflect problems with larval development more strongly. The ratio of juveniles/tadpoles should primarily reflect intrinsic post-zygotic barriers (e.g. developmental problems).

STATISTICAL ANALYSES

In Panama, we established a total of 96 pairs (Table 1) that were held together 448 ± 113 days (range: 95–535 days). We compared the number of tadpoles produced among pair types using a generalized linear model with a negative binomial error distribution (which provided better overall model fit, *sensu* Pedan, 2001, than a Poisson distribution). We included the fixed effects of male lineage, female lineage, pair type (within- or between-lineage), and the number of days paired. This approach accounts for potential differences in tadpole production among lineages regardless of pairing type, and increases power by pooling within- and among-lineage observations into only two groups. However, this pooling might also mask an effect of a single between-lineage cross that suffers reduced fertility/fecundity. To address this, we split the data by male and female lineage, and for each, assessed the effect of mate lineage on tadpole production.

In the second phase of this study, at Tulane, we established a total of 73 pairs with 61 unique females and 66 unique males; these pairs were together 643 ± 375 days (range: 115–1368 days). We

Table 1. Total number (number producing tadpoles) of within-lineage, between-lineage, and F1 backcross pairs established to assess the potential for reproductive isolation among three lineages of *Oophaga pumilio* from Bocas del Toro, Panama: Aguacate peninsula (Agua, A), Isla Bastimentos (Bast, B), and Isla Popa (Popa, P). Within- and between-lineage pairs were established in both the Panama and Tulane phases of this study, while F1 backcrosses were established only at Tulane

Male lineage	Female lineage	n	
		Panama	Tulane
Agua	Agua	21 (12)	5 (2)
Bast	Agua	12 (7)	10 (9)
Bast	Bast	23 (15)	0
Bast	Popa	10 (10)	8 (8)
Popa	Popa	22 (16)	12 (11)
Popa	Agua	8 (6)	2 (2)
Agua	B+A	n/a	1 (0)
Agua	P+A	n/a	1 (1)
Bast	B+P	n/a	2 (2)
Bast	B+A	n/a	2 (2)
Popa	B+P	n/a	7 (6)
Popa	P+A	n/a	5 (5)
B+A	Agua	n/a	3 (2)
B+P	Popa	n/a	7 (6)
P+A	Agua	n/a	3 (3)
P+A	Popa	n/a	1 (1)

assessed potential differences among pair types in the production of: (1) tadpoles; and (2) juveniles and, as an index of potential developmental success; also considered (3) the ratio of juveniles/tadpoles. Because some individuals (seven males, ten females) participated in more than one pair, we used generalized linear mixed models and included the random effects of male and female identity to address the non-independence of these observations. When comparing tadpole and juvenile production, we included the fixed effects of male and female lineage (or lineage make-up for admixed individuals) and the effect of pair type: (1) within-lineage, (2) between-lineage, backcross in which the (3) male was admixed, or (4) the female was admixed; we again included the number of days paired as a covariate. For all models, model fit (Pedan, 2001) was nearly identical when we specified Poisson and negative binomial error distributions, and we present the results from the latter models for consistency. We compared juvenile/tadpole data with a similar approach, using the events/trials syntax and specifying a binomial distribution; in this case, we did not include the covariate 'number of days paired'. These models again benefit from the increased power of pooling F1 backcrosses into groups, especially important because we prioritized

breadth of combinations over replication within combination type (Table 1). Reduced fertility/fecundity of one particular admixed offspring type could again be masked in the overall effect of pair type, but any such reduction should manifest as a significant effect of male or female lineage/admix type. We followed up on significant effects of male or female lineage with pairwise comparisons of backcrosses to within-population pairs (we considered only crosses with ≥ 3 replicates). Because these tests were post-hoc examinations of significant effects and because we expected differences to be difficult to detect with small sample sizes, we did not correct for multiple comparisons.

Degrees-of-freedom for fixed effects were calculated using Satterthwaite's approximation. We used Proc GENMOD and GLIMMIX in SAS v9.2 (SAS Institute, Cary, NC, USA) for all analyses.

RESULTS

In Panama, all types of within- and between-lineage pairs produced tadpoles (Table 1, Fig. 2). Tadpole production did not differ among male lineages (likelihood ratio chi-square: 1.42, d.f. = 2, $P = 0.492$), female lineages (likelihood ratio chi-square: 3.52, d.f. = 2, $P = 0.172$), or between within- and between-lineage pairings (likelihood ratio chi-square: 0.75, d.f. = 1, $P = 0.386$), and the number of days paired was not significantly associated with the number of tadpoles produced (likelihood ratio chi-square: 2.78, d.f. = 1, $P = 0.095$). Follow-up analyses did not suggest any effect of mate population-of-origin for males or females of any of the populations we considered (Table S1).

At Tulane, we again found that all types of within- and between-lineage pairs successfully reproduced, and all types of admixed offspring successfully reproduced as well (Table 1). The number of tadpoles pairs produced differed significantly among male lineages ($F_{4,42.7} = 4.43$, $P = 0.004$) but not among female lineages ($F_{3,52.5} = 2.34$, $P = 0.084$), and was positively associated with the number of days paired ($F_{1,50} = 43.2$, $P < 0.001$). Tadpole production did not differ among pair types ($F_{1,53.2} = 0.04$, $P = 0.847$). Similarly, the number of juveniles pairs produced differed among male lineages ($F_{4,56.1} = 3.07$, $P = 0.023$) and was positively associated with the number of days paired ($F_{1,51.4} = 38.65$, $P < 0.001$), but did not differ with respect to female lineage ($F_{3,61} = 1.72$, $P = 0.353$) or pair type ($F_{1,57.1} = 0.88$, $P = 0.353$). Parameter estimates for both tadpole and juvenile production suggested that Popa and Popa × Aguacate (P+A) males were particularly productive, while Aguacate and Bastimentos × Aguacate (B+A) males produced relatively few offspring
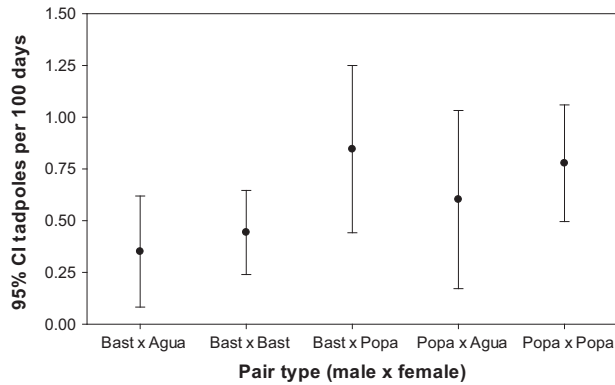
Figure 2. Tadpoles produced by *Oophaga pumilio* pairs per 100 days held in captivity in Panama. Pairs were established using wild-caught individuals from the Aguacate peninsula (Agua), Isla Bastimentos (Bast), and Isla Popa (Popa). Error bars indicate 95% confidence intervals (CI).

(Table 2). Pairwise comparisons between backcrosses and parental pairs did not suggest any differences, although P+A × Aguacate backcrosses may have been more productive than Aguacate pairings (Table S2). Finally, the proportion of tadpoles that successfully transitioned from the tadpole to the juvenile stage was unrelated to male lineage ($F_{4,50.8} = 0.68$, $P = 0.609$), female lineage ($F_{3,51} = 1.28$, $P = 0.292$), or pair type ($F_{1,34.6} = 1.38$, $P = 0.248$).

We have yet to formally analyse the coloration and patterning of admixed offspring and backcrosses, but can comment on general patterns. Overall, the offspring of between-lineage matings tended to be intermediate in coloration (Fig. 3), and offspring resulting from F1 backcrosses most resembled the over-represented lineage. Crosses between red Bastimentos and blue Aguacate frogs sometimes produced offspring that looked remarkably similar to the ancestral *O. pumilio* phenotype, with red body coloration (albeit muted red) and blue limbs. The offspring of Popa and Aguacate matings were more similar to Popa parents in coloration, and the offspring of F1 backcrosses were largely indistinguishable from the over-represented lineage, at least by eye. Admixed offspring of Bastimentos and Popa crosses were variably coloured, ranging from yellow to orange, and the offspring of F1 backcrosses remained easy to distinguish from individuals from parental lineages.

DISCUSSION

We found little data to suggest reproductive isolation among these three allopatric, phenotypically distinct

Table 2. Parameter estimates associated with male and female type; in both cases, Popa was estimated as the reference category. Estimates were drawn from a generalized linear mixed model in which number of tadpoles or juveniles was entered as the dependent variable, male and female type, pair type (within-lineage, between-lineage, backcrosses involving male or female admixed offspring), and number of days paired were entered as fixed effects, and male and female identity were included as random effects. Male type and number of days paired were the only significant terms in the model (see Results). Three allopatric lineages of *Oophaga pumilio* from Bocas del Toro were used in this study: Aguacate peninsula (Agua, A), Isla Bastimentos (Bast, B), and Isla Popa (Popa, P)

	Number of tadpoles produced		Number of juveniles produced	
	β	SE	β	SE
Intercept	1.23	0.50	0.73	0.54
Male type				
Agua	−1.43	0.47	−1.05	0.51
Bast	−0.33	0.32	−0.53	0.37
Popa	0.00		0.00	
B+A	−1.65	0.75	−2.02	1.12
B+P	−0.24	0.48	−0.45	0.51
P+A	0.08	0.45	0.09	0.48
Female type				
Agua	−0.38	0.30	−0.57	0.30
Popa	0.00		0.00	
B+A	−0.89	0.71	−1.19	0.88
B+P	0.24	0.40	−0.35	0.43
P+A	−0.67	0.59	−0.95	0.65

lineages of *O. pumilio*. All types of between-lineage crosses produced fertile male and female offspring, and between-lineage pairs and backcrosses were as productive as within-lineage pairs, at least under captive conditions. Admixed offspring might suffer reduced fitness in ways undetectable in a captive breeding study (discussed below), and such a circumstance is suggested by reproductive character displacement in at least one *O. pumilio* population (Richards-Zawacki & Cummings, 2011). However, the current study demonstrates convincingly that reproductive isolation is unlikely to be driven by intrinsic mechanisms. Overall, these data support the hypothesis that Bocas del Toro *O. pumilio* populations currently constitute a single biological species (Summers *et al.*, 2004; Hagemann & Pröhl, 2007), and similar work could test the hypothesis that Bocas del Toro lineages as a whole should be considered a separate species from other *O. pumilio* (Hagemann & Pröhl, 2007).
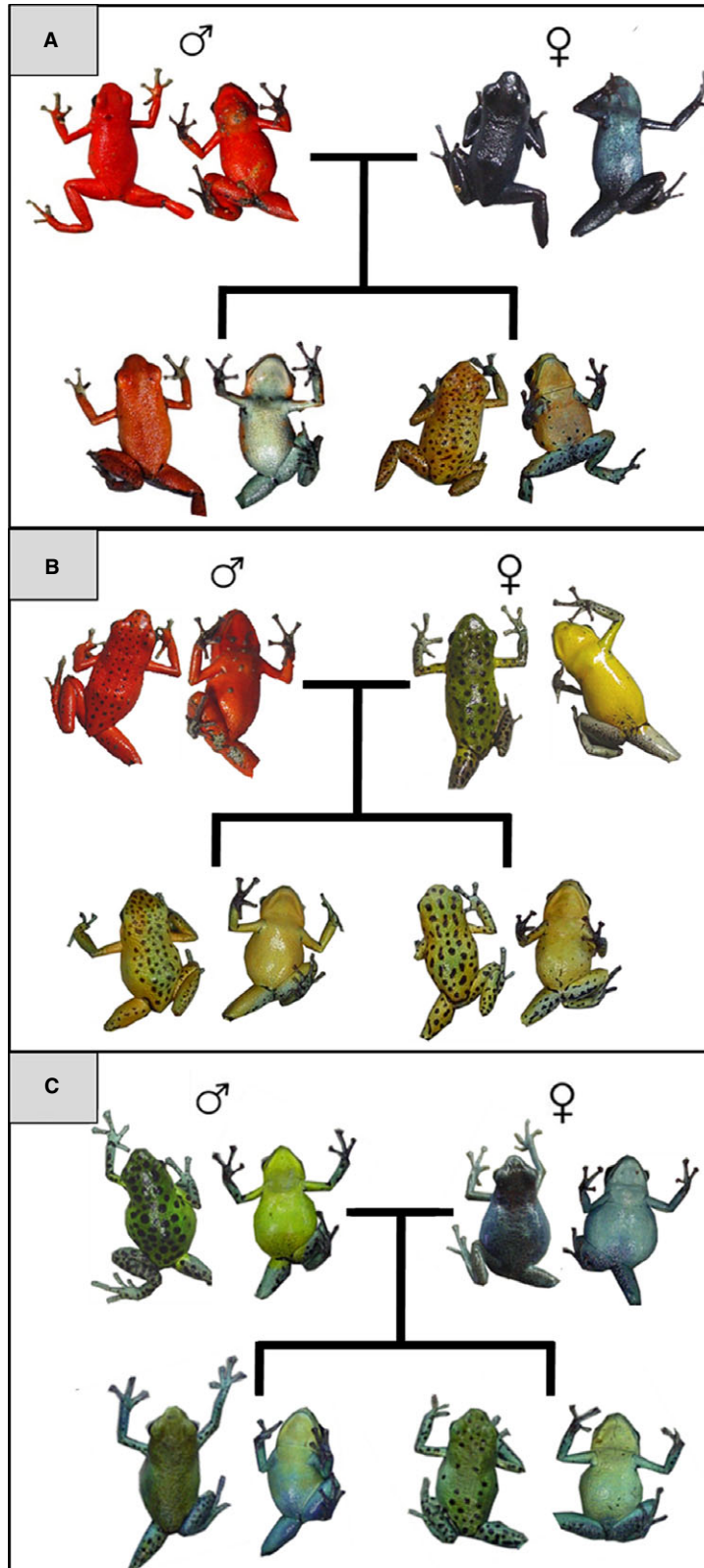
Figure 3. Photographs of representative crosses between *Oophaga pumilio* from Isla Bastimentos and the Aquacate peninsula (A), from Isla Bastimentos and Isla Popa (B), and from Isla Popa and the Aguacate peninsula (C). Each panel shows the parents and two unsexed offspring that have reached adult size.

Although this study did not explicitly and separately address potential behavioural pre-zygotic isolating mechanisms (e.g. mate preferences), absolute or strong assortative preferences should have resulted in less frequent mating in between-lineage pairs, and thus reduced reproductive output (Wade *et al.*, 1994; Shackleton, Jennions & Hunt, 2005). In a subset of Bocas del Toro lineages, including Isla Popa, females have been allowed to visually assess, but not interact with, males from their own and another population. In the majority of cases, females display assortative association preferences, suggesting that sexual selection on coloration has played an important role in driving or maintaining colour polytypism in this species (reviewed by Gehara *et al.*, 2013). However, we found no evidence that the actual mating decisions of female (or male) *O. pumilio* were influenced by the population-of-origin of their partner, at least in a no-choice scenario. Like dichotomous preference tests, no-choice experiments test one logical extreme of the task facing choosy females in the wild, and can thus offer only limited insights into the role of mate choice in mediating reproductive isolation (Wade *et al.*, 1994). However, our finding of incomplete isolation is consistent with studies of a polymorphic population in which females display assortative preferences in laboratory trials but there is incomplete and asymmetric isolation in the wild (Richards-Zawacki & Cummings, 2011; Richards-Zawacki *et al.*, 2012). This is perhaps unsurprising, as mate preference is only one of numerous factors shaping mate choice, and thus the extent of any choice-mediated reproductive isolation (Shackleton *et al.*, 2005; Jennions & Petrie, 1997). For example, female *O. pumilio* in a Costa Rican population make mating decisions based primarily on male proximity (Meuche *et al.*, 2013), suggesting that search costs in the wild could overwhelm any assortative colour preferences, especially when available mates are limited (as would be the case for an immigrant female: Hubbs, 1955). Testing the hypothesis that divergent female preferences are important isolating mechanisms early in speciation requires a multi-faceted approach, and the role of preference in effecting isolation can be examined best with realistic assessments of female choice (Gerhardt, 1992; Mendelson *et al.*, 2007; Rutstein *et al.*, 2007; Richards-Zawacki *et al.*, 2012; Martin & Mendelson, 2013).

When pre-zygotic isolating mechanisms have not evolved in allopatry and between-lineage matings are costly, reinforcement can strengthen pre-zygotic isolation during secondary contact (Servedio & Noor, 2003). Although most *O. pumilio* lineages are currently allopatric, future contact seems inevitable given the close proximity of populations and altered connectivity that comes with relatively small changes in sea level and/or climate in this region (Gehara *et al.*, 2013). The apparent absence of intrinsic post-zygotic isolation suggests that reinforcement would have to be driven by extrinsic costs of matings among lineages. Such extrinsic costs are likely when F1 offspring are intermediate in the trait that has diverged (Hatfield & Schluter, 1999; Jiggins *et al.*, 2001; Hobel & Gerhardt, 2003), as can be the case in *O. pumilio* (Summers *et al.*, 2004; Fig. 3). Because dart frog (Dendrobatid) coloration may be under both natural and sexual selection, this group seems an especially promising one in which to assess extrinsic post-zygotic barriers and concurrent selection for assortative mating preferences. In the case of *O. pumilio* in Bocas del Toro, however, neither a natural nor sexual selection cost to an intermediate phenotype is strongly indicated by current evidence. Experimental studies suggest that unusually low predation pressure on island populations may have permitted colour diversification in the first place (Hegna, Saporito & Donnelly, 2013; Richards-Zawacki, Yeager & Bart, 2013), so predators seem an unsatisfying proximate source of selection against intermediates. Sexual selection on coloration within lineages has been assessed in only one population, but suggests that skin brightness, which can vary independently of hue (i.e. colour) in integument (e.g. Dugas & McGraw, 2011), is under directional selection (Maan & Cummings, 2009; Crothers, Gering & Cummings, 2011). If brightness and hue were assessed independently by females (as suggested by Tazzyman & Iwasa, 2010; Maan & Cummings, 2009), the offspring of crosses between bright and dull lineages (e.g. between Popa and Bastimentos: Wang & Shaffer, 2008; Fig. 3) should be more attractive than their dull parents, creating asymmetric costs and benefits to between-lineage mating. Although intermediate individuals can be less fit in a number of ways (reviewed by Coyne & Orr, 2004), these may be particular to the pair of differently coloured lineages in *O. pumilio*, providing exciting opportunities to identify the specific circumstances under which colour divergence will lead to reproductive isolation.

Testing for reproductive isolation in newly diverged lineages is central to uncovering the patterns that govern speciation. Our understanding of this process grows with examples where such isolation is found as well as those in which it is not (Coyne & Orr, 2004). The lack of intrinsic post-zygotic isolation in *O. pumilio*, taken with a body of mate preference work in this system, adds to the weight of evidence suggesting that behavioural isolation is likely to evolve earlier than post-zygotic mechanisms (Coyne & Orr, 2004). However, our study

also highlights the need to consider not only whether preferences have diverged, but whether they will actually prevent gene flow before concluding that populations are moving towards speciation. While among-population colour and pattern differences are common in nature, the extent of this in *O. pumilio* and other poison frogs stands as an extreme example, and thus an excellent opportunity to understand how natural selection, sexual selection and the interplay between the two generate diversity and perhaps new species.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Comparison of genetic distances (Nei's D, from Wang & Summers, 2010) between focal *Oophaga pumilio* lineages and those at which previous breeding studies of anurans have found intrinsic post-zygotic isolation. Post-zygotic isolation indices and data (average ± SE) are from the meta-analysis in Sasa *et al.* (1998): Striped bars are IPO1, an index of post-zygotic isolation that considers only reciprocal crosses, and solid bars are IPO2, an index that considers both unidirectional and reciprocal cross data. Index values (*sensu* Sasa *et al.*, 1998) are: 0.25 = at least one sex sterile or inviable in one direction of cross, 0.5 = at least one sex sterile or inviable in both directions of crosses, 0.75 = only one sex considered viable or fertile in one direction of cross, 1.00 = all offspring sterile or inviable.

Table S1. Results of Type III comparisons from generalized linear models comparing tadpole production by different types of *Oophaga pumilio* pairs held in captivity in Bocas del Toro, Panama. We conducted analyses separately for each male and female population-of-origin, asking whether mate population-of-origin and number of days paired influenced tadpole production. Pairs were formed using individuals from populations initially collected on the Aguacate peninsula (Agua, A), Isla Bastimentos (Bast, B), and Isla Popa (Popa, P). For sample sizes, see Table 1 in text.

Table S2. Results of generalized linear models comparing tadpole or juvenile production between F1 backcrosses and within-pair matings from parental populations-of-origin. All models included the fixed effects of pair type and number of days paired; when an individual participated in more than one pairing, we included male and/or female identity as random effects. Populations used in this study were from the Aguacate peninsula (Agua, A), Isla Bastimentos (Bast, B), and Isla Popa (Popa, P); for sample sizes, see Table 1 in text.