

Variation in sexual dimorphism and assortative mating do not predict genetic divergence in the sexually dimorphic Goodeid fish *Girardinichthys multiradiatus*

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Abstract Sexual dimorphism is often used as a proxy for the intensity of sexual selection in comparative studies of sexual selection and diversification. The Mexican Goodeinae are a group of livebearing freshwater fishes with large variation between species in sexual dimorphism in body shape. Previously we found an association between variation in morphological sexual dimorphism between species and the amount of gene flow within populations in the Goodeinae. Here we have examined if morphological differentiation within a single dimorphic species is related to assortative mating or gene flow between populations. In the Amarillo fish *Girardinichthys multiradiatus* studies have shown that exaggerated male fins are targets of female preferences. We find that populations of the species differ in the level of sexual dimorphism displayed due to faster evolution of differences in male than female morphology. However, this does not predict variation in assortative mating tests in the laboratory; in fact differences in male morphology are negatively correlated with assortative mating. Microsatellite markers reveal significant genetic differences between populations. However, gene flow is not predicted by either morphological differences or assortative mating. Rather, it demonstrates a pattern of isolation by distance with greater differentiation between watersheds. We discuss the caveats of predicting behavioural and genetic divergence from so-called proxies of sexual selection [*Current Zoology* 58 (3): 440–452, 2012].

Keywords Sexual dimorphism, Assortative mating, Genetic distance, Speciation, Viviparous fish

Speciation is the cornerstone of biological diversity and studies of the processes that contribute to the divergence of populations and species are central to understanding speciation. The study of speciation has probably never been more topical, partly because of advances in comparative biology and genetics, which allow old questions to be addressed with more resolution as well as the formulation of new questions (Coyne and Orr, 2004; SPECIATION-Network, 2011; Wolf et al., 2010). Nevertheless, the major questions in speciation biology are familiar; what are the relative roles of selection, drift and gene flow in promoting or retarding divergence? Selection can arise due to ecological adaptation and the role of natural selection or ecological specialisation in reducing gene flow between taxa has been highlighted in many recent reviews (Nosil et al., 2009; Sobel et al., 2010). Selection can also arise due to variation in mating success and sexual selection has been suggested to be a particularly important cause of speciation in animals (e.g. Panhuis et al., 2001). Nevertheless, finding

strong evidence to support a particular role of sexual selection in speciation has proven to be far from straightforward, and some authors have questioned its importance (Ritchie, 2007; Kraaijeveld et al., 2010; Maan and Seehausen, 2011).

At one level a potential link between sexual selection and speciation seems obvious; many animals seem to differ primarily in sexually dimorphic traits such as the plumage of many birds, antlers of deer, beetles or trilobites, or behaviours such as courtship song or other signals. Comparative genomics is showing that sexual dimorphism extends to the genome, with genes that show sexually dimorphic expression evolving more quickly and displaying stronger signals of adaptive divergence (Ellegren and Parsch, 2007). But these apparent associations may be misleading, as many are largely anecdotal and non-quantitative. Sexual dimorphism is often thought to be a key signature of sexual selection and if dimorphism is obviously related to a trait primarily involved in sexual communication, the link may be more

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safely established. However, natural selection may act to promote or constrain divergence on dimorphic traits, potentially making such measures misleading. Further, ecological specialisation is often not studied where there is elaborate sexual dimorphism, and niche partitioning may occur between sexes as well as species. While numerous theoretical studies demonstrate that sexual signals and mate preferences rapidly evolve and could cause sexual isolation (e.g. Lande, 1981; Higashi et al., 1999; Kirkpatrick and Lavigne, 2002), it has been argued that sympatric species that only differ in behaviour are unlikely to persist without niche specialisation (Sobel et al., 2010). More quantitative studies are required to assess in detail the rates of evolution of ecological and sexual isolation, and both acting in concert may be a much more powerful force than either acting alone, especially if natural selection also acts on traits involved in mating behaviour. Comparative analyses suggest that reproductive isolation is greater where species show ecological differentiation (Funk et al., 2006).

The most common methodology adopted when asking if sexual selection promotes speciation is comparative analyses of rates of speciation in organisms differing in inferred levels of sexual selection. Sexual dimorphism, especially in plumage colouration in birds, was a major focus of early studies, which found a positive correlation between plumage dimorphism and species richness (Barraclough et al., 1995; Møller and Cuervo, 1998). Curiously, Owens et al. (1999) found an effect of plumage dimorphism but no effect of polyandry, presumably a more direct measure of the levels of sexual selection. In lizards, speciation rate is increased in sexually dichromatic lineages but reduced in lineages with greater size dimorphism (Stuart-Fox and Owens, 2003). In insects, some studies find a greatly increased rate of divergence in more polyandrous clades (Arnqvist et al., 2000) but arguably the most detailed studies across organisms failed to find any association between species richness and either size dimorphism or measures of polyandry (Gage et al., 2002; Morrow et al., 2003). Most recently, a meta-analysis of the comparative evidence found modest support for a role of sexual selection in speciation (Kraaijeveld et al., 2010). Interestingly, the strength of an association varied between study organisms (with fish having the strongest effect, though there were few studies) and between the measures used to infer the extent of sexual selection; sexual dichromatism had the most pronounced effect, but size dimorphism had an overall effect size of zero. Clearly there are many potential confounding variables in broad

comparative studies, including ascertainment biases (are we more likely to define species in elaborately dichromatic lineages?), the reliability of relatively simple indices of the strength of sexual selection, and confounding factors such as the probability of extinction, which may be increased for more sexually selected species (Morrow and Pitcher, 2003).

Less intensively used in this area are studies of phylogeography and direct assessment of predictors of gene flow in the field. Boul et al. (2007) demonstrated less gene flow at neutral loci between populations of the Amazonian frog *Physalaemus petersi*, which differ in calls and preferences. Calls seem to predict species in some closely related groups of insects (Henry and Wells, 1990, Mendelson and Shaw, 2005) but do not always accurately correlate with genetic differences or patterns of reproductive isolation (Gleason and Ritchie, 1998). It seems unfortunate that there are fewer studies of this type; correlating gene flow with traits would seem a more direct method of assessing the importance of traits to divergence than broad comparative studies.

There has been one broad comparative study of the role of sexual selection in speciation in fish. Mank (2007) that sexually selected traits (mainly dichromatism or exaggerated male fin shape) are associated found across ray-finned fish with a higher rate of divergence. Similarly, closely related species flocks of cichlid fish in Nicaragua lakes are genetically structured more by nuptial colour than by trophic or niche diversification (Wilson et al., 2000). We have been studying speciation within one family of fish, the Goodeinae, live-bearing freshwater fish, endemic to Mexico. These show extreme variation in sexual dimorphism related to sexual selection, with some extremely dimorphic species and others virtually sexually monomorphic (Ritchie et al., 2005). Copulation requires female cooperation, and the range of sexual ornamentation in the males seems to explain the variation in morphological sexual dimorphism, at least anecdotally. We have previously completed a comparative study across all the species and a directed comparison of population differentiation between contrasting species. Here we concentrate on a new study of morphological variation amongst populations of one of the most sexually dimorphic species of the family *Girardinichthys multiradiatus*.

1 Materials and Methods

1.1 The studied species

The Goodeinae consist of around 34 extant species of fish, mainly from shallow fresh water drainages of cen-

tral Mexico. The group radiated in the Miocene and occupies the basins to the north of the Mexican neovolcanic belt. These are small (usually < 10 cm in length) omnivorous topminnows, except for a large (ca. 20 cm) piscivorous specialist (*Allophorus robustus*), and a few species with a degree of specialization such as *Amea splendens* (a plant feeder), the benthivorous *Zoogoneticus* spp. and the riverine genera *Ilyodon*, *Xenotaenia* and *Allodontichthys* (also a benthivore) (see Miller et al., 2005).

Molecular (mtDNA) phylogenies of the group (Doadrio and Domínguez, 2004; Webb et al., 2004) confirm their monophyly and that the group is around 15 million years old, suggesting their radiation was related to the complex volcanic history of the region. This radiation was predominantly allopatric, but ranges have subsequently changed dramatically due to volcanism (Webb, et al., 2004) allowing interactions between divergent species.

Previously, we quantified morphological measures of sexual dimorphism across wild caught samples of 25 species of the group and tested for an association between sexual dimorphism and time to speciation within the clade using comparative approaches. Based on reconstructed levels of sexual dimorphism, time till speciation was shorter in more dimorphic lineages in 10 vs. 6 comparisons, a non-significant difference (Ritchie, et al., 2005). In a different study (Ritchie, et al., 2007) we examined gene flow between populations within species. Our logic was that if gene flow was influenced by sexual selection, the drift-selection balance would differ between populations of species with contrasting levels of sexual selection. We compared two pairs of species with relatively high and low levels of sexual dimorphism. F_{ST} was higher for a given geographic distance (approximately 0.26 vs. 0.16 adjusted for distance) between both of the more dimorphic species. Furthermore, there was evidence of sex-biased gene flow between populations of the dimorphic species, consistent with female preference against immigrant males (Ritchie et al., 2007). Hence our studies were consistent with a potential influence of sexual selection, as evidenced by sexual dimorphism, being correlated with gene flow at the level of paired comparisons, although there was not a strong signal of sexual selection influencing speciation at the level of the family.

Girardinichthys multiradiatus, or Amarillo fish, is amongst the most sexually dimorphic goodeid species. Males have much larger and colourful median fins than females (Fig. 1), and perform complicated courtship

displays (Zuarth and Macías García, 2006; Zuarth et al., 2011). Studies of the behavioural ecology of this species suggest that sexual selection must be particularly important. Fin morphology influences female mate choice; females preferentially associate with males possessing larger fins (Macías García et al., 1994), and males with reduced sexual dimorphism in body shape are discriminated against (Arellano-Aguilar and Macías García, 2008). Fin morphology also increases predation risk (Macías García et al., 1994; Macías García et al., 1998). Colour, including UV, is also a criterion for female mate choice (Macías García and Burt de Perera, 2002). Ornaments in the Amarillo are condition-dependent, because embryonic exposure to pesticides leads to suboptimal expression of sexually dimorphic fins, colour, and courtship displays (Arellano-Aguilar and Macías García, 2008).

Because male morphology is heritable (Arellano-Aguilar and Macías García, 2008), influences reproductive success via female mate choice, and is opposed by local natural selection (Macías García et al. 1998) the species appears to be a good candidate with which to explore the possible link between sexual selection, sexual dimorphism and gene flow at the level of a phylogeographic analysis. If sexual dimorphism evolves as



Fig. 1 Male (above) *G. multiradiatus* have larger and more colourful fins than females (below: this is a pregnant female)

Forced inseminations cannot occur in this family of fish, since sperm transfer is only aided by the small spermatopodium (at the front of the male's anal fin), which is not an intromittent copulatory organ.

a response to sexual selection and divergence influences gene flow, we predicted that a) sexual dimorphism would be variable between populations b) males may differ more between populations than females (if female preferences have selected for exaggeration of male morphology, including ornaments) c) either sexual dimorphism or male morphology would predict patterns of behavioural sexual isolation between populations d) genetic differentiation between populations would be correlated with sexual dimorphism or behavioural sexual isolation between populations. Here we test each of these predictions.

1.2 Sampling and morphology

Girardinichthys multiradiatus were sampled at several localities in Central Mexico, which encompass the majority of its geographic range (Gesundheit and Macías García 2005; Fig. 2). These include ponds, reservoirs and rivers in three major catchments, although the bulk of the populations are found in the upper Lerma River basin. Topography suggests that the few localities occupied by the Amarillo in the Balsas basin were colonised independently, whereas the small area of the

Panuco where this species is found was probably reached through a single region lying adjacent to one of the lowest points of the watershed divide. Collections were conducted with 3m seine nets hauled towards the shores (where adults congregate; Macías García et al., 1994). Fish were anaesthetised and photographed upon capture, when a small portion of the tail fin was collected for DNA extraction. Fish were given commercial anti-stress treatment before being transported to the laboratory, where they were housed in 40L tanks provided with filters and aeration (see González Zuarth and Macías García, 2006).

Morphology was examined from digital photographs taken on anaesthetised fish; as reported in González-Zuarth and Macías García (2006). We included six measures that have been implicated in female mate choice (standard length, body depth, and length and breadth of the dorsal and anal fins), and four that have not (eye and pupil diameter, depth of the caudal peduncle and length of the tail fin). Morphological variables were standardised to a mean of zero and a standard deviation of one then subject to a canonical discriminant

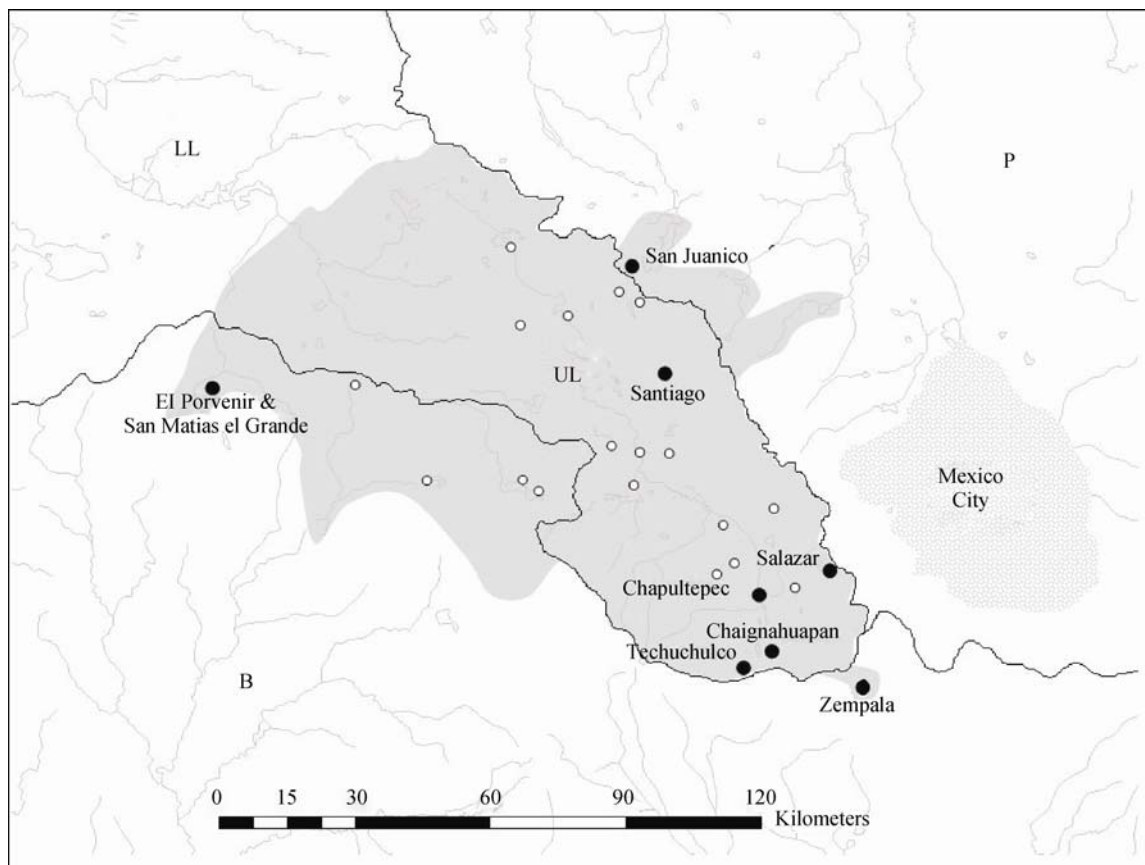


Fig. 2 Hydrographical map of Central Mexico

Black lines run along the watershed divides between the Panuco (P), Balsas (B) and Lerma (upper Lerma, UL; lower Lerma, LL) basins. Circles indicate localities containing substantial populations of *G. multiradiatus* known to us; larger, solid circles show the locations we have studied in more detail.

analysis (with population and sex giving 14 discriminant groups, i.e. population 1 males, population 1 females, population 2 males, and so on for all populations). This was in order to 1) quantify global sexual dimorphism as the canonical distance between males and females from each population, 2) quantify differences in sexual dimorphism between populations, as the pairwise population comparisons of sexual dimorphism, 3) assess whether male morphology diverges more rapidly than female morphology, and 4) assess whether the differences in male or female morphology between populations are related to the amount of genetic and/or behavioural divergence (assortative mating). We did not correct for size variation before completing the multivariate analysis because differences in size can be an important component of sexual dimorphism, and is one of the most widely used proxies for sexual selection (e.g. Gage et al., 2002; Kraaijeveld et al., 2010). However, our conclusions remain unchanged if size is not included in the analysis.

1.3 Behavioural analyses

Data on behavioural isolation and male and female morphologies were collected from seven populations, including those described by González Zuarth and Macías García (2006), with the addition of Techuchulco and Chignahuapan. Briefly, each of between 30 and 35 females from each population was exposed to a pair of males; one of her own population and another from a different population. Every female was presented a different pair of males (which were not re-used), and we performed 5–7 replicates of each combination. The mean of these replicates was used in the analysis. Males were introduced into the observation tank within plastic bags containing water from their home tanks, thus avoiding the stress associated with netting and water changes. Presentation of males within plastic bags allows the females to approach each male from a variety of directions as they do in the field, and also facilitates the occurrence of copulation attempts (copulatory embraces), which do not occur across rigid walls. Males were located at opposite ends of the tank and after 10 min of habituation the female was introduced and her behaviour recorded. We quantified the number and duration of visits (approaches to within one female body length) to each male (previously the frequency of copulation attempts was used to demonstrate that the duration of visits is a good predictor of willingness to mate, which translates into paternity; see González Zuarth and Macías García, 2006; Macías García and Saborío, 2004). Since females from each of the seven populations were

tested with six possible combinations of sympatric - allopatric males, we obtained 42 scores of pre-mating isolation, which were calculated as the difference between the mean time (in seconds) spent with sympatric versus allopatric males. We then added the scores of reciprocal tests (i.e. females of population A choosing between males from population A and B, and females of population B choosing between males of the same two localities). This generated 21 measures of the strength of pre-mating isolation ranging from -67 (negative values indicating that females of at least one of the two localities discriminate against their own males) to 142 (mean = $34.5 \pm 12.8 SE$). These 21 scores satisfied assumptions of normality and thus were not transformed before the analyses.

1.4 Genotyping

DNA was extracted from fin clips of individuals from ten populations of *G. multiradiatus* (Fig. 2) using the Purgene DNA isolation method. Primers for microsatellite loci have been previously designed for various fish species and were obtained for this study from Boto and Doadrio (2003) and Hamill et al. (2007). In total fifteen loci were surveyed using primers designed for the Goodeinae, these were: XC18, XC25, AS2, AS4, AS5, CA6, CA8, CA10 CA12, IW193 IW196 developed by Hamill et al. (2007) and ZT1.3, ZT1.6, ZT1.7 and ZT1.9 by Boto and Doadrio (2003). Hamill et al. (2007) found only 7 of these loci to be polymorphic in *G. multiradiatus* with three displaying little variation. Here we tested all fifteen loci and found only five (XC18, IW196, ZT1.6, ZT1.9 and CA12) polymorphic enough for population genetic analyses. In total 408 individuals from the 10 populations were analysed for five microsatellite loci (see Table 4a for numbers per population). One primer for each locus was fluorescently tagged and PCR was carried out in multiplexed groups using the Qiagen Multiplex PCR Kit and genotyped on a Beckman Coulter CEQ 8000XL.

1.5 Population genetic analyses

Micro-Checker (Van Oosterhout et al., 2004) was used to check the reliability of the microsatellite genotype data and test for Hardy-Weinberg Equilibrium (HWE). Tests of HWE for each population and each locus, linkage disequilibrium, population differentiation (F-statistics) as calculated by Weir and Cockerham (1984) and migration estimates (N_m) were calculated with GenePop (Raymond and Rousset, 1995; Rousset, 2008) and Arlequin version 3.1 (Excoffier et al., 2005) using Fisher's exact tests (Guo and Thompson, 1992) with Bonferroni correction when multiple tests were

performed.

Metrics of genetic differentiation, such as F_{ST} and G_{ST} , have been criticized as inaccurate measures of population differentiation when gene diversity is high because they approach zero, even when subpopulations are highly structured (Jost, 2008). Jost (2008) suggested an estimator of actual differentiation, D_{est} that takes into account gene diversity, which we calculated using the online program SMOGD (Crawford, 2010). An isolation-by-distance (IBD) analysis was performed using the isolation-by-distance service (IBDWS) version 3.15 (Jensen et al., 2005) through a Mantel test with 30,000 permutations and custom software. Due to our sampling both within and between watersheds we examined IBD over the entire dataset as well as looking at only within the upper Lerma River basin. Finally, matrices of simple linear geographic distance), and our measures of sexual isolation and sexual dimorphism between the seven populations in common were constructed. A partial mantel test was used to examine the relationships between genetic distance (D_{est}) and geographic distance, sexual isolation and sexual dimorphism. A sex biased dispersal analysis was carried following Goudet et al. (2002) as implemented in FSTAT version 2.9.3 (Goudet, 2001).

2 Results

2.1 Evolution of sex dimorphism

Sexual dimorphism, calculated as the between sex differences for each population in multivariate measures of morphological variation, differed between populations (range 7.2–8.5). Standard length and dorsal and caudal fin size had the largest loadings on the canonical variates (Table 1), so both size dimorphism and relative fin size contribute to morphological divergence between sexes and populations. Measures derived from males and females were highly correlated between the sexes (Fig. 3) but values for males were higher. In order to assess whether the rate at which populations differ in morphology between males and females differs significantly, we compared a) the mean between-population canonical distances of males with that of females (males; 2.79 ± 1.8 ; females 0.92 ± 0.43 ; $t_{\text{paired}} = 5.8$, $n=21$, $P < 0.0001$; Fig. 3 [also, the slope is significantly > 1]; Table 2), and b) the scores from the first two canonical variables between sexes and populations (Table 3). There were significant differences both between sexes (CV1) and between populations (CV1 and CV2), and the interaction was also significant for both canonical variables, confirming that the extent of differences were

not the same between sexes.

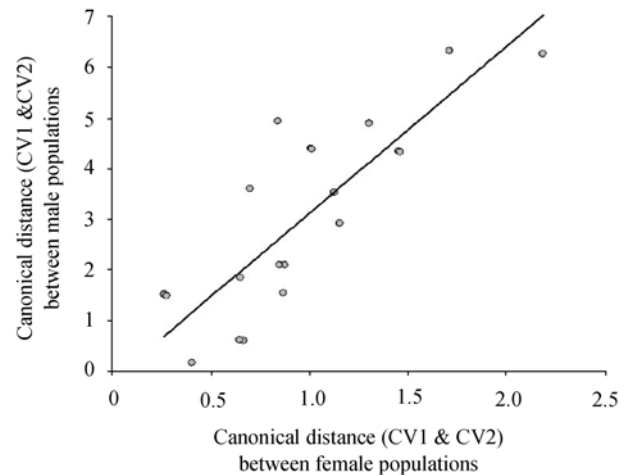


Fig. 3 Canonical distances from the first two canonical variates (92.5% of variance explained; Table 1) of a discriminant analysis of morphological variation amongst populations and sexes

Table 1 Standardised canonical coefficients from the first two canonical variates of a discriminant analysis to calculate sexual dimorphism and morphological divergence between populations of males and of females

Variable	Canonical Variate	
	CV1	CV2
Standard length	1.024009	1.323412
Length of anal fin	-0.020288	-0.841586
Length of caudal fin	0.188106	-1.355198
Length of dorsal fin	-0.689712	2.128972
Breadth of anal fin	-0.473283	-0.461595
Breadth of dorsal fin	-1.005069	-1.164784
Eye diameter	0.113949	0.311531
Pupil diameter	0.175604	-0.36217
Body depth	-0.072345	0.155999
Depth of caudal peduncle	0.619857	0.401072
Eigenvalue	16.10705	2.400353
% of variance explained	80.5	12
Cumulative %	80.5	92.5
F-value	11.4	5.4
Numerator DF	130	108
Denominator DF	1576	1439.4
P	0	0
Wilk's Lambda	0.004907	0.083937

Table 2 Classification of males and females per population of origin based on the canonical analysis reported in Table 1

Classified in	Belonged to													
	♀							♂						
	Ch	P	Sal	Sgo	SJ	T	Z	Ch	P	Sal	Sgo	SJ	T	Z
♀														
Chignahuapan	9	0	0	0	0	2	0	0	0	0	0	0	0	0
Porvenir	1	13	1	1	1	0	1	0	0	0	0	0	0	0
Salazar	0	2	4	5	3	0	3	0	0	0	0	0	0	0
Santiago	0	1	3	5	1	1	3	0	0	0	0	0	0	0
San Juanico	1	0	2	2	10	0	2	0	0	0	0	0	0	0
Techuchulco	4	0	0	2	1	12	0	0	0	0	0	0	0	0
Zempoala	0	0	0	1	2	0	3	0	0	0	0	0	0	0
♂														
Chignahuapan	0	0	0	0	0	0	0	10	0	0	1	0	5	0
Porvenir	0	0	0	0	0	0	0	0	15	0	4	1	0	2
Salazar	0	0	0	0	0	0	0	0	0	10	2	0	0	1
Santiago	0	0	0	0	0	0	0	0	1	0	11	1	0	3
San Juanico	0	0	0	0	0	0	0	0	3	4	4	8	0	0
Techuchulco	0	0	0	0	0	0	0	2	0	0	0	0	10	0
Zempoala	0	0	0	0	0	0	0	0	0	1	10	2	0	5
Total	15	16	10	16	18	15	12	12	19	15	32	12	15	11
Proportion correct	0.6	0.8	0.4	0.3	0.56	0.8	0.25	0.8	0.8	0.67	0.3	0.67	0.67	0.45

Table 3 ANOVAs of CV1 and CV2 (from Table 1) examining the consistency of sex differences amongst populations

Analysis of Variance Table, CV1			
Source Term	df	F ratio	P level
Sex	1	686.21	<0.0001*
Population	6	3.38	0.0033*
Sex*Population	6	4.32	0.0004*
Error	204		
Total (Adjusted)	217		
Total	218		
Analysis of Variance Table, CV2			
Sex	1	0.59	0.473
Population	6	62.34	<0.0001*
Sex*Population	6	19.19	<0.0001*
Error	204		
Total (Adjusted)	217		
Total	218		

2.2 Sexual isolation and sexual dimorphism

To explore the relationship between sexual isolation and the degree of morphological differentiation we regressed our measure of assortative mating on the population differences in sexual dimorphism and on the ca-

nonical distances between male morphology across populations. Differences in sexual dimorphism were unrelated to pre-mating isolation (Fig. 4a Mantel $P=0.2$), whereas, curiously, the magnitude of the differences in male morphology between populations was negatively correlated to the degree of pre-mating isolation (i.e. the greater the difference in male morphology, the lower the pre-mating barriers; Fig. 4b; Mantel $P=0.03$).

2.3 Genetic differentiation

The five polymorphic microsatellite loci surveyed were found to have an allelic diversity ranging from nine to 35 alleles and an observed heterozygosity of 0.038–0.921 (Table 4b). Locus CA12 had a large number of alleles and demonstrated significant deviation from HWE in half the populations (Table 5), however results were comparable with or without this locus. No other loci demonstrated consistent significant deviations from HWE overall, though 4 of the 5 loci showed significant heterozygote deficit in the Chignahuapan and San Pedro Techuchulco populations. Population substructure is possible for the Chignahuapan population as individuals came from four geographically close sampling localities. Possible null alleles were detected by Micro-Checker in only three populations for locus CA12, two populations for locus XC18 and one for loci IW196 and ZT1.9, with no evidence of large allele

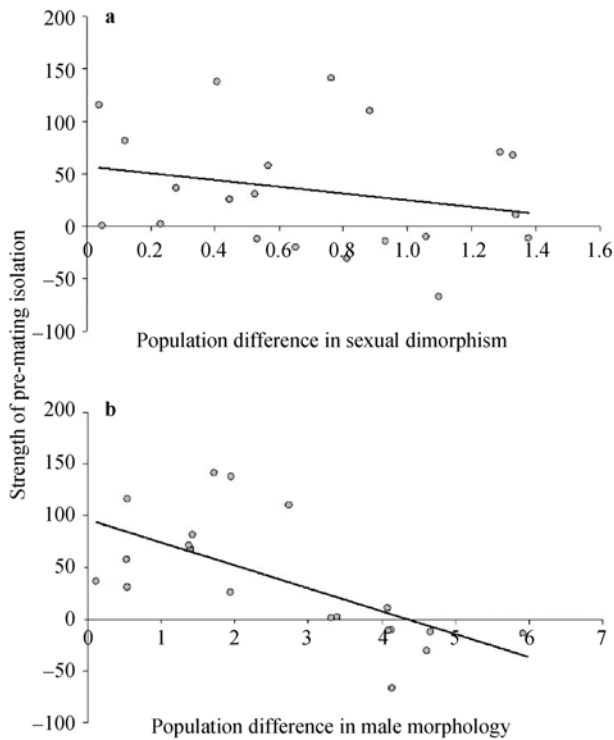


Fig. 4 The strength of pre-mating isolation against population differences in sexual dimorphism (a) and differences in male morphology (b)

dropout or scoring errors due to stuttering in any population.

Estimates of F-statistic genetic differentiation (F_{ST}) for each population comparison were significant (after Bonferroni correction) except for that between El Porvenir and San Matias el Grande. Measures of D_{est} were taken as the harmonic mean across all loci for each population pair and ranged from 0.011 to 0.598 with El Porvenir and San Matias el Grande populations being highly differentiated from all else, but not each other (Table 6). The Zempoala population was also found to highly differentiated from all groups except the Salazar lake population (Table 6).

Comparison of the genetic and trait data showed that morphological differentiation between populations appeared arbitrary, in the sense that it is not significantly related to genetic distance in either sex (Fig. 5: Mantel $P=0.85$ for males, 0.29 for females). Results of isolation-by-distance analyses showed a significant correlation of genetic distance (D_{est}) to geographic distance (Mantel $P=0.0047$; Fig. 6). However, this is clearly superimposed on a larger difference between populations from different watersheds (Fig. 2). IBD within the Lerma basin alone was non-significant (Mantel $P=0.46$).

Table 4 Summary of microsatellite sampling site locations, including sample size per population (n) (a), microsatellite variability in *G. multiradiatus* (b)

(a)

Population	Location	n
Lake of Chinahuapan	19 08' N, 99 29' W	94
Chapultepec	19 13' N, 99 33' W	30
El Porvenir	19 40' N, 100 38' W	30
Salazar	19 17' N, 99 24' W	28
Santiago	19 44' N, 99 41' W	44
San Juanico	19 55' N, 99 43' W	42
San Matias el Grande	19 42' N, 100 42' W	29
San Pedro Techuchulco	19 06' N, 99 31' W	37
Temascalcingo	19 55' N, 100 0' W	30
Zempoala	19 19' N, 99 19' W	44

(b)

Locus	Size range	Number of Alleles
XC18	248-298	22
ZT1.6	232-250	9
IW196	191-221	9
ZT1.9	357-429	17
CA12	127-243	35

Partial Mantel tests comparing genetic distance to sexual isolation and sexual dimorphism were also non-significant ($P=0.35$ and $P=0.1$ respectively). Further, a pattern of sex-biased dispersal was not seen in our data ($P>0.45$). Thus, the only variable that significantly explains genetic differentiation of *G. multiradiatus* populations is geographic distance, in a manner compatible with isolation across watersheds.

3 Discussion

Testing for a potential role of sexual selection in speciation and population divergence has been undertaken using a variety of approaches and at a number of scales, from broad comparative studies to analysis of gene flow in individual species. Results provide at best limited support for a clear role of sexual selection in speciation (Ritchie 2007; Kraaijeveld et al., 2010). This may be because the importance of sexual selection in causing reproductive isolation has been overestimated and natural selection may be more effective (Sobel et al., 2010). Mating signals may diverge quickly only because of an interaction with ecological selection, if they allow assortative mating between entities under divergent

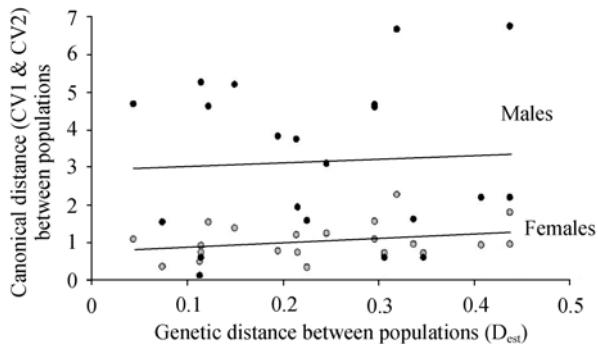


Fig. 5 Morphological divergence between populations of males (filled circles) and females (shaded circles) against genetic distance

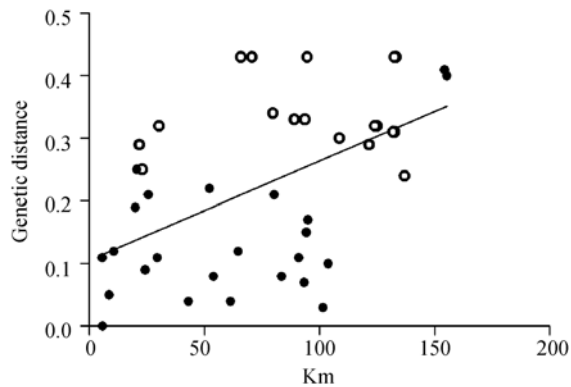


Fig. 6 Isolation by distance

Genetic distance (D_{est}) plotted against geographic distance (Km). Open circles represent between watershed comparisons and filled circles within watershed comparisons.

ecological selection (Van Doorn et al., 2009). Sexual selection may actively oppose speciation under some conditions, for example if male competition is a more powerful force than assortative female preferences (Parker and Partridge, 1998). Alternatively, a problem that has been highlighted with comparative studies of sexual selection and speciation is that the proxies used to estimate variation in the intensity of sexual selection may be misleading (Gonzalez-Voyer and Kolm, 2011; Kraaijeveld et al., 2010). Our study has allowed a detailed examination of the evolution of divergence in sexual dimorphism and assortative mating, yet neither has shown any correlation with gene flow. Our prediction was that greater sexual dimorphism would evolve in populations where female preferences were stronger and that such populations would also build up genetic differences due to more restrictive matings (Ritchie et al., 2007).

We quantified sexual dimorphism among multiple populations. This dimorphism was due to both sexual size differences, but also differences in fin morphology.

Size dimorphism could occur for a number of reasons, including sexual selection, but differences in fin shape seem almost certainly to be influenced by sexual selection. Fins are used extensively during courtship interactions and the exaggerated fin shape (and colour) of male fins in this species suggests they at least partly function as an ‘ornament’, i.e. a target of female mating preferences. We therefore expected to find an association between our measure of mating isolation and either the magnitude of the morphological divergence between populations (i.e. differences in sexual dimorphism or in male morphology) or the degree of genetic divergence. In general, local divergence of male mating traits is expected to arise due to 1) differences in magnitude of an ornament that can be expressed given the local ecology, and 2) differences in the magnitude of an ornament preferred by the majority of the local females. Indeed, from classical Fisherian models, it is expected that populations are driven apart by female mate choice only to the point where they are counter-selected by natural selection, where they are expected to remain. It is possible that variation in sexual dimorphism reflects ecological variation in counter-selection rather than variation in female preferences. *Girardinichthys multiradiatus* is an endangered fish with their natural habitat being lost (for example, since we began our studies on this species several populations have disappeared through desiccation, e.g. at Ignacio Ramirez, Ixtlahuaca and Acambay) rapidly through increased land use, tourism and pollution (De La Vega et al., 1997). Pollutants such as methyl parathion can affect the expression of male traits and influence male attractiveness to females (Arellano-Aguilar and Macías García, 2008). Recent change in selection pressures, such as pollution, may have led to changes in the interplay of natural and sexual selection and thus create a disjunction between more historical genetic variation and our proxies for sexual selection. Further, invasive species such as the poeciliid *Heterandria bimaculata* (Ramírez Carillo and Macías García, 2012) have played a big part in influencing the conservation status of *G. multiradiatus* through reproductive interference and conceivably may have caused a more recent shift in mate choice than the genetic data could detect.

It is also possible that the expectation of different levels of sexual selection and assortative mating may not be met in many systems. All populations could have directional female preferences for exaggerated fins, so it is not necessarily appropriate to translate differences in sexual ornamentation to potential assortative mating

(Price, 1998). There are very few systems where female preference functions amongst populations have been examined and then compared to variation in male traits, and those that have do not always show the covariance that would be predicted by correlated coevolution (Prum, 2010), which is necessary to underpin assortative mating. The fact that we see male morphology diverge between populations more quickly than female morphology supports the assumption that male morphology is under stronger selection than female morphology, however our expected link between morphological divergence and assortative mating is not supported. Indeed, the variation in sexual isolation we find is in fact negatively correlated with divergence in male morphology and sexual dimorphism. If females all have open-ended preferences for extreme males, they could show disassortative preferences when paired with males from more dimorphic populations. Perhaps our results also suggest more complicated preferences such as an attraction to novel male morphologies, which would more explicitly predict disassortative mating.

Although our measure of genetic divergence is derived from relatively few microsatellite markers we do detect isolation by distance and an analysis of mtDNA sequence variation showed a similar pattern, though there was limited variation (unpublished data). There is no suggestion that gene flow is related to either morphological variation or potential assortative mating. This is surprising as a previous study of F_{ST} amongst populations of four species of Goodeids did suggest that genetic differences between populations were greater in more dimorphic species. We believed the pattern would be greater in *G. multiradiatus*, as this is among the most sexually dimorphic species of the group. The first and most obvious explanation for these results is that sexual selection does not influence population divergence in this species. However, it is also possible that we did not assess a trait that predicts assortative mating. The exaggerated fins of male *G. multiradiatus* are highly colourful, and we have not assessed colour variation. Also, there is evidence in birds and some fish to suggest that colour patterns in the UV range of the light spectrum can be detected by females and can influence mate choice (Bennett and Cuthill, 1994; Smith et al., 2002). Further, we did not allow courting couples to have physical contact thus discounting the use of pheromones in mate choice. Other possible traits might mediate mate choice and thus be under sexual selection and more detailed analyses of courtship behaviour could be more revealing, as subtle changes in the behavioural courtship

elements may be involved. However, the steady pattern of isolation by distance superimposed on divergence between watersheds is simply most compatible with genetic differences at these neutral markers building up by genetic drift despite the obvious (and quite striking) variation in sexual morphologies and behaviour in this sexually dimorphic species.

In general, our studies exploring a potential link between sexual dimorphism, behaviour and evolutionary divergence in the Goodeid fish point to the contrasting results seen at different levels of analysis. They highlight that simple measures of predictors of the extent of sexual selection amongst species or populations may be misleading. While some trait comparisons may predict gene flow in one context, the complex dynamics of sexual selection may mean that correlations are not maintained across different comparisons or scales of analysis (in their review of comparative evidence, Kraaijeveld et al., 2010, found that dichromatism and mating system had significantly greater mean effect sizes for an association with speciation than simple size dimorphism). Variation in a frog call or an elaborate fin may predict gene flow between certain populations, but why should this be generally true across multiple species? Furthermore, the divergence in sexually selected male traits may not accurately predict assortative mating, which depends on the interaction between variation in male traits and female preferences, both of which may be under multiple sources of selection.

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