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Sexual isolation and extreme morphological divergence in the Cumaná guppy: a possible case of incipient speciation

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speciation.

Abstract

Theory predicts that sexual selection can promote the evolution of reproductive isolation and speciation. Those cases in which sexual selection has led to speciation should be characterized by significant differentiation in male display traits and correlated female preferences in the absence of post-zygotic isolation, accompanied by little genetic or other morphological differentiation. Previous evidence indicates that a cluster of populations of the guppy (*Poecilia reticulata* Peters) from Cumaná, Venezuela, the 'Cumaná guppy', differs significantly in female preferences from a nearby guppy population (A. Lindholm & F. Breden, *Am. Nat.*, **160**: 2002, S214). Here, we further document sexual isolation between these populations. In addition, these populations exhibit significant divergence in male display traits correlated to differences in between-population mating success, little mitochondrial genetic differentiation, and we find no evidence for genetic incompatibility between a Cumaná population and several geographically isolated populations. These results suggest that divergent sexual selection has contributed to differentiation of the Cumaná guppy, and this may be the first example of incipient speciation in the guppy.

Introduction

Theoretical studies have shown that sexual selection can drive speciation by producing divergence between populations in sexual traits (female preferences and male signals) (Lande, 1981; Schluter & Price, 1993; Pomiankowski & Iwasa, 1998; Higashi *et al.*, 1999; Turelli *et al.*, 2001). Comparative studies have also suggested that sexual selection can contribute to and accelerate speciation (Barraclough *et al.*, 1995; Mitra *et al.*, 1996; Möller & Cuervo, 1998; Price, 1998; Panhuis *et al.*, 2001). Whereas several empirical studies have directly demonstrated the evolution of reproductive isolation by natural selection (Hendry *et al.*, 2000; Higgie *et al.*, 2000; Rundle *et al.*, 2000; Schluter, 2000; Nosil, 2002; Greenberg *et al.*, 2003), there are few empirical studies which provide direct evidence of speciation driven by sexual selection (reviewed in Panhuis *et al.*, 2001).

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Recently, several authors have outlined criteria that would demonstrate speciation by sexual selection and rule out alternative scenarios (Gray & Cade, 2000; Panhuis *et al.*, 2001). First, sexual selection is indicated when substantial differences among populations in male sexually selected traits and female preferences directly influence between-population mating success. Secondly, those taxa likely to have undergone speciation by sexual selection are expected to show substantial prezygotic isolation. Thirdly, populations that differ in secondary sexual characters should show little neutral genetic differentiation, comparable with that normally found between populations, suggesting divergence only in the secondary sexual characters (Coyne & Orr, 1989; McCune & Lovejoy, 1998; Gray & Cade, 2000; Uy & Borgia, 2000). Finally, there should be little or no post-zygotic isolation because of genetic incompatibility or ecological selection against hybrids. This fourth criterion implies that prezygotic isolation is the initial cause of speciation, rather than having arisen secondarily. In this study, we test whether the divergence of a highly differentiated form of the guppy (*Poecilia reticulata* Peters)

is consistent with sexual selection according to these four criteria as outlined by Gray & Cade (2000) and Panhuis *et al.* (2001).

The guppy is a small, live-bearing fish in the family Poeciliidae, endemic to streams in north-eastern South America (Venezuela, Guyana and Suriname), and the adjacent Lesser Antilles islands, including Trinidad (Rosen & Bailey, 1963). The guppy is a model system for evolutionary studies, including both natural and sexual selection (see Houde, 1997 for review). Male guppies vary greatly within and among populations in several secondary sexual traits in response to predation pressure, sexual selection, and other environmental factors (Endler, 1983, 1995; Endler & Houde, 1995; Grether *et al.*, 1999). Female preferences and male colouration, which play key roles in the promiscuous mating system of the guppy, are heritable and vary geographically among populations (Breden & Stoner, 1987; Houde, 1988, 1997; Stoner & Breden, 1988; Houde & Endler, 1990; Endler & Houde, 1995).

Guppy populations are capable of rapid differentiation because of strong divergent natural selection (Endler, 1995; Magurran *et al.*, 1995; Houde, 1997; Reznick *et al.*, 1997; Magurran, 1998). This might be expected to lead to reproductive isolation (Hendry *et al.*, 2000; Higgie *et al.*, 2000; Rundle *et al.*, 2000). However, there is currently no evidence of post-zygotic reproductive isolation (Magurran, 1998) and little evidence of sexual isolation among the well-studied Trinidadian guppy populations (Endler, 1995; Endler & Houde, 1995; Magurran, 1998). Here, we examine the divergence and potential speciation of a highly differentiated, extremely colourful morphotype of the guppy from Venezuela. This form was first collected by Franklyn Bond in 1937, and later by John Endler in 1975, in the coastal town of Cumaná, north-eastern Venezuela. Endler's attempts at crossing this form with guppies from nearby populations resulted in few hybrid offspring, and he suspected them to be a species distinct from the guppy (J. Endler, personal communication). This morphotype of guppy has since become widely known in the aquarium trade as 'Endler's Livebearer', but has never been systematically described. Our results suggest that it is a highly differentiated form of the species *P. reticulata*, and has not attained strong enough isolation such that it would be considered a species according to most species concepts. Thus, we identify this morphotype according to its geographical location, namely the 'Cumaná guppy'.

Our first goal was to sample populations around Cumaná and adjacent drainages to determine the geographical distribution of this morphotype. We then tested for differentiation in characters known to be under sexual selection in Trinidadian guppies (Endler & Houde, 1995), differentiation in neutral genetic markers, and post-zygotic isolation. Female preference tests by Lindholm & Breden (2002) had previously suggested asymmetric prezygotic isolation. We directly examined whether sexual selection in terms of female preferences contributed to differences in male mating success between the Cumaná guppy and a high predation guppy population, and whether these differences were correlated to male display traits. As many of these populations come from similar high-predation selection regimes, our cumulative evidence suggests that sexual selection has likely played a significant role in this first report of incipient speciation in the guppy.

Materials and methods

Geographical distribution

More than 20 streams and canals in the Cumaná region (Estado Sucre, Venezuela) and in adjacent drainages were surveyed in May 2000 and 2001 (Fig. 1). The end of the dry season in north-eastern Venezuela occurs in the months of May and June, therefore, any creek beds containing water at this time would sustain guppy populations year round. Each stream or canal was haphazardly sampled by dip net. All males in each sweep of the net were collected so samples were not biased towards more conspicuous males. The live fish were transported to a temporary laboratory where they were anaesthetized in a approximately 0.03% aqueous solution of MS-222 (tricaine methanosulphonate), placed on a paper towel to remove excess water and the right side of each fish was photographed with a scale and colour standards. Fish were subsequently fixed for later molecular analysis in a solution of 95% ethanol saturated with

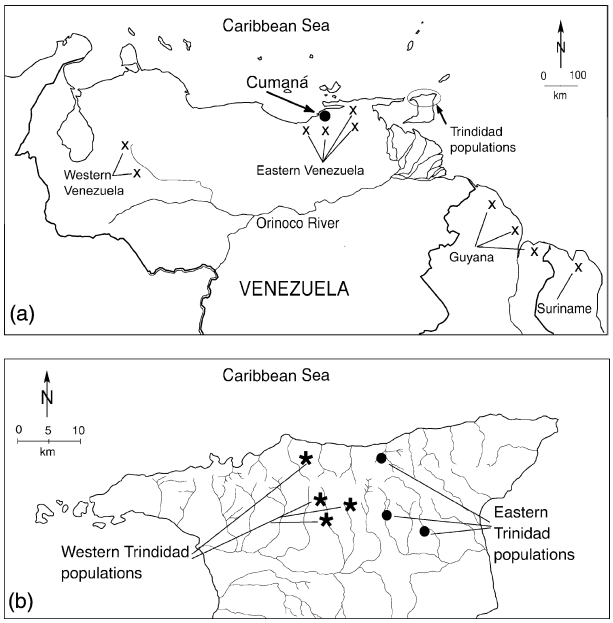

Fig. 1 Collection sites in north-east South America. (a) North-east South America including Venezuela, Guyana, Suriname and Trinidad. (b) Northern Trinidad.

ethylenediaminetetraacetic acid (EDTA). Colour prints and slides were digitized at 150 dpi.

The geographical coordinates of each sampling site were recorded, and temperature and salinity of the water were measured. Two of the streams in the Cumaná drainage (CC and WC) were seined three times each to survey potential guppy predator species. Fish captured in the seine net were anaesthetized in MS-222, photographed on site with a scale and colour standard, and released.

In order to examine whether the Cumaná guppy comprises a group distinct from other guppy populations, adult male individuals from 12 populations from across the range of *P. reticulata* were measured for colour, body size and shape. Cumaná is bisected by the Rio Manzanares. Three populations were sampled on the western side of this river [Central Cumaná (CC), Central Cumaná East (CCE) and West Cumaná (WC)], and two on the eastern side, [Calle Caripe (CCAR) and Calle Margarita (CMARG)]. We concentrated on populations from Cumaná, as it is the only reported locality for this unique morphotype. Two populations were sampled in adjacent drainages; to the south, Mira Flores (MF), and to the west of the Cumaná drainage, Yaguaracual (YCUAL). The remaining populations used in this study were sampled in previous expeditions by F. Breden and J. Taylor: Pozo Azufre (PA) (Winemiller *et al.*, 1990) and 6 km north of Pozo Azufre (PA6) located approximately 250 km south-east of Cumaná in 1998; the Upper Aripo River (APU) in Trinidad, and Springlands (SPL) in Guyana in 1997. These populations were sampled and photographed using the same sampling method, camera, scale and colour standard as in the May 2001 expedition. Paria River (PAR) males were collected and photographed by M. Gilchrist in 1997.

The first step in our analysis was to determine whether there are morphological features that identify a separate group in the Cumaná area distinct from other guppy populations across the range.

Male colour pattern variation

Body area and colour patch areas were measured from digitized images using the public domain NIH Image Version 1.63 (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). Colour patch areas and the number of patches were standardized relative to total body area (including the caudal fin, but not the dorsal fin, an area that is difficult to quantify). The number and area of colour patches on the caudal fin were standardized to caudal fin area.

Colour patches were categorized into the following classes: orange (including red), yellow, solid black, and fuzzy black (Baerends *et al.*, 1955). The colour area per unit of total body area (mm^2) and the number of patches of a particular colour class per unit of total body area were calculated and compared across populations. For colour area measurements, a sample of fish ($n = 25$) was measured twice and repeatabilities were high ($r = 0.90$, $P < 0.001$). The number and shape of black patches were scored as round spots, bars, crescents or 'other'. The presence or absence of orange double swords on the caudal fin (orange patches on the dorsal and ventral margins of the caudal fin) and colouration on the dorsal fin was recorded.

As many of the populations were not normally distributed despite transformation, nonparametric tests (Kruskall–Wallis) were conducted. Those populations that passed the Kolmogorov–Smirnov test ($P < 0.05$) for normal distribution were examined using parametric analyses. Univariate ANOVA were run to examine the patterns of variation among populations for each colour variable separately, and Tukey's *post hoc* tests were conducted to examine pairwise differences among populations. Statistical analyses were performed using SPSS version 11 (SPSS Inc., Chicago, IL, USA).

Colour quality of orange patches: hue, saturation and brightness

To examine variation in colour quality among populations, colour characteristics of orange patches were obtained from the digitized images described above. Hue, saturation and brightness values were measured from the digitized images using the Adobe Photoshop™ v.5.0 (Adobe Systems Inc., San Jose, CA, USA) colour-picker function. Hue is defined as the name of the colour such as red, orange, or green, and is the colour reflected from or transmitted through an object. It is measured as a location on the standard colour wheel, expressed as a degree between 0 and 360. Saturation, sometimes called chroma, represents the amount of grey in proportion to the hue, and is the strength or purity of the colour. Saturation is measured as a percentage from 0 (grey) to 100% (fully saturated). Brightness is the relative lightness or darkness of the colour, usually measured as a percentage from 0 (black) to 100% (white) (Adobe Systems Inc.). All three characteristics contribute to orange spot colouration as perceived by humans. Only populations that were sampled and photographed in May 2001 (CC, CCE, WC, CMARG, CCAR, MF and YCUAL) were measured in this analysis, as the other populations were photographed using different film types and/or colour standards.

To adjust for variation among images, three replicate measurements of each colour characteristic (hue, saturation and brightness) were taken for each red colour standard for each fish. In order to control for variation within and among photographs in the colour standard values, the mean value of three random spots taken from the red standard for each photo was subtracted from the orange patch measurements.

For each fish, three measurements of each orange patch were obtained using Photoshop's colour-picker function, and the mean of these measurements for each colour characteristic was used as the colour value for each patch. A sample of fish ($n = 52$) was measured twice and repeatabilities were high (hue, $r = 0.88$, $P < 0.001$; saturation, $r = 0.61$, $P < 0.001$; brightness, $r = 0.77$, $P < 0.001$). Discriminant function analyses (DFA) were performed using colour quality variables (corrected for colour standard variation) as the dependent variables, and populations as the grouping variable. First, we examined how well individuals could be classified into their natural populations, and identified the factor (or discriminate function) that best characterizes the differences among populations (Tabachnick & Fidell, 2001). Based on the results of our analysis of colour pattern variation, the DFA was repeated grouping populations containing males with high yellow and orange area, presence of double swords, and orange and black on dorsal fin, as 'Cumaná guppy'. All other populations were categorized as 'guppy'. This tested whether the Cumaná guppies formed a distinct group based on colour quality.

Body shape variation

Variation in body shape among 12 natural populations (CC, CCE, WC, CMARG, CCAR, MF, YCUAL, PA, PA6, APU, SPL and PAR) (342 individuals) was analysed using geometric morphometric methods (Bookstein, 1991; Rohlf & Marcus, 1993). This is a powerful tool for analysing shape variation, and has recently been applied to fish shape (Walker, 1997; Walker & Bell, 2000; Douglas *et al.*, 2001). This method consists of fitting an interpolating function (the thin-plate spline) to the *x*- and *y*-coordinates of landmarks for each individual in a sample (Rohlf & Marcus, 1993; Rohlf, 1993). This method allows analysis of shape while controlling for body size.

Nine *x, y* coordinates of homologous landmarks (discrete points that can be recognized as the same point in all individuals at all sizes) were measured using the same digitized images as were used for the male colour pattern and colour quality analyses. The landmarks were chosen such that a two-dimensional grid would outline a guppy shape (Fig. 2). Landmarks 1–7 occur at the meeting of two or more tissues. Landmark 8 was measured at the closest projection onto the fish from the halfway point between the dorsal fin and caudal fin (landmarks 3 and 4) and similarly landmark 9 was the projection halfway between the anal fin and caudal fin (landmarks 5 and 6). Landmarks 8 and 9 were chosen to obtain information on shape variation in the functionally important area of the fish body that determines the fast-start, or predator escape swimming behaviour (Webb, 1984). The landmark coordinates were determined using TpsDig (Rohlf, 2000a).

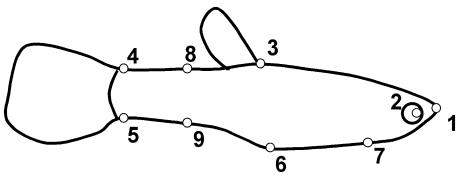

Fig. 2 Landmarks used for geometric morphometric analyses.

The generalized orthogonal least-squares (GLS) procedure was used to compute the Procrustes average configuration of landmarks (consensus configuration) (Rohlf & Slice, 1990). The GLS superimposition procedure computes an average shape, aligns the specimens to this average shape, and serves as a reference for the computation of thin-plate spline transformations (Bookstein, 1989; Rohlf & Slice, 1990; Bookstein, 1991). The *x*- and *y*-coordinates of the 342 aligned specimens projected onto the principal warps [deformations at different geometric scales in the nonuniform (nonaffine) component of shape variation] yields the partial warp scores for each fish (Rohlf *et al.*, 1996). The resulting $n \times 2(p - 3)$ **W** matrix of partial warp scores is referred to as the weight matrix, which is used in multivariate analyses to examine the uniform (affine) and nonuniform component of shape variation (n = number of specimens; p = number of landmarks). Nonaffine transformations describe local deformations. Affine components of shape variation describe uniform spatial covariation in the *X* and *Y* planes, and are described as shearing (uniform *X*), and stretching (uniform *Y*). Shearing shifts all points parallel to one another, for example, a square into a parallelogram. Stretching shifts points parallel with each other but without shearing, such as when a square is stretched into a rectangle (interpretation of geometric morphometrics follows Langerhans & DeWitt, 2004). All morphometric computations were performed in TpsRegr (Rohlf, 2000b).

Discriminant function analyses were performed using partial warp scores as the dependent variables and populations as the grouping variable. First, we examined how well individuals can be classified into their natural populations based on body shape, and to identify the factor (or discriminate function) that best characterizes the differences among populations (Tabachnick & Fidell, 2001). Based on the results of this analysis and the previous colour analysis, a DFA was repeated grouping populations into 'Cumaná guppy' and 'guppy' to measure how well the Cumaná guppies form a distinct group based on body shape.

A nested MANCOVA was performed using partial warp scores as a multivariate measure of body shape to examine how variation in body shape was partitioned between Cumaná guppy and guppy morphotypes, relative to among-population variation nested within

morphotype (Tabachnick & Fidell, 2001). The following standard nested MANCOVA model was used:

$$\text{Body shape} = \text{Constant} + \text{centroid size} + \text{morphotype} + \text{population (morphotype)}$$

Centroid size was used as a covariate, and is defined as the square root of the sum of squared distances from all of the landmarks to the centroid, and controls for differences in size between populations (Rohlf *et al.*, 1996). A canonical analysis was also conducted to examine the nature of shape variation between Cumaná guppy and guppy populations (morphotype effect).

Statistical analyses on shape were conducted using TpsRegr, TpsRelw (Rohlf, 2000b,c), SPSS version 11 and SAS version 8 (SAS Institute Inc., Cary, NC, USA).

Divergent female preferences between populations

This experiment tested for differences in female preferences between the Cumaná guppy (CC) and another geographically isolated guppy population (PA6). An open aquaria design was used to more realistically replicate social groupings. As naïve virgin females may initially mate indiscriminately, paternity tests were used to determine the mating success of males in a relatively long-term social grouping (Houde, 1988, 1997).

Juvenile fish were randomly selected from stock populations and raised as individuals in 3.8 L aquaria to obtain adult virgin females and inexperienced males. For each replicate, four mature females from the same population (CC or PA6) were placed in a 120-L aquarium (hereafter referred to as a 'group tank'), and allowed to acclimate for 24 h. After 24 h, two inexperienced CC males and two PA6 males were anaesthetized in a approximately 0.03% aqueous solution of MS-222, placed on a paper towel to remove excess water and the right side of each fish was photographed with a scale and colour standards, and then introduced to the group tank. The four males and the four females were held together for 21 days, following which time the females were removed from the group tank and isolated in 20-L aquaria. Each female was allowed to give birth to at least two broods of offspring, following which the original female was removed from the 20-L aquarium. The offspring were raised under standard conditions to adults. Once females were identified from the broods, they were removed. Male colour patterns have previously been used to determine paternity (Houde, 1997; Brooks, 2000), as they are extremely variable, and a large proportion of the genes controlling these colour patterns are permanently linked to the Y-chromosome (Lindholm & Breden, 2002). Our crosses with CC and PA6 have also shown male colour patterns to be highly heritable (H. J. Alexander and F. Breden, unpublished observations). Males, once mature, were therefore photographed as above and scored for paternity. All male parents were measured for body size (total length) and several characters including those that differentiate CC from PA6 males: orange area, tail orange area, number of black round spots per area, presence of black crescents, presence of dorsal fin colouration and body shape.

The proportion of male offspring sired by each male parent for each female in each group tank was recorded. First, a two-tailed *t*-test was used to test for a difference in the mean proportion of CC male offspring in each group tank ($n = 7$ for both CC and PA6) between CC and PA6 female treatments.

We examined whether male mating success was associated with male colour pattern, body shape and body length. A principal components analysis (PCA) was used to examine the correlation structure among male characters and to extract a male morphological variable. We then tested whether this variable explained mating success among group tanks. For each female, the number of male offspring sired by each male was regressed against each male's PCA male morphological variable.

If male mating success were because of male–male competition, then it would not vary between female treatments (CC vs. PA6). Therefore, differences in the standardized regression coefficients (β) between female treatments estimate differences in female preferences. To avoid pseudoreplication, the mean regression coefficient for each group tank was used as the female preference function. Two-tailed *t*-tests were used to examine whether the group tank female preference functions for the male morphological variable differed between CC and PA6 treatments. We also tested whether the female preference functions differed from zero using two-tailed *t*-tests. All statistics were conducted using SPSS Version 11 (SPSS Inc., 2002).

Molecular analysis

Guppies were sampled from across their natural range to examine population differentiation in mitochondrial DNA (mtDNA) control region (Table 1). Guppies from Trinidad, Guyana, and the Venezuelan populations of BTB, SUS, PA and PA6 populations were sampled in 1997 by F. Breden and J. Taylor. Other Venezuelan and Suriname populations were sampled in May 2000 and May 2001 (Fig. 1, Table 1).

Total genomic DNA was extracted from the tail fin or tail muscle from male fish using standard methods (Fajen & Breden, 1992). A 100 μL polymerase chain reaction (PCR) contained 100 ng genomic DNA template, each dNTP at 1 mM, each primer at 0.5 μM, 3 mM $MgCl_2$, 10 μL of 10X PCR buffer (200 mM Tris, pH 8.4, 500 mM KCl) and 2 U *Taq* DNA polymerase (Rose Scientific, Edmonton, AB, Canada). Reactions were amplified for 35 cycles: 94 °C for 60 s, 50 °C for 30 s and 72 °C for 90 s.

An approximately 850-bp segment of the control region was amplified using L15926 as the forward primer and MRT2 as the reverse primer. These primers and

Table 1 Location of sampling sites used in morphological and molecular analyses.

Location	Population	Abbreviation	P	S	GenBank accession	M	Geographical coordinates
West Trinidad	Arima River, Churchill	ACR-1	High	3	AY135452		10°36.0′N, 61°15.8′W†
	Roosevelt Highway	ACR-2			AY135450		
		ACR-9			AF170265		
West Trinidad	Arima River, Asa	ASA	Low	3	AF228623		10°42.3′N, 61°17.8′W†
	Wright Nature Centre	ASA-2			AY135460		
		ASA-3			AY135476		
West Trinidad	Guanapo River	GUA6-1	High	3	AF170267		10°29.9′N, 61°14.9′W†
		GUA6-2			AY135449		
		GUA6-3*			AY373762		
West Trinidad	Upper Aripo River	APU	Low	1	AF170268	23	10°40.7′N, 61°13.9′W†
East Trinidad	Upper Quare River	QMD-1	Low	3	AF193898		10°40.7′N, 61°11.7′W†
		QMD-9*			AY373763		
		QMD-10			AF529252		
East Trinidad	Aqui River (tributary of Madamas)	AQUI-3	Low	3	AF170262		10°45.5′N, 61°13.0′W†
		AQUI-5			AF529254		
		AQUI-6			AF529248		
East Trinidad	Lower Oropuche River	ORL-89	High	1	AF529249		10°39.0′N, 61°7.8′W†
North Trinidad	Paria River	PAR-9	Low	3	AY135453	24	10°44.5′N, 61°15.6′W†
		PAR-10			AY135448		
		PAR-71			AF193902		
East Venezuela	Central Cumaná East	CCE	High	3	*	25	10°26.9′N, 64°11.1′W‡
East Venezuela	Central Cumaná	CC	High	6	*	34	10°26.5′N, 64°11.5′W‡
East Venezuela	West Cumaná	WC	High	6	*	28	10°24.6′N, 64°13.3′W‡
East Venezuela	Calle Caripe	CCAR	High	3	*	36	10°28.5′N, 64°09.8′W‡
East Venezuela	Calle Margarita	CMARG	High	2	*	36	10°28.6′N, 64°10.5′W‡
East Venezuela	Yaguaracual	YCUAL	Low	3	*	36	10°18.4′N, 64°20.6′W‡
East Venezuela	Ajies	AJIES	?	3	*		10°31.4′N, 63°07.1′W‡
East Venezuela	Yaguaraparo	YPARO	?	3	*		10°34.4′N, 62°49.9′W‡
East Venezuela	Guarapiche	GUAR	Low	5	*		10°30.7′N, 63°20.1′W‡
East Venezuela	Pozo Azufre	PA7*	Low	3	AY373790	24	10°17′N, 63°7′W§
		PA8			AY135454		
		PA8-4*			AY373794		
East Venezuela	6 km N. Pozo Azufre	PA6-1	High	2	AF538280	12	10°15′N, 63°22′W†
		PA6-3*			AY373789		
East Venezuela	Arenas	AREN	Low	3	*		10°18.8′N, 64°57.1′W‡
East Venezuela	Mira Flores	MF	Low	4	*	36	10°10.3′N, 63°42.2′W‡
East Venezuela	Cumanocoa	COA	?	3	*		10°13.9′N, 64°54.6′W‡
West Venezuela	Upper Guanare River	BTB-1	Low	3	AY135457		9°10′N, 69°53′W†
		BTB-2			AY135451		
		BTB-3			AF170255		
West Venezuela	Lower Guanare River	SUS-1	High	3	AY135473		9°27′N, 69°55′W†
		SUS-2			AY135466		
West Venezuela	Lower Guanare River	SUS-3			AF228615		
Guyana	New West Amsterdam	NWA-3	High	1	AF228609		6°15′N, 57°31′W¶
Guyana	Springlands	SPL-3	High	1	AF228608	24	5°54′N, 57°8′¶
Suriname	Lelydorp	LYD-3	High	1	AF228605		5°42′N, 55°13′W¶

P, predation regime; S, number of individuals sequenced for the mtDNA control region; M, number of individuals used in morphological analyses; mtDNA, mitochondrial DNA.

*Sequences new to this study are deposited in GenBank under accession numbers AY373762–AY373814.

†Longitude and latitude from the state map of Trinidad, 1/150 000; Venezuela (various scales) from the Ministry of Public Works.

‡Global positioning system (GPS) coordinates.

§Corresponds to locality of Winemiller *et al.* (1990).

¶http://www.calle.com.

several internal primers were used for sequencing (Ptacek & Breden, 1998). PCR products were purified using Qiaquick PCR purification columns (Qiagen, Inc., Mississauga, ON, Canada) to remove primers. This purified product was used as template in sequencing reactions with the Cycle Sequencing Kit (Applied

Biosystems Inc., Foster City, CA, USA). The thermocycler profile for sequencing was 25 cycles at 95 °C for 20 s, 50 °C for 15 s, 60 °C for 60 s.

Mitochondrial DNA sequences were aligned using CLUSTAL X (Thompson *et al.*, 1998) (see Table 1 for accession numbers). Individual sequences were collapsed to common haplotypes. Phylogenetic relationships among sequence haplotypes were examined using maximum parsimony, maximum likelihood and neighbour-joining analyses, with *P. parae* (accession number AF033050) designated as the outgroup (Breden *et al.*, 1999). Maximum parsimony analysis was performed using a branch and bound search algorithm in PAUP Version 4.0b10 (Swofford, 2002). A distance matrix (uncorrected 'p') was used to estimate a neighbour-joining tree. Support for the resulting maximum parsimony and neighbour-joining topologies was assessed using a full heuristic search criterion with 500 bootstrap replicates and random stepwise addition. A strict consensus tree for the maximum parsimony analysis was calculated using PAUP Version 4.0b10 (Swofford, 2002).

For the maximum likelihood analysis, the collapsed sequence data were tested to determine the best-fit model of DNA nucleotide substitution using the hierarchy of likelihood ratio tests implemented in MODELTEST 3.06 (Posada & Crandall, 1998). The maximum likelihood analysis was performed using the 'Likelihood Ratchet' (Vos, 2003), a tree-searching algorithm that extends the concept of the 'Parsimony Ratchet' (Nixon, 1999). Rate heterogeneity among lineages was tested using the maximum likelihood clock model (PAUP* 4.0b10) (Swofford, 2002). A Shimodaira–Hasegawa (SH) test (Shimodaira & Hasegawa, 1999) was conducted by comparing likelihood ratios with 1000 replicate bootstraps, to examine whether trees constrained with Cumaná guppy as a monophyletic group were more likely than an unconstrained phylogeny.

To test whether morphological differentiation occurs without appreciable neutral genetic differentiation, we tested for correlations between genetic pairwise distances and morphometric distances [orange and black patch, colour quality, shape (canonical loadings) and standard length variables] between populations, using univariate Mantel tests (Manly, 1997).

Genetic incompatibility among populations

To test for genetic incompatibility, a Cumaná guppy population (CC) was crossed with a high predation guppy population from eastern Venezuela (PA6), but from outside of the range of the Cumaná guppy. Males and virgin females from CC and PA6 were crossed in all possible combinations, and both male and virgin female F_1 offspring were backcrossed to each parental type. Control, within-population crosses for CC and PA6 populations were set up in a similar manner. In a second set of crosses, the CC and PA6 populations were crossed with a geographically and genetically distant, high predation population from Suriname (SUR). In this set, we did not raise within-population crosses as controls, or were sample sizes as large. Males and virgin females from CC, PA6, and SUR were crossed in all possible combinations, and both male and virgin female F_1 offspring were backcrossed to each parental type. In a third set, the CC population was crossed with an eastern Trinidad population (QUL); F_1s were crossed to produce F_2s.

In all three sets of crosses, juvenile fish were randomly selected from stock populations and raised individually in 3.8 L aquaria to obtain virgin females. Virgin females were housed with a single adult male (obtained from stock populations) in 3.8 L aquaria for 3 weeks, after which time the males were removed. Each female was maintained in the 3.8 L aquarium until she gave birth to up to two broods. F_1 offspring were isolated to obtain virgin F_1 females for backcrosses to male paternal types. Juveniles from stock populations were raised individually to obtain virgin females for backcrosses with male F_1s. For the first set of CC/PA6 crosses, Kruskal–Wallis tests were used to compare the number of offspring from each cross: controls, $n = 3$–8; interpopulation crosses, $n = 2$–7; and reciprocal backcrosses (female $F_1 \times$ parental male), $n = 1$–3, and (parental female $\times$ male F_1), $n = 1$–4. Because sample sizes were low in the second set of crosses, between CC, PA6 and SUR, the number of offspring from each cross type (interpopulation, female backcross, and male backcross) were pooled and a one-way ANOVA was used to test for differences in offspring number. For the third set, between CC and QUL, adult F_1s and F_2s were raised from several families, but total number of offspring was not recorded.

Results

Geographical distribution of colour morphs and identification of the 'Cumaná guppy'

Three populations (CC, CCE and WC) on the western side of the town of Cumaná, Venezuela formed a distinct group based on several components of male colour; these populations had increased orange area on the body and caudal fin, increased yellow area (Figs 3 and 4), increased number of black crescents, and fewer round spots on the body (Figs 3 and 5), compared with other guppy populations measured. We did not locate any other populations containing male fish with these distinctive colour pattern elements, despite a thorough survey conducted in May 2000 and 2001 of an approximately 100 km radius around Cumaná, Venezuela, including all adjacent drainages. There are no other published reports of this distinct form despite extensive sampling by biologists across the guppy range. Two of these distinct populations (CC, WC) were found in the presence of the characid, *Astyanax bimaculatus*, considered to be a major predator,

Fig. 3 Males from CC (a) and YCUAL (b) (see Table 1 for collection coordinates). Scale in mm.

and *Rivulus hartii*, a less dangerous predator that feeds primarily on juveniles (Houde, 1997).

The average relative orange area for male colour patterns varied significantly among populations ($F_{11,337} = 22.32$, $P < 0.001$), ranging from 12 (CC, CCE) to 2.9% (APU). Two of the three western Cumaná populations of CC and CCE, as well as the Trinidad population of PAR all had significantly more orange colouration per unit of body area than all other populations measured (Table 2, Fig. 4a). On average, WC males had more orange area than males from other guppy populations, but not significantly so (Fig. 4a).

The number of orange spots (per unit of total body area) also differed significantly among populations ($F_{11,337} = 25.172$, $P < 0.001$; Table 2). The western Cumaná populations (CC, CCE and WC) had significantly more spots (including spots on the caudal fin) than all other guppy populations. Orange spot size varied significantly among populations ($F_{11,337} = 10.484$, $P < 0.001$; Table 2) but did not distinguish between Cumaná guppy and guppy males. Paria males had significantly larger orange spots than all other populations ($P < 0.05$, Tukey's *post hoc*) with APU males having the small-sized orange spots (Table 2).

Males from western Cumaná populations had greater orange area and number of orange spots on the caudal fin than did all other populations (Figs 3 and 4b, Table 2; area; $\chi^2_{11} = 134.4$, $P < 0.001$; number: $\chi^2_{11} = 148.0$, $P < 0.001$); these traits clearly distinguish the Cumaná populations from other guppy populations.

Double swords (orange patches on the dorsal and ventral margins of the caudal fin) were present only in males sampled from the three western Cumaná populations (Table 2). Colouration on the dorsal fin (orange, black, orange and black, yellow and blue) was present among western Cumaná males, as well as in males from

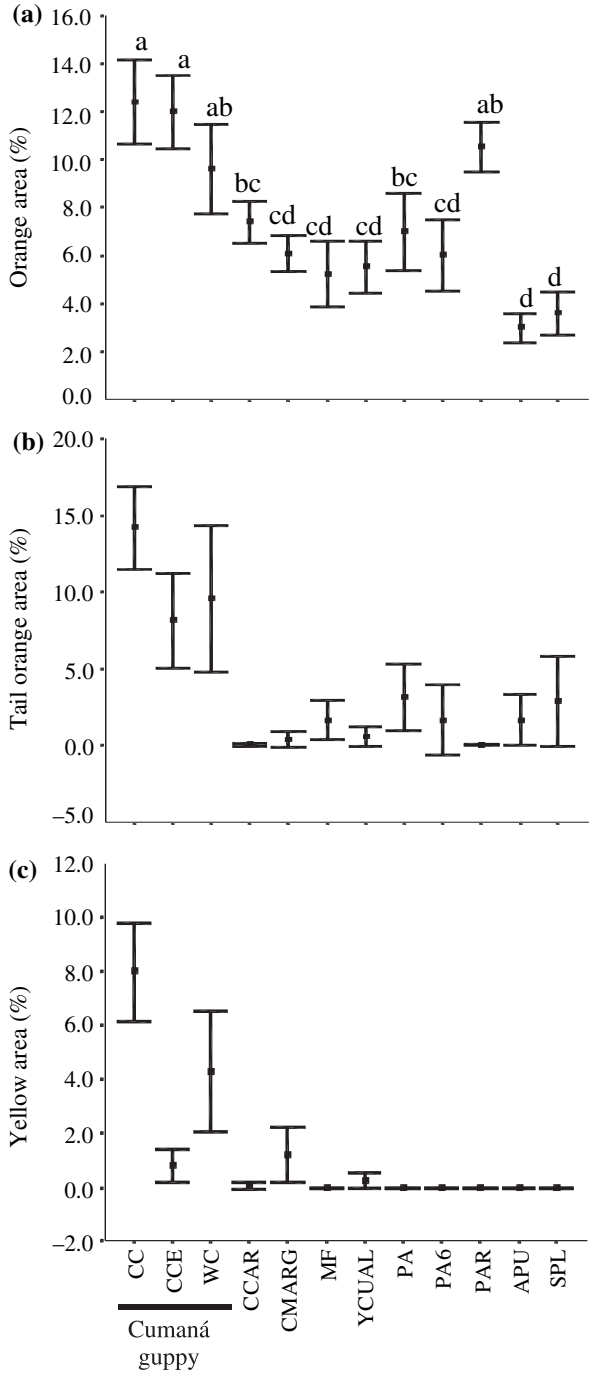

Fig. 4 Colour patterns of wild-caught males from Venezuela, Trinidad and Guyana (see Table 1 for population abbreviations and number of samples). Error bars represent two standard errors. The same letters above the error bars indicate no significant difference at $P < 0.05$ level. (a) Mean relative orange area. (b) Mean relative orange area on the caudal fin. (c) Mean relative yellow area.

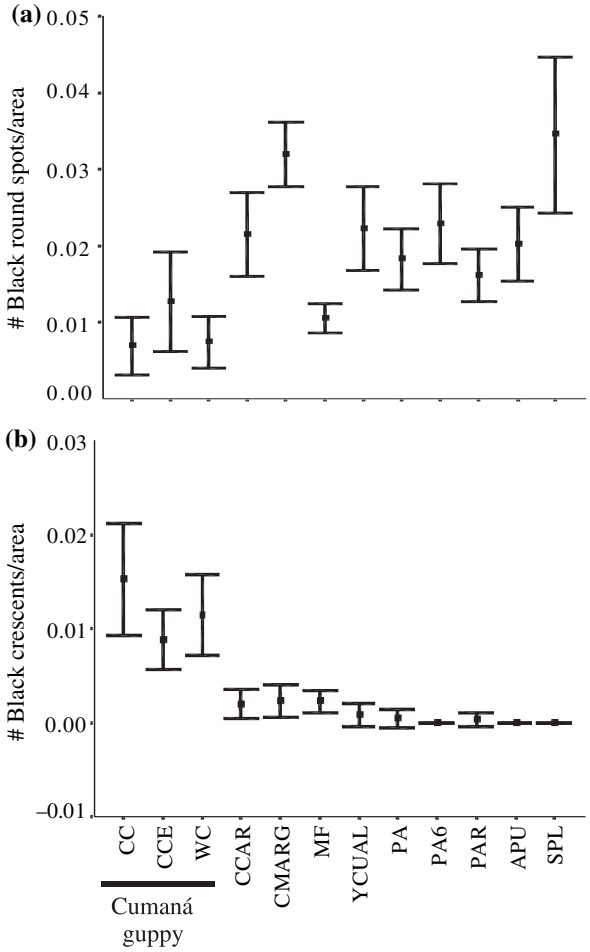

Fig. 5 Mean number of spots per unit of body area (not including the caudal fin) in wild-caught males from Venezuela, Trinidad and Guyana (see Table 1 for population abbreviations and number of samples). Error bars represent two standard errors. The same letters above the error bars indicate no significant difference at $P < 0.05$ level. (a) Black, round spots. (b) Black, crescent-shaped patches.

several other guppy populations (Table 2). More than half of the males collected from CC (59%), CCE (72%) and PA (48%) had dorsal fin colouration, which was significantly more than all other populations ($P < 0.05$, Mann–Whitney multiple comparisons tests). Colour on the dorsal fin was significantly associated with the presence of double swords ($\chi^2_{11} = 43.29$, $P < 0.001$) and these colour traits co-occurred only in males from western Cumaná populations.

The area and number of yellow patches varied significantly among populations (area: $\chi^2_{11} = 175.96$, $P < 0.001$; number: $\chi^2_{11} = 183.6$, $P < 0.001$). CC males had significantly more yellow area and number of yellow patches than all other populations (Figs 3 and 4c, Table 2). Ninety-one percentage of males sampled from the CC population had yellow patches, whereas 40% of WC males and 28% of CCE males sampled had yellow patches.

Table 2 Mean values (top) and SE (bottom) for standard length and colour pattern variables of wild-caught males from Venezuela, Trinidad and Guyana (see Table 1 for population abbreviations).

Population	CC	CCE	WC	CCAR	CMARG	MF	YCUAL	PA	PA6	PAR	APU	SPL
N	34	25	28	36	36	36	36	24	12	36	23	24
Standard length (mm)*	17.91	17.26	16.21	17.43	17.88	21.92	15.13	17.62	15.58	16.99	17.89	13.71
	0.18	0.23	0.22	0.20	0.27	0.17	0.17	0.27	0.20	0.15	0.31	0.29
Number of orange spots mm^{-2} ($\times 10^2$)*	7.03	6.16	6.42	5.23	3.75	1.72	4.61	3.21	3.80	3.50	2.44	3.
	0.39	0.39	0.53	0.30	0.24	1.89	0.38	2.85	3.33	0.24	0.19	3.81
Number of orange spots mm^{-2} on tail fin	1.94	1.38	0.95	0.04	0.09	0.14	0.08	0.38	0.21	1.19	0.26	0.17
	0.14	0.32	0.22	0.04	0.05	0.05	0.05	0.12	0.11	0.21	0.11	0.07
Orange spot size mm^{-2} ($\times 10^2$)*	2.57	2.81	2.67	1.83	2.13	2.26	2.19	2.81	2.55	4.00	1.45	2.37
	0.13	0.22	0.22	0.10	0.15	0.28	0.22	0.25	0.24	0.26	0.16	0.27
Number of yellow spots mm^{-2} ($\times 10^2$)	3.28	0.51	1.63	0.01	0.04	0	0.35	0	0	0	x	x
	0.32	0.02	0.40	0.01	0.02	0	0.01	0	0	0		
Percentage black area*	12.19	18.20	8.10	8.10	12.01	12.34	12.93	6.83	13.44	12.38	9.45	6.34
	1.11	1.90	0.98	0.66	0.84	1.25	1.19	1.05	1.49	1.17	0.95	0.65
Number of black bars mm^{-2} ($\times 10^2$)	2.30	1.06	1.65	0.21	0.71	0.71	1.32	0.21	1.10	0.41	0.80	0.63
	0.27	0.27	0.28	0.09	0.17	0.15	0.29	0.13	0.36	0.13	0.24	0.30
Double sword												
N	19	6	6	0	0	0	0	0	0	0	0	0
Percentage	56	25	17	0	0	0	0	0	0	0	0	0
Dorsal fin colour												
N	20	18	3	0	0	0	0	7	2	0	10	0
Percentage	59	72	9	0	0	0	0	20	5	0	42	21
Saturation*	17.86	18.82	6.60	−8.64	4.74	−21.10	−1.12	x	x	x	x	**x**
	0.58	0.80	1.06	1.51	1.85	2.04	1.74					
Hue*	11.93	18.56	20.71	14.86	29.55	10.30	19.24	x	x	x	x	x
	0.52	0.65	0.93	1.16	1.40	2.00	1.34					
Brightness*	−3.65	−8.79	1.65	−7.14	−6.40	13.79	10.88	x	x	x	x	x
	0.50	0.97	0.91	0.67	0.69	0.76	0.60					

N, number of individuals in sample; x, no measurements were taken.
*Normally distributed. Kolmogorov–Smirnov test ($P > 0.05$).

Although there were significant differences in total black area in male colour patterns among populations ($F_{11,337} = 7.795$, $P < 0.001$), this was not a character that contributed to differences between western Cumaná and guppy males (Table 2). However, the shape of black patches contributed to differentiating the western Cumaná populations from other guppy populations; these populations (CC, CCE and WC) tended to have fewer round spots and more crescent shaped patches than most other guppy populations (Figs 3 and 5a,b).

In summary, orange, yellow and black colour pattern elements in the three western Cumaná populations clearly define these populations as a distinct group, designated as 'Cumaná guppy' populations.

Colour quality of orange patches: hue, saturation, and brightness

Hue, saturation and brightness of orange patches also contributed to defining the Cumaná populations as a distinct group (Table 2). Colour quality values were significantly correlated (saturation/hue: Pearson correlation = 0.535, $P < 0.001$; saturation/brightness: Pearson correlation = 0.258, $P < 0.001$; hue/brightness: Pearson correlation = 0.257, $P < 0.001$). DFA showed that saturation correlated most highly with the first discriminant function, and accounted for 80.4% of the variation, and 51.9% of individuals were correctly classified into their assigned populations. A second DFA was conducted grouping individuals as either 'Cumaná guppy' (CC, CCE and WC) or 'guppy' (all other populations), based on male colour pattern variation. In this analysis, 89.3% of individuals were properly classified into their assigned group.

Body shape variation

Geometric morphometric analysis revealed that males from natural populations exhibit significant morphological differences among populations (nested MANCOVA: between morphotypes, $F_{14,316} = 61.21$, $P < 0.001$; among populations within morphotypes, $F_{140,2610} = 10.32$, $P < 0.001$). The morphotype-effect (Cumaná guppy vs. other guppy populations) canonical variate explained 73% of the shape variation in males. DFA correctly assigned 84.5% of individuals to their designated populations, and the majority of incorrect assignments were among the guppy populations. When

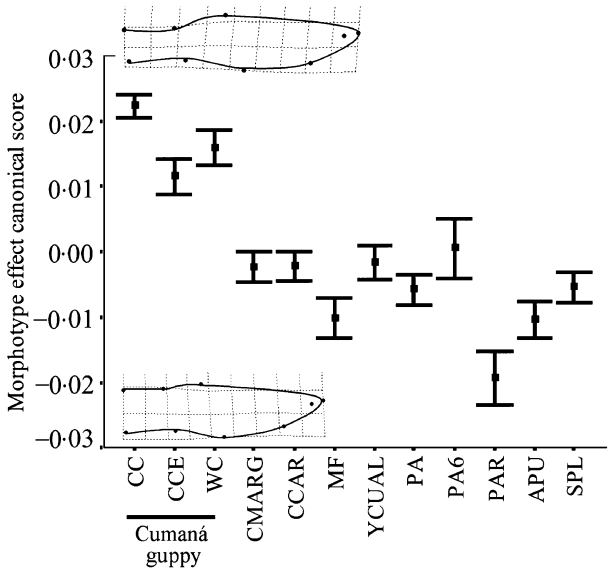

Fig. 6 Mean morphotype effect canonical scores for each population. Error bars represent two standard errors. Grids and outlines represent the body shape at each end of the morphotype axis, enhanced three-fold.

Table 3 Canonical loadings of the morphotype effect of Cumaná and guppy populations from MANCOVA models using geometric morphometric shape variables.

Independent shape variable	Interpretation	Loadings
PW 1X	Eye-snout length	0.0861
PW 1Y	Eye position	−0.0625
PW 2X	Insertion of anal fin relative to mid-caudal region	−0.0846
PW 2Y	**Eye and anal fin position**	**0.3082**
PW 3X	Insertion of anal fin relative to gill	−0.0066
PW 3Y	Thickness of head	0.0719
PW 4X	Length of caudal region/dorsal fin insertion	0.1904
PW 4Y	**Thickness of caudal peduncle**	**0.5468**
PW 5X	Length of caudal and head region	0.0496
PW 5Y	**Thickness of caudal peduncle and head region**	**0.4917**
PW 6X	Length of caudal peduncle	−0.1240
PW 6Y	Thickness of caudal peduncle/snout and eye position	−0.0030
Uniform X	Shearing	0.1318
Uniform Y	**Aspect ratio (elongation)**	**−0.4999**

Interpretations describe the nature of respective partial warps. Partial warps were interpreted using TPSRelw and TPSRegr (Rohlf, 2000b,c). All loadings >0.2 in absolute value are in bold type.

populations were categorized as either guppy or Cumaná guppy morphotype, based on our previous analysis of colour characters, an average of 94.4% of individuals were correctly classified, indicating that the Cumaná guppy populations form a distinct group in terms of body shape as well as colour characters.

Examination of canonical loadings from the MANCOVA model and visualization of shape shifts for each partial warp reveals that the major morphological shift between Cumaná guppy and guppy populations involves changes in thickness and length of the caudal peduncle and head (Fig. 6; Table 3). Male Cumaná guppies tend to be narrower through the caudal peduncle and overall more elongate than male guppies (Fig. 6). Qualitatively, these differences persist in laboratory stocks.

Divergent female preferences between populations

The CC females gave birth to a significantly higher proportion of CC male offspring than did PA6 females ($t_{12} = 3.630$, $P < 0.01$). In the CC female treatments, 80.2 ± 10.4% (mean ± SE) of male offspring were sired by CC males, whereas in the PA6 female treatments, only 30.5 ± 9.0% of male offspring were sired by CC males.

As body shape and all colour traits measured on males in this experiment (except body length) were significantly correlated with each other (black spots – negatively; tail orange area, body orange area, dorsal fin colouration, black crescents, body shape – positively; Pearson's correlation, $P < 0.05$), a PCA was conducted in order to extract a single variable to describe male morphological variation among individuals. The first component accounted for 53.7% of the variance, and body shape and all colour pattern variables loaded high (0.60–0.92) and positive, except black spots, which loaded high (0.83) and negative. There was a significant difference in mean preference functions between CC and PA6 female treatments ($t_{12} = 3.53$, $P < 0.01$); preferences of CC females were significantly positively associated ($t_{22} = 3.06$, $P < 0.01$) to the male morphology variable, whereas PA6 were significantly negatively associated ($t_{23} = -2.96$, $P < 0.01$). Body length was not significantly correlated to any colour pattern or body shape variable, and there was no difference in the mean preference functions for body length between CC and PA6 female treatments ($t_{12} = -1.89$, $P =$ ns).

Molecular analysis

Sequence analysis resulted in an 835 bp alignment of mitochondrial control region for 78 individuals. A total of 127 characters were variable, with 56 being parsimony-informative. The best-fit model of nucleotide substitution was the HKY + I + G model (I = 0.5184, G = 0.5397) (Hasegawa *et al.*, 1985). The Ti : Tv ratio estimated by maximum likelihood was 2.46.

Maximum likelihood, maximum parsimony and neighbour-joining analyses produced similar patterns of

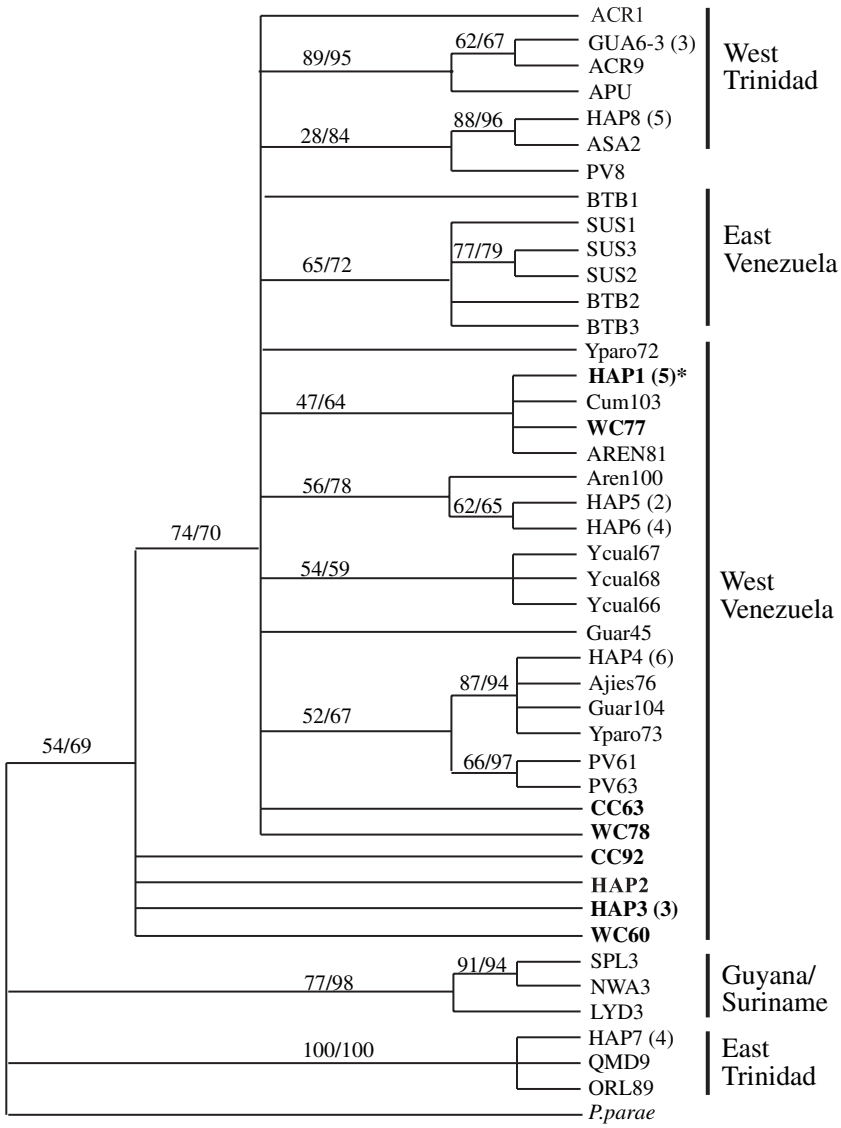

Fig. 7 Strict consensus tree based on 5000 most parsimonious trees generated by branch and bound algorithm as implemented in PAUP* Version 4.0b10 (Swofford, 2002). Bootstrap values are from maximum parsimony/neighbour-joining analysis. Populations in bold are individuals collected from western Cumaná populations (Cumaná guppy). Numbers in brackets are the number of identical haplotypes. Asterisk (*) indicates both guppy and Cumaná guppy individuals sharing a single haplotype. HAP followed by a number indicates populations sharing a single haplotype, as in Table 4.

haplotype relatedness. Suriname (LYD) and Guyana (NWA, SPL) populations form a well-resolved monophyletic group, as do the East Trinidad populations (AQUI, ORL, QMD) (Fig. 7). Haplotypes from the West Trinidad and Venezuela populations are less clearly resolved, and haplotypes from the Cumaná guppy populations, although all from a single highly morphologically differentiated group, are scattered among the eastern Venezuelan guppy haplotypes (Fig. 7). A maximum likelihood molecular clock model was rejected in favour of unconstrained rate heterogeneity among lineages ($\chi^2_{43} = 424$, $P < 0.001$), precluding any estimates of haplotype divergence times. However, phylogenetic analyses indicate that the Cumaná guppy populations do not form a monophyletic group (Shimodaira–Hasegawa test, -ln likelihood for constrained = 2615, -ln likelihood for unconstrained = 2550; $P < 0.05$).

Of the 78 individuals sequenced, we detected 44 unique haplotypes. Cumaná guppy populations and guppies collected from streams within the Cumaná drainage shared identical haplotypes (Table 4). Among the haplotypes from Cumaná guppy populations, sequence divergence ranged from 0 (identical haplotypes) to 2.56%, a range that is similar to genetic distances among haplotypes from Venezuelan populations (Table 5).

Genetic distances, in all cases but two, were not significantly correlated with morphometric distances (Mantel tests, $P =$ ns). Those significant associations (Mantel tests, $P < 0.05$) between genetic and morphometric distances (orange tail area and black crescent patches) were negatively correlated, indicating that increased genetic distance equates to less morphometric distance. These results suggest that morphological differ-

Population	Form	1	2	3	4	5	6	7	8
CC	CG	1	2	1					
CCE	CG	2	1						
WC	CG	1		2					
CCAR	G		3						
CMARG	G		2						
COA	G	1				1			
AREN	G						1		
MF	G					1	3		
AJIES	G				2				
GUAR	G				3				
YPARO	G				1				
AQUI	G							3	
QMD	G							1	
ASA	G								2
PAU	G								3
Total		5	8	3	6	2	4	4	5

Table 4 Frequency of identical haplotypes (columns) at each sampled population (rows).

Abbreviations as in Table 1. CG, Cumaná guppy morphotype; G, guppy morphotype.

Table 5 Percentage divergence in mitochondrial DNA (mtDNA) control region sequences.

	Cumaná	Venezuela	West Trinidad	East Trinidad	Guinanas
Cumaná	0–2.56	0–2.44	1.18–1.95	2.08–4.39	1.58–3.78
Venezuela		0–2.32	1.05–1.95	2.68–4.28	2.20–3.66
West Trinidad			0–1.34	3.29–4.27	2.56–3.54
East Trinidad				0–0.73	2.93–4.03
Guianas					0.24–1.46

entiation occurs without appreciable neutral genetic differentiation.

Genetic incompatibility

There was no evidence of genetic incompatibility in the first set of crosses between the tested Cumaná guppy population (CC) and a geographically isolated guppy population (PA6). Females gave birth to offspring in all combinations of within- and between-population crosses, and in all combinations of backcross, but one [(CC × PA6) × PA6]. There was no significant difference in the number of offspring arising from all cross-types ($\chi^2_{11} = 15.1$, $P =$ ns; pooled mean and SE: within CC controls, 11.4 ± 2.5; and within PA6 controls, 4.3 ± 2.2; CC/PA6 crosses, 9.3 ± 1.9; backcross to CC, 9.2 ± 1.8; backcross to PA6, 6.0 ± 2.3).

In the second set of crosses (CC, PA6 and SUR), offspring were produced in all combinations of interpopulation crosses and from most backcross combinations. There was no significant difference in the number of offspring produced among interpopulation, female and male backcrosses ($F_{2,24} = 0.840$, $P =$ ns). There was also no difference in the number of offspring between the first (CC/PA6) and second (CC/PA6/SUR) sets of crosses ($t_{70} = 0.452$, $P =$ ns). In the third set, two families of CC/QUL-F_1 crosses produced a total of 89 adult F_2s.

Discussion

Morphological, genetic, and behavioural evidence suggests that the Cumaná guppy has most likely differentiated from other guppy populations because of divergent sexual selection. Male fish from west Cumaná, Venezuela, form a cluster of populations that have significantly differentiated in what are inferred to be sexually selected colour patterns from other wild populations of guppies sampled from across Venezuela, Trinidad and Guyana. Body shape is also highly differentiated between these groups. Despite this morphological and behavioural divergence, the neutral genetic distances among haplotypes from Cumaná populations and between Cumaná and other guppy populations are similar to distances among Venezuelan guppy populations. Although our study suggests no evidence for post-zygotic isolation in terms of genetic incompatibility among a Cumaná guppy population (CC) and several geographically isolated guppy populations (PA6, QUL and SUR), differences in female preferences between the Cumaná and PA6 populations, correlated to male colour pattern divergence, indicate sexual isolation between these populations. These results, combined with dichotomous choice female preference tests conducted by Lindholm & Breden (2002) also showing divergent female preferences between CC and PA6 populations, suggest that this may be a case of incipient speciation in the guppy.

Males from the Cumaná guppy populations had significantly more orange pigment in their colour patterns than all other populations measured, with the exception of the Trinidadian Paria River population. The Paria River population is recognized for its extreme

development of large orange patches in male colour patterns (Houde, 1987, 1997). In contrast, Cumaná populations have an increased number of orange patches, and orange patch size is, on average, similar to other guppy populations (Table 2). Orange patch size differences and extreme neutral genetic differentiation between the Cumaná and Paria populations (Table 5) suggest that these populations have independently evolved high levels of orange colouration. The presence of relatively large yellow patches on the body and orange double swords (and consequently higher orange area) on the caudal fin also contribute to characterizing the west Cumaná populations as a distinct group. Double swords on the caudal fin and colouration on the dorsal fin among Cumaná males may serve to enhance the perceived caudal fin size. In some guppy populations, caudal fin size has been shown to be important in female preferences when size was used as a mate choice criterion (Endler & Houde, 1995).

In guppies, elements of male colour patterns, including the relative area of orange patches, are highly heritable (Houde & Torio, 1992), and are largely determined by genes linked to sex chromosomes (Winge, 1927; Haskins *et al.*, 1961; Lindholm & Breden, 2002). This also appears to be the case for Cumaná populations, for which male offspring colour patterns, including tail and dorsal fin colouration, closely match those of the male parents (H. J. Alexander and F. Breden, unpublished observations). However, several studies of birds and fishes (including guppies) suggest that the expression of carotenoid- and pteridine-based pigments in terms of brightness varies among populations depending upon environmental conditions (Grether *et al.*, 1999). Carotenoid-based sexual colouration may be indicative of the health and vigour of individuals (Kodrick-Brown, 1989; Milinski & Bakker, 1990; Zuk *et al.*, 1990; Hill, 1991; Houde & Torio, 1992; Grether *et al.*, 1999), but see (Dale, 2000), because vertebrates must obtain carotenoids from their diet (Goodwin, 1984). Conversely, Craig & Foote (2001) suggest that distinct morphs of the Pacific salmon (*Oncorhynchus nerka*) differ genetically in their efficiency at sequestering dietary carotenoid pigments into the skin. Because the Cumaná populations cluster as a group based on colour quality variables (in particular saturation and hue), it is possible that Cumaná guppies sequester carotenoid pigments more efficiently than do males from other guppy populations examined (Craig & Foote, 2001). Alternatively, Cumaná males may inhabit streams with higher carotenoid availability than other guppy populations measured, or even have greater health and vigour than guppy males. Although laboratory-reared Cumaná guppy males appear to maintain highly saturated orange patches, only a quantitative comparison of colour quality of carotenoid-based orange patches of laboratory-reared males raised on controlled diets could distinguish between these hypotheses.

Black colour patches are highly variable within-and between-guppy populations (Baerends *et al.*, 1955). Previous studies have examined female preferences for specific colour patterns, including black patches; however, results have been inconsistent, possibly as a result of genetic variation for preferences across populations (Houde, 1997). Although females prefer black colouration in a few populations (Endler & Houde, 1995), other studies suggest that mate choice is affected by the interaction of black and orange colouration (Brooks & Caithness, 1995; Brooks, 1996). Black patches may amplify or improve the detectability of orange patches (Hasson, 1989; Schluter & Price, 1993) and may allow females to discriminate among males based on orange colouration (Brooks, 1996). In our study, the relative number and area of black patches among populations did not contribute to differentiating the Cumaná guppy from the guppy populations. However, when solid black patches were partitioned in terms of shape, Cumaná guppy populations were distinct in that they had significantly more crescent shaped patches and fewer round patches other than guppy populations measured, suggesting that crescent-shaped black patches may be important for sexual selection in the Cumaná guppy.

Although previous studies have shown that female preferences vary geographically among Trinidadian guppy populations, females both generally prefer to mate with males with increased colouration, and tend to respond more to native rather than alien males (Houde, 1987, 1997; Endler & Houde, 1995). In dichotomous choice tests, Lindholm & Breden (2002) showed that females from a west Cumaná population (CC) associated approximately three-fold more frequently with CC males than with males from a geographically isolated guppy population (PA6), whereas PA6 females spent an equal amount of time associating with either CC or PA6 males. Here, we show that Cumaná males sired more offspring with Cumaná females, suggesting that new preferences may have evolved among Cumaná females for traits characteristic of the Cumaná male type.

Under the speciation by sexual selection hypothesis (Gray & Cade, 2000; Uy & Borgia, 2000; Panhuis *et al.*, 2001), divergence in male display traits and female preferences should be associated with little neutral genetic differentiation. That Cumaná populations do not form a monophyletic group indicates a low level of mtDNA differentiation, despite significant changes in male morphology. However, genetic distances within and among Cumaná guppy and other Venezuelan guppy populations range up to 2.56%. Such large genetic distances within populations suggest the maintenance of old lineages. The presence of these old lineages may be explained by at least two possible scenarios. First, a high degree of ancestral polymorphism may have existed in Venezuelan guppy populations, and the Cumaná morphotype has become fixed more quickly than the time

necessary for sorting mtDNA lineages. If so, the amount of sequence divergence within and among the Cumaná and other Venezuelan populations also argues against the founder effect, which would have depleted within-population variation. Secondly, introgression of guppy haplotypes into Cumaná populations may have occurred, thus obscuring any signal of mtDNA divergence. The important point with regard to the speciation by sexual selection hypothesis is that neutral genetic differentiation between Cumaná guppy populations and Venezuelan guppy populations is similar to that observed between Venezuelan guppy populations.

The more elongate body shape observed in the Cumaná guppy compared with other guppy populations has been shown to be an adaptation to predation in *Gambusia affinis, Brachyrhaphis rhabdophora* and among Trinidadian populations of *P. reticulata*. In these systems, fish inhabiting a predator environment tend to be larger in the caudal region and are generally more elongate than those in an environment free of predators (Langerhans & DeWitt, 2004). In *P. reticulata* and *G. affinis*, these more elongate individuals from predator populations exhibit higher fast-start escape speeds than those from populations without predators (O'Steen *et al.*, 2002; B. Langerhans, personal communication, 2002). Enhanced predator avoidance ability associated with the change in body shape may have allowed brightly coloured Cumaná males further success in a high predation environment.

Panhuis *et al.* (2001) proposed that if sexual selection has driven speciation, then closely related groups are expected to differ substantially in mating signals and preferences, but little in other traits, as reported in the field crickets *Gryllus texensis* and *G. rubens* (Gray & Cade, 2000), and the African cichlid fishes (Seehausen & van Alphen, 1999). The shape shift in a character normally responding to high predation raises the possibility that the sexual isolation and colour pattern divergence in the Cumaná guppy are pleiotropic effects of divergent natural selection (Schluter, 2000; Nosil, 2002), rather than direct effects of sexual selection. Although coastal streams appear similar across the northern coast of Venezuela, we have not quantified potential differences in abiotic and biotic factors in these streams extensively. However, similar predator communities were found in both Cumaná and other high predation guppy populations. Also, responses to predation among guppy populations typically involves less conspicuous colour patterns (Haskins *et al.*, 1961; Endler, 1980), whereas in the Cumaná guppy populations, males are extremely colourful. Therefore, we find it unlikely that natural selection is the major explanation for the diversification of colour and sexual isolation of the Cumaná guppy.

It has been hypothesized that members of the subgenus *Lebistes*, of which *P. reticulata* is a member (Breden *et al.*, 1999), have speciated largely because of sexual selection (Liley, 1966). Among these species, females are difficult to distinguish; however, male colouration, courtship display, and female preferences for these signals differ (Liley, 1966). This lack of divergence of the female morph along with extreme differentiation in male sexually selected characters among these clearly defined species further supports the speciation by sexual selection hypothesis.

Several hypotheses have been proposed to explain the lack of reproductive isolation among guppy populations despite the speed and magnitude of evolutionary change reported among Trinidadian populations (Endler, 1995; Reznick *et al.*, 1997). Endler (1995) suggests the lack of pre- and post-zygotic isolation among guppy populations may due to (1) the ephemeral nature of many guppy habitats, which may not persist long enough to allow reproductive isolation to evolve, (2) the decline of female preference in high predation habitats, and (3) the scale of gene flow, which may be greater than the scale over which selection takes place. Magurran (1998) argues that gene flow, in the form of sneaky copulations by males, may be a critical factor undermining the isolating effects of female preference. However, Cumaná males are highly differentiated and Cumaná females exhibit preferences for the distinctive Cumaná males, suggesting unidirectional sexual isolation and little gene flow in these characters. In conclusion, we suggest the Cumaná guppy may be the first documented case of incipient speciation in the guppy, and this group may be a likely candidate as a vertebrate model for studies of speciation by sexual selection.

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