Evolution of reproductive isolation in the Trinidadian guppy, *Poecilia reticulata*

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Thesis submitted for the degree of Doctor of Philosophy, University of St. Andrews

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ABSTRACT

Due to the rarity of observed speciation events, speciation research has concentrated on processes that are currently operating between species. But it is uncertain whether these processes accurately reflect what happens during speciation. The guppy, *Poecilia reticulata*, may help to resolve this problem since some populations, from the Caroni and Oropuche Drainages of Trinidad, appear to be in the initial stages of speciation. This thesis examines various issues related to the evolution of reproductive barriers in the Trinidadian guppy.

Laboratory crossing experiments evaluated intrinsic post-zygotic isolation between Caroni and Oropuche guppies. Male behavioural sterility was observed in the F_1 generation – this is the first documentation of behavioural sterility within a species, indicating that it is feasibly involved in causing speciation. The crosses also uncovered hybrid breakdown for embryo viability, brood size and sperm counts in the BC_1 or F_2 generations. Hybrid unfitness has a greater effective magnitude than behavioural isolation, suggesting that it is more likely to contribute to the speciation of these populations.

Complementary insights into the relative importance of different reproductive barriers were obtained by the genetic analysis of rates of inter-specific insemination between sympatric populations of guppies and their putative sister species, *P. picta*. Insemination rates were low, but indicated that behavioural isolation based on male mating preferences is incomplete. These crosses do not result in fertilisation; hence, post-copulatory barriers again seem to have evolved more rapidly than behavioural isolation. The data also support the efficacy of the alternative male mating tactic, "sneaky mating", in inseminating females.

Finally, genetic analysis of the effects of an artificial introduction involving Caroni and Oropuche guppies documented extensive displacement of the native nuclear genome. These consequences caution against the use of introductions as an experimental tool, although the resulting admixture may provide fruitful ground for future speciation research.

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CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION: Speciation and the guppy

1.1 Summary

Much is known about speciation, though many uncertainties remain. One of the most important concerns the relative significance of different reproductive barriers in causing speciation. Another is the generality of phenomena that have been characterised largely from a single genus, *Drosophila*. Emerging work suggests that the guppy, a small neotropical freshwater fish species, may offer a useful intra-specific perspective on these problems.

1.2 Introduction

The guppy, *Poecilia reticulata*, may provide a badly required view of speciation. Ever since 1859, when Charles Darwin published '*On the Origin of Species*' and speciation biology entered mainstream scientific consciousness, various fundamental issues have proved intractable. This is because speciation takes a long time, typically millions of years (Coyne & Orr 2004, p. 420). Instances of ongoing speciation are thus difficult to detect, obliging researchers to study current phenomena amongst inter-specific taxa that may poorly reflect what happens during speciation. The guppy is potentially useful here since some Trinidadian populations are highly divergent, implying that they might provide insights into the early stages of speciation.

Speciation is a key engine of biodiversity, responsible for many millions of lineages that have inhabited Earth. Considerable advances in understanding the mechanics of speciation occurred following the Modern Synthesis (1926-1947; Berlocher 1998) and the formulation of the Biological Species Concept (Mayr 1970). It is now understood that reproductive isolation (RI) barriers, which prevent introgressive hybridisation between populations, must somehow contribute to the formation of biological species. Otherwise, incipient species would lose their integrity following gene flow.

But many RI barriers exist, and it is uncertain what their relative importance is in causing speciation. This doubt stems from several factors. One is the use of taxa that have already speciated (Presgraves 2003). Another is the common failure to assay multiple barriers in crossing experiments. For instance, although hybrid behavioural dysfunction has been documented in inter-specific hybrids, it has not yet been assayed in intra-specific hybrids, and so its potential contribution to speciation remains unclear. A final constraint has been the preferential use of *Drosophila* in hybridisation experiments (Coyne & Orr 1998) - although these experiments have uncovered general principles, such as the disproportionately large contribution of X-linked loci to hybrid unfitness (Wu & Ting 2004), their applicability to other taxa remains largely uncertain.

Guppies from the Caroni and Oropuche drainages of northern Trinidad are geographically adjacent, but amongst the most genetically divergent populations currently known from the species's natural range. Because mitochondrial sequence data suggest they separated up to 2 mya (see Appendix I), a period equivalent to 4-6 million generations, the existence of partial reproductive isolation between them is highly feasible. Trinidadian guppies may therefore constitute an ideal vertebrate model for examining the evolution of reproductive isolation *prior* to speciation.

In addition, a considerable literature detailing the behavioural ecology and evolutionary biology of Trinidadian guppies has accumulated over the last ~ 60 yrs (see Houde 1997 and Magurran 2001 for reviews). This allows the evolution of reproductive barriers to be considered within particular ecological and genetic contexts. For instance, due to geographical variation in the sexual behaviour of males, Trinidadian guppies could shed light on behavioural causes of lineage specificity in the rates of evolution of different RI barriers. Also, because they lack strongly differentiated sex chromosomes, guppies could indicate lower thresholds for the effects of sex chromosomes on hybrid unfitness. Another advantage of the Trinidadian system is the presence of an admixed population of Caroni and Oropuche guppies, which resulted from an artificial introduction. This introduction allows the effective magnitude of RI barriers in the wild to be evaluated – a key test if speciation is to be interpreted in terms of reproductive isolation. Finally, Trinidadian guppies occasionally co-occur with their putative sister species, *P. picta* (Breden et al. 1999), allowing the effective strength of RI barriers between closely related species to be examined following secondary contact. Thus, a range of population divergences and

distribution patterns exists that involve the Trinidadian guppy, and they are exploited in this thesis to further our understanding of speciation.

This chapter initially summarises what is currently known about speciation (section 1.3), concentrating on the evolution of intrinsic isolation between allopatric populations, whose study constitutes the main body of this thesis. In particular, studies examining the genetic architecture and the rate of evolution of intrinsic isolation are discussed, since they may have bearing on the nature and magnitude of any intrinsic isolation that exists between guppy populations. Next (sections 1.4), I introduce various components of guppy biology that are pertinent to the study of population differentiation, including geographical trait variation, reproductive biology, dispersal and phylogenetics. Finally (section 1.5), I present the objectives of the research that is described in subsequent experimental chapters.

1.3 Speciation

1.3.1 The Biological Species Concept

Speciation is the splitting up of one species into several. Countless species definitions and diagnostic tools now exist to evaluate species status (Mayden 1997; Lee 2003; Sites & Marshall 2003; Turner 2000 provides a perspective from fish biology), and it seems unlikely that any one definition can be universally applicable (Nelson 1989). This is 'The Species Problem'. One factor contributing to this problem is the difficulty in determining whether the organismal group designated as a species by taxonomists can justifiably be considered a species using evolutionary criteria (Hey et al. 2003). To a large extent this problem is mitigated by the Biological Species Concept (BSC), which posits that species are "groups of (potentially) interbreeding natural populations, that are reproductively isolated from other such groups (Mayr 1970)." This definition therefore explicitly allows hypothetical species groupings to be tested by examining the reproductive compatibility amongst them. The BSC is the most widely accepted and practised species definition amongst biologists studying sexually reproducing organisms, especially amongst zoologists. Under the BSC, species boundaries are not sharply demarcated; rather, until the development of complete reproductive isolation, boundaries are typically porous such that recently evolved sister species may experience gene flow at loci unimportant for maintaining species distinctiveness (e.g., Rieseberg & Wendel 1993; see Wu 2001 for

review). Reproductive isolation is perhaps effectively complete when incipient biological species are not subject to selective sweeps, which are based on advantageous mutations arising in sister taxa (Rieseberg et al. 2003).

Under the BSC, speciation research becomes the study of geographical and biological barriers to gene flow.

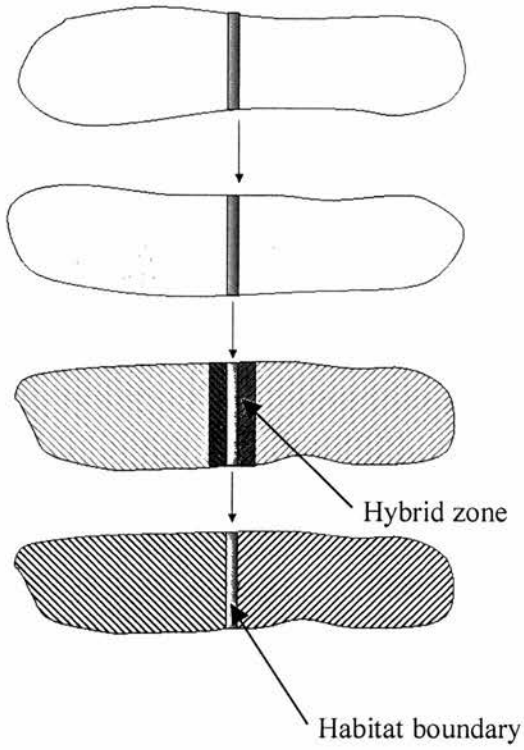
Geographical barriers to gene flow

Many modes of geographical speciation theoretically exist, though substantive evidence for some is lacking. For instance, in parapatric speciation, RI evolves between lineages having abutting ranges; these lineages are distributed according to clinal or stepping- stone models, but experience gene flow only incompletely because of environmental differences between populations (Fig. 1.1a; Rieseberg et al. 2003). Although parapatry is occasionally purported to be the most common geographical speciation mode (Endler 1995; Gavrilets 2003), it is presently uncertain whether this is the case (Coyne & Orr 2004, p. 112).

A similar situation exists with sympatric speciation, where RI evolves between populations that breed in the same area. Because models of sympatric speciation generally lack biological realism, the plausibility of sympatric speciation must be deduced from empirical case studies (Turelli et al. 2001). But despite attracting considerable theoretical attention, compelling examples of non-polyploid based sympatric speciation are currently non-existent.

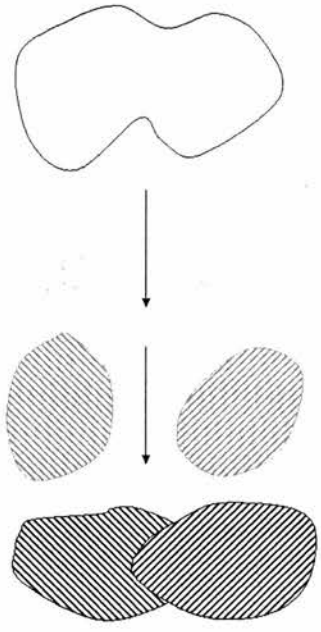
Substantive evidence for parapatric or sympatric speciation is presently weak due to the general lability of species ranges, which renders either mode difficult to distinguish from allopatric speciation. A further difficulty with sympatric speciation is that it is uncertain how the evolutionary factors causing population divergence could overcome the homogenising effects of gene flow (Slatkin 1987; Coyne & Orr 2004, p. 123).

In contrast, allopatric speciation is conceptually simple and, on empirical grounds, seems very common (Lynch 1989; Rice & Hostert 1993). Here, RI is an inevitable consequence of sustained geographical isolation. No assumptions exist regarding the evolutionary forces driving population divergence; rather, such divergence may result from differential



A. Parapatric speciation

Geographically adjacent populations are subject to divergent selective regimes. Despite some initial hybridisation, reproductive isolation eventually ensues.



B. Allopatric (vicariant) speciation

A physical barrier splits a formerly contiguous population into separate components that, having evolved independently for sufficient time, can no longer hybridise upon subsequent admixture.

Fig. 1.1. Cartoons illustrating parapatric (A) and allopatric vicariant (B) speciation.

selection regimes, the evolution of different adaptations to identical environments, or genetic drift.

Allopatric speciation may occur vicariantly or peripatrically. In vicariant speciation, geographical barriers emerge within formerly contiguous distributions to isolate large populations, between which RI then evolves unimpeded by gene flow (Fig. 1.1b). Evidence for vicariant speciation comes mainly from the concordance of species range boundaries with geographical barriers. For example, allopatric speciation is implicated in the divergence of multiple pairs of sister species that are separated by the Isthmus of Panama, since their divergence seems to postdate the appearance of the Isthmus (Lessios 1998).

In peripatric speciation, reproductive isolation evolves after the colonization of an isolated area by a small number of individuals ("founder events"). Thus, population divergence may entail much greater drift than in vicariant speciation. Substantive evidence for peripatric speciation comes from the existence of endemic taxa in isolated islands (Emerson 2002). It can often be difficult to distinguish peripatric from vicariant speciation due to lability in range sizes and to the emphemerality of some geographic barriers.

Finally, Wiens (2004) has recently suggested that the evolution of RI barriers may only constitute one component of allopatric speciation, and one that is only important in maintaining lineage distinctiveness by preventing introgressive hybridisation. According to Wiens (2004), other factors are more important in actually causing lineage divergence; that is, in the formation of new species. These factors shape habitat specialisation within a species, and so ultimately constrain an individual's ability to disperse between allopatric populations that share the same habitat, but which are separated by a different habitat populated by a related taxon. Whilst intuitively appealing, Wien's conceptualisation does not have universal applicability. For instance, it can not often be used with freshwater fishes, since the habitat specialisation that precludes their interpopulation dispersal (i.e., the inability to cross land) evolved millions of years before any lineage splitting. In such cases, the study of RI barriers is the study of speciation.

Biological barriers to geneflow

Biological barriers can either exist pre- or post-zygotically. Pre-zygotic barriers include behavioural isolation that is caused by divergent mating preferences between populations, functional incompatibilities involving genitalia and gametic isolation. Components of gametic isolation are relatively poorly studied, but may include reduced inseminate volumes (e.g., Price et al. 2001), behavioural inviability of sperm in a heterospecific's reproductive tract, coevolution of genes that mediate egg-sperm contact (e.g., Kresge et al. 2001) and inefficiencies in sperm storage (e.g., Price et al. 2001). In contrast, post-zygotic isolation can be developmentally mediated ("intrinsic isolation"), in which case it is manifested as reductions in hybrid fertility or viability. Or, it can be ecologically mediated ("extrinsic isolation"), wherein selection occurs against hybrids possessing an intermediate phenotype. In extrinsic isolation, selection promoting population divergence is functionally related to selection against hybrids, whereas these selection modes are just indirectly related to each other in intrinsic isolation.

Several lines of evidence suggest that there are typically many biological barriers isolating species in nature. For instance, several often operate in single hybrid zones (e.g., Barton & Hewitt 1985), and laboratory hybridisation experiments nearly always uncover multiple barriers between single pairs of taxa (see Table 1.1 for some examples that I compiled from the literature). In fact, most crossing experiments do not evaluate all possible barriers, suggesting that the number currently involved in isolating taxa is usually underestimated. For example, behavioural sterility during mating is rarely assayed, even though it is probably a significant component of intrinsic isolation (Chapter 2.5.1).

However, the general importance (and number) of individual barriers in *causing* speciation is essentially wholly unknown (Tregenza 2002; Mendelson 2003). One problem has been the traditional concentration of crossing studies on inter-specific taxa. Comparative phylogenetic studies, which consider the relative rate of evolution of different barriers, offer one means for resolving this problem (e.g., Coyne & Orr 1997; Mendelson 2003). Yet, such studies are rare, and have not always been appropriately conducted (see Chapter 2.2). So, in order to draw more robust conclusions regarding the relative importance of RI barriers, one must analyse multiple barriers across a wide range of population divergences, within many different species.

Cross	Habitat isolation	Behavioural isolation	Gametic incompatibilities	Hybrid sterility	Hybrid inviability	Extrinsic isolation	Other	References
Mimulus lewisii – *M. cardinalis* (monkey flowers)	Yes	-	Conspecific pollen precedence	Yes	Yes		Pollinator isolation	11
Platanthera bifolia – *P. chloranthia* (orchids)	-	Asynchronous flowering	-	-	-	Hybrids less attractive to pollinators	Mechanical isolation in flower-pollinator compatibility	7
Drosophila simulans – *D. mauritiana*	-	Assortative mating; reduced copulation times	Heterospecific sperm incapacitation; inefficiencies in heterospecific sperm storage	Yes	Yes	-		9, 10, 12, 13
Drosophila simulans – *D. sechelia*	-	Assortative mating	Smaller heterospecific inseminates	Yes	Yes	-		2, 4, 10
Echinometra oblonga – *E. mathaei* (sea urchins)	Yes	Asynchronous spawning	Disruptions to sperm-egg interactions	-	-	-		5, 8

Cross	Habitat isolation	Behavioural isolation	Gametic incompatibilities	Hybrid sterility	Hybrid inviability	Extrinsic isolation	Other	References
Mytilus edulis – *M. galloprovincialis* (mussels)	Yes	Asynchronous spawning	Assortative fertilisation	-	Yes	-		1
Heliconius cydno – *H. melpomme* (butterflies)	-	Assortative mating	-	Yes	-	Increased predation susceptibility of hybrids		6
Anartia fatima – *A. anathea* (butterflies)	-	Assortative mating	-	Yes	Yes	-	Behavioural inviability of female F_1s	3

Key: -, data unavailable. 1, Bierne et al. 2002; 2, Coyne 1992; 3, Davies et al. 1997; 4, Hollocher & Wu 1996; 5, Metz et al. 1994; 6, Naisbit et al. 2002; 7, Nilsson 1983; 8, Palumbi & Metz 1991; 9, Price et al. 2000; 10, Price et al. 2001; 11, Ramsey et al. 2003; 12, Tao et al. 2003a; 13, Tao et al. 2003b.

Table 1.1 (continued). Representative crosses that have documented the presence of multiple reproductive barriers between single pairs of taxa.

1.3.2 The evolution and genetic architecture of intrinsic isolation

The Dobzhansky-Muller model

Various processes can cause intrinsic isolation, including differences in ploidy between related taxa (Stebbins 1950) and chromosomal rearrangements that result in mis-segregation during meiosis (Edmands 2002). But it seems likely that genic incompatibilities, which may evolve according to the Dobzhansky-Muller model (see below), effect intrinsic isolation more often than any other factor, at least in animals (Howard et al. 2002). For example, strong evidence for the role of chromosomal rearrangements in animal speciation is only available from mammals (Searle 1993). Both chromosomal and polyploid speciation are, however, more important in plants.

Dobzhansky (1936) and Muller (1940) considered that hybrid sterility and inviability result as pleiotropic byproducts of independent evolution in allopatric populations. In the Dobzhansky-Muller (D-M) model, alleles that enhance fitness on the normal genetic background may lower fitness when brought together in hybrids with alleles from another taxon. Thus, intrinsic isolation results from the accumulation of deleterious epistatic interactions in hybrids (Fig. 1.2).

Although D-M incompatibilities are generally assumed to act intrinsically, environmental conditions can affect their expression (e.g., Maside et al. 1998; Wade et al. 1999; Willett & Burton 2003; see Bordenstein & Drapeau 2001 for review). Indeed, when the effects of genotype-by-environment interactions are entered into D-M models, the number of negative epistatic interactions increases considerably over that predicted by the standard D-M model (Bordenstein & Drapeau 2001). So, GxE interactions may enhance the speed at which RI is attained, and it may be necessary to examine reproductive barriers under different environments to properly appreciate their potential significance.

Lynch & Force (2000) proposed a RI mechanism related to the D-M model. This entails divergent resolution of gene duplicates, wherein copies that are located at different chromosomal locations between populations undergo loss of function or subfunctionalisation. As with the D-M model, incompatibilities accumulate with no loss of fitness within populations. However, only very circumstantial evidence currently exists for the hypothesis (Taylor et al. 2001), which, at any rate, may only have limited utility for

explaining hybrid unfitness (Coyne & Orr 2004, p. 313).

Finally, the D-M model is perhaps more appropriately termed the Bateson model, since Bateson (1909; quoted in Coyne & Orr 2004) was the first to elaborate upon the contribution of genic incompatibilities to hybrid unfitness.

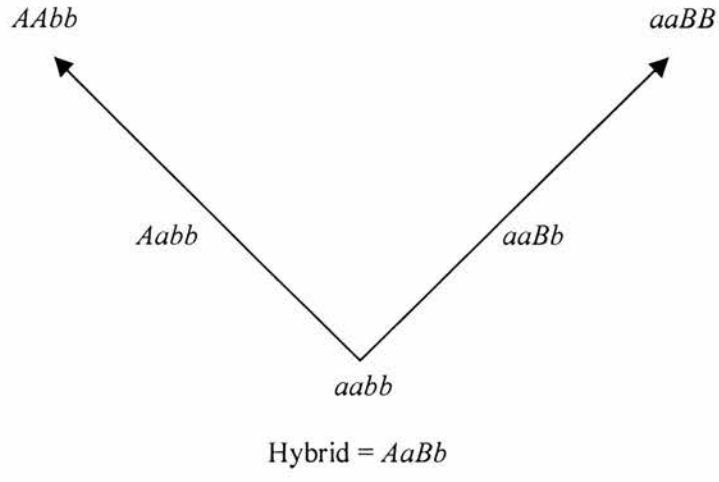


Fig. 1.2. The evolution of a simple two-locus Dobzhanky-Muller incompatibility. Independent evolution within two lineages leads to the fixation of alternative alleles at two loci, which subsequently interact epistatically in hybrids to reduce fitness. Taken from Coyne & Orr (2004).

Theoretical and empirical studies of the genetic architecture of intrinsic isolation

The genetic architecture of a quantitative trait encompasses many components, including (1) the number and chromosomal locations of loci affecting that trait; (2) the relative magnitudes of the effects of these loci both intra- and inter-specifically; (3) the extent of additive and epistatic gene actions, and of pleiotropy; (4) variation in dominance, epistatic and pleiotropic relations between sexes and environments; (5) the molecular basis of quantitative trait loci (QTL); and (6) QTL allele frequencies within and between populations. The genetic architecture of any trait has yet to be elucidated in such detail (Mackay 2001), and that of reproductively isolating traits remains particularly poorly characterised (Presgraves 2003). Studies investigating the genetic architecture of intrinsic isolation employ backcross introgression experiments or QTL-mapping (Coyne & Orr

1998; Orr & Irving 2001), and their results have often corroborated those from recent mathematical explorations of the D-M model.

The severity of hybrid unfitness and the number of loci underlying incompatibilities should increase as the square of time separating two taxa (Orr 1995; Orr & Turelli 2001). Consistent with this effect, in *Drosophila*, few genetic factors contribute to hybrid male sterility between recently diverged subspecies (Orr & Irving 2001), whereas many more are implicated in inter-specific crosses (e.g., Cabot et al. 1994; Davis & Wu 1996). Theoretical work has also suggested that complex incompatibilities involving three or more genes should evolve more easily than pair-wise incompatibilities (Orr 1995; Orr & Turelli 2001). This prediction has been substantiated by many studies describing complex patterns of epistasis (e.g., Orr & Coyne 1989; Cabot et al. 1994; Carvajal et al. 1996; Orr & Irving 2001) and the apparent relative scarcity of pair-wise incompatibilities. Nevertheless, simple incompatibilities do exist, as evidenced in *D. melanogaster* - *D. simulans* hybridisations, where hybrid fitness can be restored upon the introduction of single hybrid rescue mutations, which are probably alleles at those loci causing hybrid unfitness (e.g., Hutter et al. 1990; Sawamura et al. 1993; Barbash et al. 2003a). Moreover, single two-locus incompatibilities have resulted in isolation in the plant genera *Mimulus* (Christie & Macnair 1984) and *Crepis* (Hollingshead 1930; quoted in Orr & Turelli 2001). So, the number of genes responsible for initially causing speciation can easily be overestimated using inter-specific crosses, and intrinsic isolation does not necessarily have a complex basis, though it often does.

Besides epistasis, another gene action underlying hybrid unfitness is reccessivity. According to the 'Dominance theory', alleles involved in hybrid incompatibilities are, on average, partially recessive (Turelli & Orr 1995). The Dominance theory may explain Haldane's rule (see below) as well as hybrid breakdown, where reductions in fertility and viability only become evident amongst post-F_1 hybrid generations (Turelli & Orr 2000). Evidence for such recessivity is compelling for hybrid inviability (e.g., Turelli & Begun 1997; Presgraves & Orr 1998), but relatively lacking for hybrid sterility (Hollocher & Wu 1996; True et al. 1996).

In relation to the chromosomal distributions of incompatibilities, they map disproportionately to the X chromosome in *Drosophila* (e.g., Tao et al. 2003b). This

'large-X effect' is probably due to elevated rates of mutation substitutions on X-linked over autosomal loci (Tao et al. 2003a), rather than to a greater density of viability- or reproduction-related genes on the X chromosome (Naveira 2003). The large X-effect has also been documented in lepidoptera (e.g., Davies et al. 1997; Jiggins et al. 2001) and anophiline mosquitos (Slotman et al. 2004).

Lastly, only four genes that contribute to intrinsic isolation have been characterised at a molecular level (Table 1.2). These genes are involved in many different kinds of processes, and three are related to transcriptional regulation (*Xmrk*, *OdsH* and *Hmr*), supporting the common postulate that species divergence is regulatory in nature (Wu & Ting 2004).

Cross	Gene	Effects on hybrids	Putative identity / function	Positive Darwinian Selection?	Reference
D. simulans - *D. melanogaster*	*Nup96*	Male lethality	Nuclear pore protein	Yes	2
D. melanogaster, *D. simulans*, *D. mauritiana*, and *D. sechellia*	*Hmr*	Lethality in both sexes; female sterility	Transcriptional regulation	?	1
D. simulans - *D. mauritiana*	*OdsH*	Male sterility	Homeobox gene	Yes	3,4
Xiphophorus maculatus - *X. helleri*	*Xmrk-2*	Lethality in both sexes	Tyrosine kinase receptor copy	?	5

Key: 1, Barbash et al. 2003b; 2, Presgraves et al. 2003; 3, Ting et al. 1998; 4, Sun et al. 2004; 5, Wellbrock et al. 2002.

Table 1.2. Genes contributing to intrinsic isolation that have been characterised at a molecular level.

In summary, the D-M model is the most widely accepted model explaining the evolution of incompatibilities causing hybrid unfitness. Yet, its acceptance is largely based on experiments with *Drosophila*. Crossing work in additional taxa is needed to evaluate the broader significance of the D-M model and of phenomena characterised from *Drosophila*.

1.3.3 Patterns in post-zygotic isolation

Because the genetic architecture of intrinsic isolation requires so much effort to elucidate, comparative phylogenetic analyses have proved useful in identifying general but relevant patterns. These analyses relate the magnitude of hybrid sterility and inviability to genetic divergence, a surrogate for time, and provide insights into what may be expected from any individual crossing experiment. These studies have detected several notable trends:

(a) In fish (Russell 2003; Fig. 1.3), *Drosophila* (Coyne & Orr 1989, 1997; Fitzpatrick 2002), birds (Lijtmaer et al. 2003) and Lepidoptera (Pregraves 2002), inter-specific hybrid unfitness increases gradually suggesting that it is usually due to the gradual accumulation of deleterious epistatic interactions amongst species. Positive correlations between sequence divergence and hybrid unfitness have also been reported from intra-specific crosses involving two crustacean (Lonsdale et al. 1988; Edmands 1999) and one ascidian taxa (Grosberg 1987). However, sequence divergence is an unreliable predictor of hybrid fitness (Coyne & Orr 1997; Vogler 2001), possibly because it inaccurately represents total-genomic divergence and because of publication biases (Edmands 2002). Nevertheless, differences among groups in the rate of evolution of hybrid unfitness are apparent. For example, total post-zygotic RI is attained most slowly in fishes and birds (Table 1.3). These differences may reflect differences in generation time (Marzluff & Dial 1991; Chao & Carr 1993), the degree of sex chromosome differentiation (Turelli & Orr 2000) or the ability of lineages to accumulate incompatibilities before RI occurs (Thorgaard & Allendorf 1988). The last possibility could result from differences in the flexibility of regulatory controls on gene expression (Prager & Wilson 1975), or from an accelerated evolution of mother-offspring conflicts in viviparous versus egg-laying organisms (Zeh & Zeh 2000). In short, intrinsic isolation generally evolves slowly, but its magnitude is impossible to predict due to the stochastic nature of its evolution and to the simultaneous operation of many different

processes (e.g., the slower evolution of hybrid unfitness in fish may be counterbalanced by the effects of viviparity).

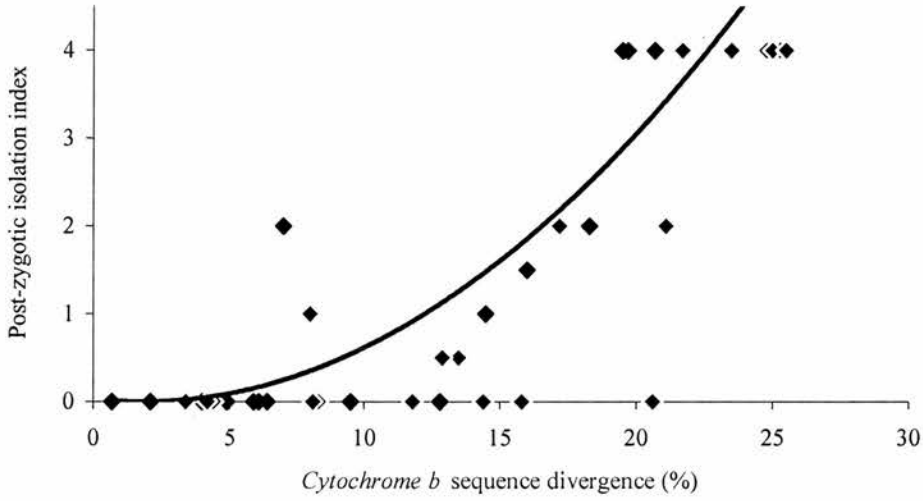


Fig. 1.3. Plot of the strength of intrinsic isolation against mitochondrial *cytochrome b* distances (Kimura-2-parameter corrected). Isolation index is as follows: 0, both sexes fertile; 0.5, one sex fertile, the other sex some individuals recorded as fertile; 1, one sex fertile, one sex viable but infertile; 1.5, one sex sometimes fertile, one sex viable but infertile; 2, both sexes viable but infertile; 2.5, one sex viable but infertile, one sex sometimes viable; 3, one sex viable, one sex missing; 3.5, one sex sometimes viable, one sex missing; 4, both sexes missing. Data have been controlled for phylogenetic non-independence (see Russell 2003 for details). Line of best fit is a second order polynomial.

(b) Hybrid sterility evolves sooner than hybrid inviability in taxa as divergent as Lepidoptera (Presgraves 2002), *Drosophila* (Coyne & Orr 1989, 1997), fish (Russell 2003) and birds (Price & Bouvier 2002). This is probably because sex- and reproduction-related genes evolve more rapidly than viability-related ones (Singh & Kulathinal 2000), perhaps because of sexual selection (Wu & Davis 1993) and sexual conflict (Howard et al. 1998; Rice & Chippindale 2002).

(c) Haldane's rule, which states that "when, in the offspring of two different animal races, one sex is absent, rare, or sterile, that sex is the heterozygous [heterogametic] sex (Haldane 1922)." Haldane's rule is very widespread and has been documented

in both groups with heterogametic males (e.g., Mammalia, Amphibia, Reptilia, Diptera), and those with heterogametic females (Orthoptera, Heteroptera, Lepidoptera, birds) (Coyne & Orr 1989, 1997; Laurie 1997; Orr 1997; Presgraves 2002; Price & Bouvier 2002; Lijtmaer et al. 2003). Haldane's rule is probably a composite phenomenon reflecting both faster male evolution and the Dominance theory (Wu et al. 1996; Laurie 1997; Orr 1997; Presgraves & Orr 1998; Turelli 1998), although the latter may have greater general significance (Orr 1997), at least in cases not involving male hybrid sterility in *Drosophila* (Naveira 2003).

Taxonomic group	Mean time to total intrinsic isolation (myr)	Maximum age for partial reproductive compatibility (myr)
Drosophila[1]	~5.0	~7.8
Birds[2]	~11.5	~16.8
Lepidoptera[3]	~3.5	~7.5
Amphibians[4]	~4.2	~9.0
Fish[5]	~11.6	~10.3

Table 1.3. Estimated mean times to complete intrinsic isolation and maximum ages for partial fertility in various taxonomic groups. Data compiled from comparative studies in: [1], Coyne & Orr 1997; [2], Price & Bouvier 2002; [3], Presgraves 2002; [4], Sasa et al. 1998; [5], Russell 2003. Estimates were generated from allozyme (Refs. 1 - 4) and mitochondrial sequence (Ref. 5) data.

In all, the evolution of intrinsic post-zygotic isolation is a complicated process, likely to exhibit numerous taxon-specific properties. It is thus necessary to investigate different taxa to identify the phylogenetic distribution of these properties. Teleost fish are particularly suitable vertebrate models for such work because they comprise the most diverse vertebrate lineage - approximately half of all bony vertebrates are teleosts, which number some 23,600 species (Nelson 1994) - and because they possess a wide range of sex-determination systems. Unfortunately, although the fish hybridisation literature is vast (Schwartz 1981), few studies have considered intrinsic isolation in relation to speciation,

for most hybridisation experiments were made prospectively by the aquacultural industry (Bartley et al. 2000), and so were rarely appropriately designed to have any incidental utility for speciation research. Guppy populations from the Caroni and Oropuche Drainages of Trinidad are well placed to redress this deficiency because they show considerable overall genomic and adaptive genetic differentiation, suggesting that some intrinsic isolation may already exist. Moreover, they belong to the same species, avoiding the restriction of having to study speciation from post-hoc analyses of phenomena amongst inter-specific taxa.

1.4 The guppy

The cyprinodontiform family Poeciliidae is endemic to the neo-tropics and neo-subtropics (Rosen & Bailey 1963; Parenti & Rauchenberger 1989). It contains various species important in reproductive biological research, including *Poeciliopsis spp.*, *Xiphophorus spp.*, and *Poecilia spp.* (see Chapter 5.1). The guppy, *Poecilia reticulata*, is a freshwater fish species naturally distributed across North-eastern S. America; namely, Guyana, Surinam, Trinidad and Venezuela (Rosen & Bailey 1963). Additional wild populations exist worldwide because of artificial introductions for mosquito control (Courtney & Meffe 1989).

1.4.1 Evolutionary biology and behavioural ecology

Despite the broad natural distribution of guppies, research into their behavioural ecology and evolutionary biology has mostly used populations from Trinidad's Northern Range (Houde 1997; Magurran & Phillip 2001) (Fig. 1.4). This is largely because these populations show considerable adaptive geographic differentiation across a whole suite of characters, including morphological, behavioural, physiological and life-history traits (Endler 1995; Magurran et al. 1995). This differentiation is mainly due to changes in predation regime within rivers (Endler 1995). Because waterfall barriers impede the upstream dispersal of fish predators, downstream sites (Fig. 1.5c) host more diverse predator communities than upstream sites (Fig. 1.5a,b), which typically lack the important cichlid predators *Crenicichla alta* and *Aequidens pulcher*. The importance of predation can easily be gauged by considering that predation risk has accounted for up to ~ 70% of the variance in life-history traits (Strauss 1990). Other environmental factors such as

temperature, light intensity and stream size may interact with predation risk to influence geographical trait variation (Endler 1995).

In general, such trait differentiation appears to have a genetic basis. For instance, variation in many behavioural traits including schooling propensity (Seghers 1974), predator inspection behaviour (Magurran & Seghers 1994) and predator escape ability (O'Steen et al. 2002) is partially heritable. Female mating preferences also vary geographically and are genetically correlated with male colour traits, leading to incomplete sexual isolation between some populations (e.g., Ballin 1973; Houde 1988; Luyten & Liley 1991; Houde 1994; Endler & Houde 1995; Magurran et al. 1996; see Lande 1981). Male colour phenotypes are less conspicuous in high predation sites, and different combinations of genes in different populations are responsible for these changes (Endler 1980). In relation to life-history traits, significant heritabilities have been established for, amongst others, brood size, offspring size, age and size of males at maturity, and age and size of females at first parturition (Reznick et al. 1990). Finally, phenotypic plasticity also contributes to phenotypic variation (e.g., Reznick 1982), perhaps through effects of the demographic environment (e.g., differences in sex-ratio, density, etc.; Rodd et al. 1997).

Much of the work on the genetic basis of trait variation comes from manipulations of predation regimes in introduction experiments. These have often induced substantial hereditary changes over only several generations (e.g., Reznick & Bryga 1987; Reznick et al. 1990, 1997; O'Steen et al. 2002). Collectively, these experiments provide important examples of contemporary evolution (Kinnison & Hendry 2001; Stockwell et al. 2003).

Guppies are also important because they provide outstanding demonstrations of the effects of sexual selection. Sexual selection in guppies mainly operates through female mate choice, although the significance of male-male competition may be greater than currently envisaged (Houde 2001). The larger cryptically coloured females (Fig. 1.6a) evaluate a plethora of male traits, including colour patterns (Houde 1997; Fig. 1.6b), body size (Reynolds & Gross 1992; but see Endler & Houde 1995), ultraviolet markings (Kodric-Brown & Johnson 2002; Smith et al. 2002) and perhaps 'sigmoid' displays (Kodric-Brown & Nicoletto 2001). Courting males perform sigmoid displays to obtain consensual copulations with receptive females, and in these displays males bend their body into a characteristic S-shape (Fig. 1.6c).

All males employ an alternative non-consensual mating tactic – 'sneaky' mating - in which they thrust their intromittant organ towards the genital opening of non-receptive females (Fig. 1.6d). In poeciliids, the intromittant organ is derived from a modified anal fin, called the gonopodium. Sneaky mating is highly prevalent, occurring at rates of up to one per minute per male (Magurran & Seghers 1994; Magurran et al. 1995). It is a facultative behaviour used opportunistically according to environmental and ecological circumstances; hence, it is more frequently used with increased predation risk (Magurran & Seghers 1990), reduced light intensity (Luyten & Liley 1985; Endler 1987) and more male-biased population sex ratios (Farr 1976; Rodd & Sokolowski 1995). By circumventing female choice, sneaky mating may retard adaptive differentiation between populations connected by dispersal. It may also affect the dynamics of sexual selection within populations, and so retard the evolution of behavioural isolation between populations too (Magurran 1996, 1998, 2001; see Chapter 4.2). However, the efficacy of the tactic for achieving inseminations and fertilisations remains contentious (see Chapters 4 and 5.2). Baerends et al. (1955) and Liley (1966) provide more detailed descriptions of male and female sexual behaviour.

1.4.2 Dispersal

Environmental factors, other than predation risk, change with river location. For example, decreases in water velocity, and increases in temperature, light intensity, water depth, productivity and water turbidty occur as one goes downstream (Reznick et al. 2001). So, populations within the same river often experience very different selection regimes. Yet, the extent to which inter-population dispersal hinders adaptive differentiation is unknown. That such effects are feasible is suggested by several considerations. First, any existing dispersal could readily be translated into gene flow by the sneaky mating of immigrant males, incomplete sexual isolation based on female mating preferences (Houde 1994) and rare male advantage in sexual selection (Hughes et al. 1999). Second, gene flow-induced constraints on adaptive differentiation are more likely when the spatial scale of gene flow is large relative to that of habitat change; a situation Endler (1977, 1995) has suggested exists for Trinidadian guppy populations.

Unfortunately, the absence of suitably variable molecular markers has hitherto constrained attempts to estimate dispersal rates. The recent development of multiple microsatellites at St. Andrews provides an opportunity to redress this deficiency (Becher et al. 2002 (presented as Appendix II), Becher & Magurran 2004).

1.4.3 Inter-population genetic divergence

In Trinidad, guppies show a hierarchical pattern of population genetic divergence that corresponds to the island's geography. Carvalho et al. (1991) found that ~ 66% of allozyme diversity exists between river drainages, 32% between rivers within drainages, and 2% within rivers. Founder effects have probably been particularly important in shaping these patterns (Shaw et al. 1994; Magurran et al. 1995; Carvalho et al. 1996).

At a broader regional level, molecular phylogenetic work using mitochondrial genes has uncovered two principal guppy clades. One clade comprises populations from Guyana, Suriname, Venezuela and Trinidad's Caroni Drainage, and the other populations from Trinidad's Oropuche drainage. Relationships within the former clade have not yet been resolved (Fig. 1.7; see Appendix I for a description of the phylogenetic methodology). So, although the Caroni and Oropuche drainages are geographically separated by as little as 70 m during the wet season, their populations are amongst the most genetically divergent known from throughout the guppy's natural range. This incongruity lacks a simple explanation because the geological history of the region is highly complex (Donovan & Jackson 1994). One hypothesis that has been advanced invokes independent colonisation of the drainages by different lineages from the Venezuelan mainland (Carvalho et al. 1991; Fajen & Breden 1992). Unfortunately, artificial introductions are posing an increasingly great threat to the genetic integrity of native populations in Trinidad (Magurran et al. 1995) and, in at least one instance, they have led to admixture between Caroni and Oropuche guppies (Chapter 3).

Either *Poecilia picta* (Fig. 1.6.e,f) or *P. parae* is the putative sister species to guppies (Breden et al. 1999). In Trinidad, guppies occasionally co-occur with *P. picta* in freshwater habitats adjacent to brackish waters. *P. parae* is not found in Trinidad.

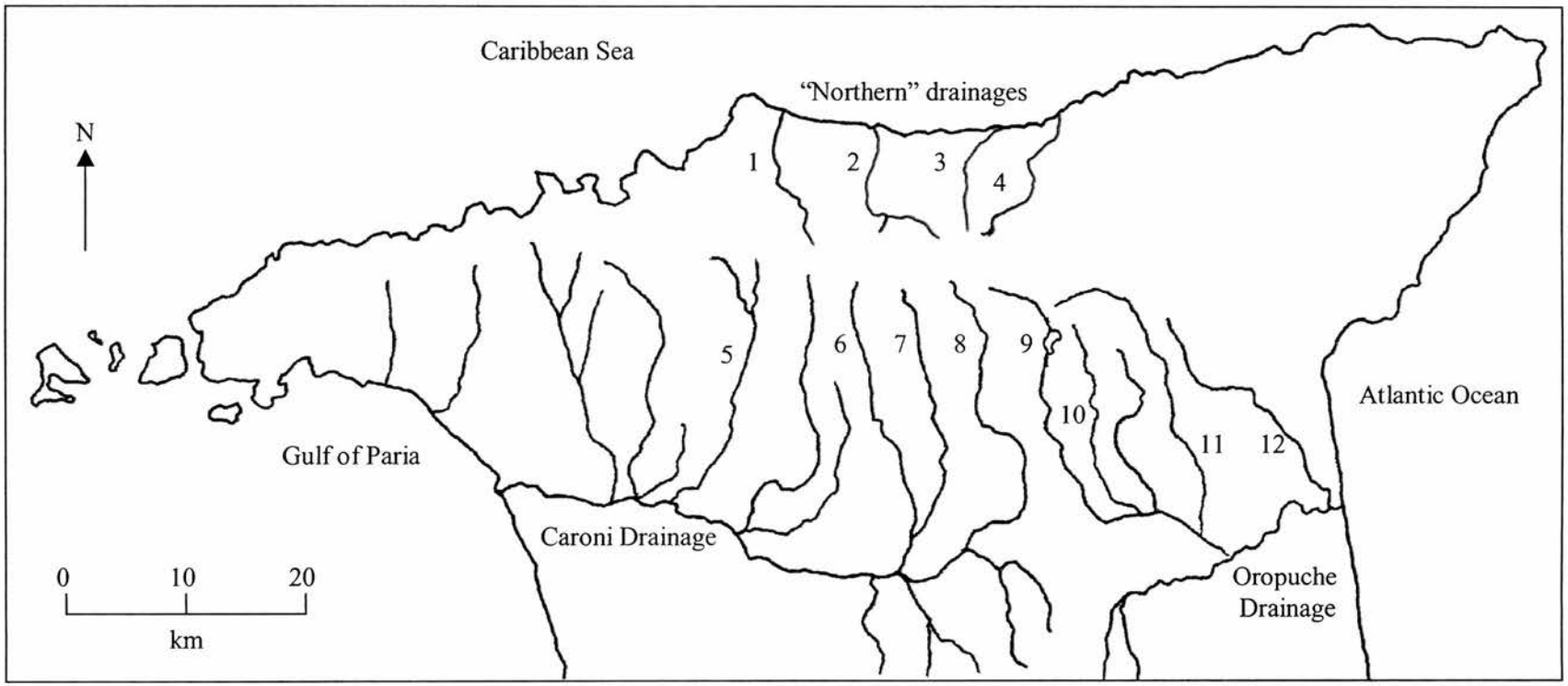
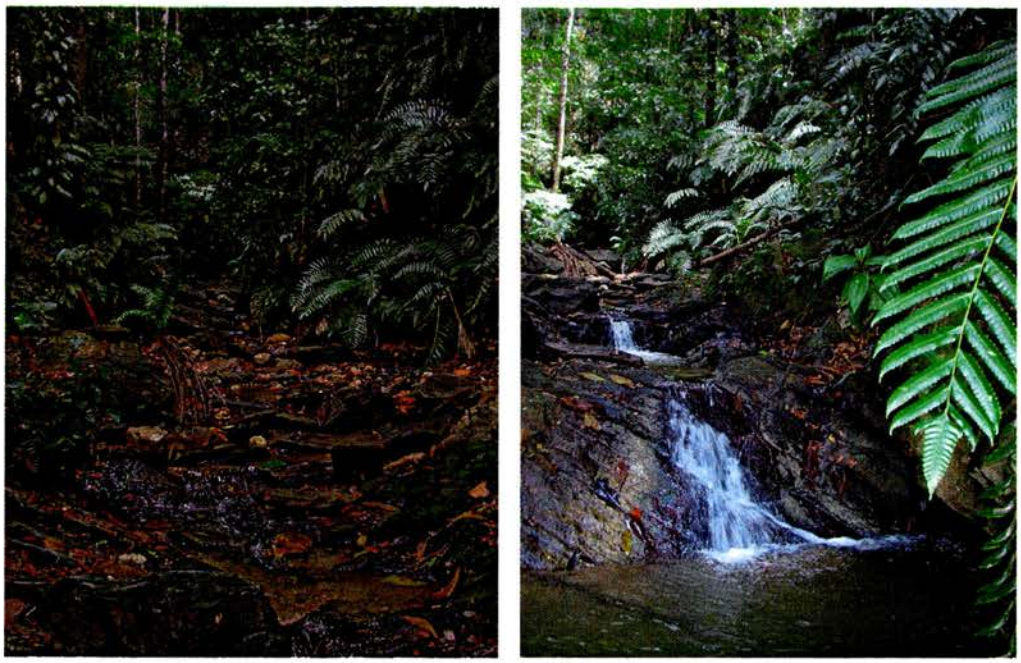
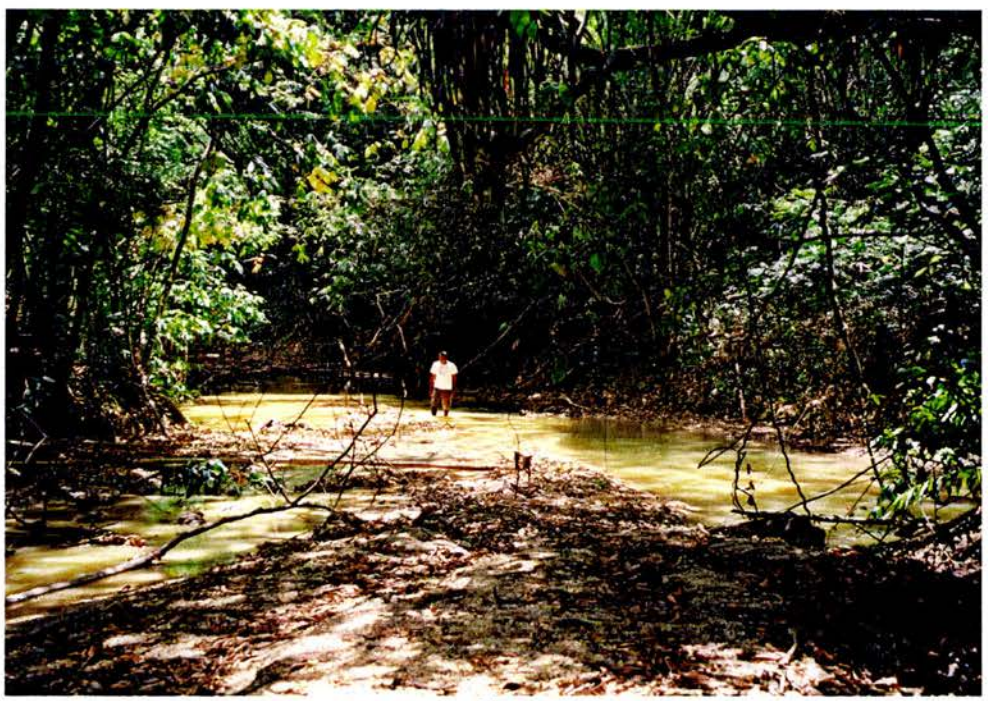


Fig. 1.4. Map of Trinidad's Northern Range showing principal drainages and rivers. 1, Yarra; 2, Marianne; 3, Paria; 4, Madamas; 5, Tacarigua; 6, Arima; 7, Guanapo; 8, Aripo; 9, Quare; 10, Turure; 11, Oropuche; 12, Rio Grande.



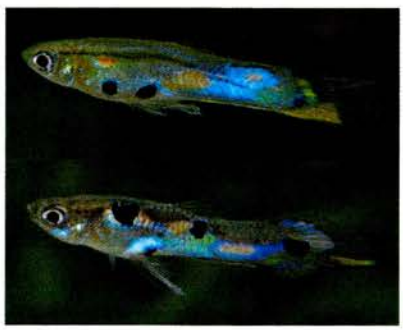
A. B.



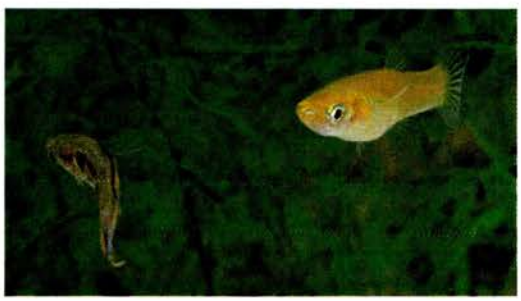
C.

Fig. 1.5. Typical guppy habitats in Trinidad's Northern Range. **A** and **B,** upstream low predation sites in the Upper Aripo River; **C,** downstream high predation site at the confluence of the Turure and Quare Rivers.

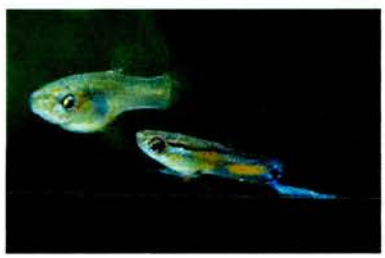
A. Female guppy



B. Male guppies



C. A male 'sigmoid' display



D. An attempted sneaky mating

E. A *P. picta* female

F. Male *P. picta*

Fig. 1.6. Photographs illustrating male and female phenotypes in guppies, and in one of their putative sister species, *Poecilia picta*.

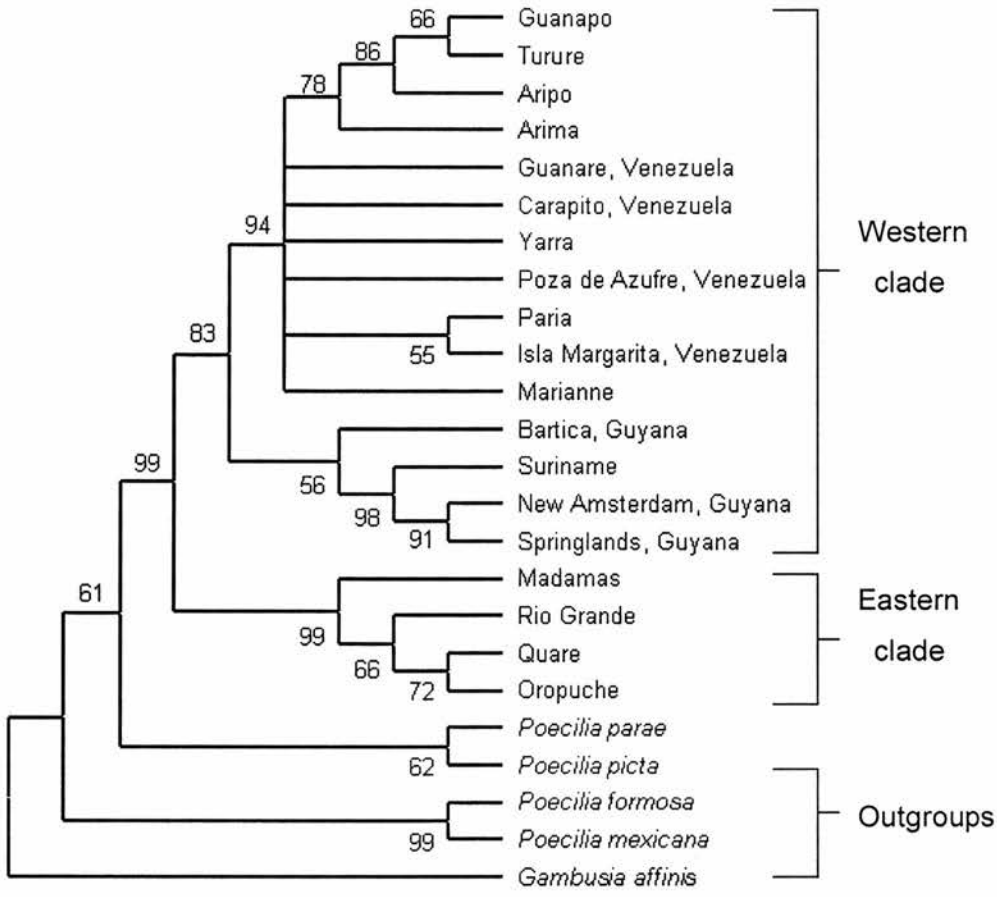


Fig. 1.7. Maximum parsimony consensus tree. Node support estimated with 1000 bootstraps. Only those nodes with ≥ 50% support are indicated. See Appendix I for further details of construction.

1.4.4 Sex-determination system

Guppies possess cytogenetically distinct sex chromosomes (Winge 1922; Nanda et al. 1990; Traut & Winking 2001). Their sex determination mechanism is essentially XX-XY, although autosomal sex determining loci also exist (Volff & Schartl 2001). Consequently, autosomal sex-determination (ASD) occasionally occurs, forming YY males and XY females. YY males are fully viable and fertile, indicating that sex chromosome differentiation is only modest and has not been accompanied by any loss of essential genes on the Y chromosome. Whether ASD is due to a polymorphism of either the Y chromosomal sex-determining locus or of the autosomal modifier is not known (Volff &

Schartl 2001). But it does not appear to result from the heavy selection and inbreeding regimes associated with laboratory rearing because individuals with ASD have been found in natural populations (Haskins et al. 1961). The frequency of ASD in wild populations is not known.

Besides comparative genomic hybridisation work (Traut & Winking 2001), crossing studies have also ascertained that the sex chromosomes are molecularly differentiated. For instance, genes encoding male courtship displays occasionally appear to be Y-linked (Farr 1983; Houde 1997), and those encoding male colour traits are X- or Y-linked (though mostly X-linked; Lindholm & Breden 2002).

The Y chromosome consists of a proximal homologous and a distal differential segment. The proximal segment pairs with its homologous X chromosome counterpart in early pachytene. The differential segment, containing the postulated sex determining region, is unpaired in early pachytene, but synapses later in an equalisation step (Traut & Winking 2001). Recombination is thus suppressed in the non-homologous region, but increases with distance from the sex-determining region (Lindholm & Breden 2002).

Finally, these cytogenetic studies have provided evidence neither for chromosomal rearrangements nor differences in ploidy between guppy populations. Guppies possess 46 chromosomes in the diploid state (Nanda et al. 1990).

1.4.5 Reproductive biology

Males possess paired testes that are fused to form a single tubular organ attached to the dorsal body wall (Billiard 1986; Constantz 1989). Mature sperm form densely packed bundles called spermatozeugmata, each containing ~ 20,000 sperm (Billiard 1969). The erection of the gonopodium during mating provides a groove to facilitate sperm transfer (Rosen and Bailey 1963).

Fertilisation is internal and broods are live-born. Several days after parturition, a mature egg batch is fertilised and gestation lasts ~ 30 days (Houde 1997). There is no maternal contribution to eggs following fertilisation (ovoviviparity), and all embryos develop simultaneously to occupy the same developmental stage (i.e., non-superfetatation).

Females are sexually receptive only when virgins or for ~ 36 hours following brood production (Liley 1966). Females typically mate with two or three males (Becher & Magurran 2004), and can store sperm for up to six months (Constanz 1984) within folds of the ovary (Constantz 1984; Kobayashi & Iwamatsu 2002). In the wild, females typically produce broods of between two and eight young once a month (Reznick & Endler 1982). The mean generation time is uncertain, although it may vary from 1.7 to 4.0 generations per year (Reznick et al. 1997; Haskins et al. 1961), and depend upon the predation regime.

1.5 Speciation and the guppy

Trinidadian guppies from the Caroni and Oropuche drainages are well placed to provide a novel and essential perspective on speciation. This is because they are of the same species, allowing inferences to be made about the relative importance of different reproductive barriers during the evolution of speciation. Previously, researchers have been obliged to infer speciation processes by the post-hoc analyses of phenomena amongst inter-specific taxa, which may poorly reflect what happens during speciation. That reproductive barriers have already evolved between Caroni and Oropuche guppies is feasible given the highly genetically divergent nature of these populations. Moreover, the considerable literature that describes their evolutionary biology and behavioural ecology allows the evolution of such barriers to be interpreted within particular ecological or genetic contexts. For instance, the slight sex chromosome differentiation of guppies might allow the identification of lower thresholds for effects of sex chromosomes on speciation. To date, these effects have only been assayed in organisms possessing highly differentiated sex chromosomes, where they are a near universal feature. Further, due to geographical variation in predation risk, and so in the incidence of sneaky mating, Trinidadian guppies could shed light on behavioural causes of lineage specificity in the rates of evolution of different barriers. This latter possibility exists because sneaky mating may constrain the evolution of behavioural isolation, although it should be emphasised that the efficacy of sneaky mating for securing fertilisations currently remains unknown.

Guppies are also advantageous because they occasionally co-occur with congenerics (*P. picta*) or highly divergent conspecifics (in the case of artificial introductions). Hence, interactions between different lineages can be directly assayed in the field – an important point if speciation is to be interpreted in terms of reproductive isolation.

Finally, it is possible that guppies will become an important model for exploring the effects of gene flow on adaptive population differentiation (A. Hendry, personal communication). This is because selection regimes differ considerably between upstream and downstream portions of rivers, which are feasibly connected by considerable gene flow. Yet, population genetic analyses necessary for the study of such effects have been hampered by a dearth of suitably variable molecular markers, meaning that information even on dispersal rates is presently very limited.

1.5.1 Aims of the thesis

This thesis aims to use the Trinidadian guppy to further our understanding of population differentiation at a variety of scales, and focuses largely on the evolution of post-zygotic reproductive isolation. The underlying research forms part of a broader research programme based in the University of St. Andrews, which is also evaluating the strength of gametic and behavioural isolation. One rationale for this programme is to test the assumption, prevalent amongst guppy biologists, that "the divergence of guppies has not proceeded beyond a preliminary stage in speciation (Houde 1997)". The rationales for the work conducted for this thesis are reported for each experimental chapter below:

Chapter 2. Laboratory assays of intrinsic isolation.
This chapter reports laboratory crossing experiments between Caroni and Oropuche Drainage guppies that were designed to:

1. Test for reductions in hybrid fitness in the reciprocal F_1, F_2 and BC_1 generations. Particular attention is paid to behavioural sterility during mating since it is a rarely considered but probably generally important component of reproductive isolation.
2. Determine whether X-linked loci contribute disproportionately to hybrid unfitness
3. Compare the rate of evolution of intrinsic isolation with that of behavioural isolation, for which information is already available. This comparison should provide insights into what barriers are most likely to drive speciation in guppies.

Chapter 3. Population genetic analysis of admixed populations

Populations from the Caroni and Oropuche drainages became admixed following an artificial introduction in 1957 (see Chapter 3.2). The admixture was analysed using recently developed genetic markers to:

1. Obtain baseline information on the degree of introgression and the spread of introduced alleles.
2. Test Endler's (1977, 1995) hypothesis that spatial scales of gene flow are large relative to those of habitat change.
3. Evaluate the effective strength of reproductive barriers to gene flow

Chapter 4. Assessing the prevalence of inter-specific inseminations

Inter-specific inseminations between sympatric guppy and *P. picta* populations result from sneaky matings (see Chapter 4.2). Their frequency in both cross directions is assessed using genetic analysis of female reproductive tract extracts, to:

1. Conservatively test the efficacy of sneaky mating in achieving inseminations
2. Compare the rates of evolution of behavioural isolation and other barriers to inter-specific gene flow.

Chapter 5. Overview: Speciation and the guppy

This chapter summarises findings of the Ph.D. research, and indicates further aspects of speciation biology that Trinidadian guppies can contribute to.

CHAPTER TWO

Laboratory assays of intrinsic isolation

2.1 Summary

Previously, intrinsic isolation between guppy populations has been investigated only cursorily. To rectify this shortage I crossed guppies from the Caroni (L. Tacarigua River) and Oropuche Drainages (Oropuche R.) in the laboratory, and examined multiple aspects of reproductive compatibility in the F_1, F_2 and BC_1 generations. In open aquarium experiments, F_1 males performed fewer mating behaviours relative to parental population controls. This is the first documentation of hybrid behavioural sterility within a species, and suggests that it may feasibly be involved in causing speciation. Biometrical analysis of this dysfunction failed to detect any underlying large-X effect, consistent with the only modest degree of sex chromosome differentiation that exists in guppies. The crosses also uncovered hybrid breakdown for embryo viability, brood size and sperm counts. In contrast, no reductions in female fertility were detected, indicating that guppies obey Haldane's rule for sterility. Intrinsic isolation currently presents a much stronger obstacle to gene flow than behavioural isolation.

2.2 Introduction

Several patterns in the genetic architecture of intrinsic isolation are evident from *Drosophila* studies, notably the large-X effect and a high frequency of gene actions that involve epistasis or recessivity (see Chapter 1.3.2). Their identification represents substantial progress in speciation biology research. But the traditional concentration on *Drosophila* has occurred to the neglect of other groups, of which only occasional use has been made. Non-drosophilids that have been considered include *Nasonia* (Gadau et al. 1999), Lepidoptera (Davies et al. 1997; Jiggins et al. 2001; Naisbit et al. 2002), anophiline mosquitoes (Slotman et al. 2004) and rats (Vrana et al. 2000). These studies have at least established that large-X effect exists outside of *Drosophila* (Jiggins et al. 2001; Naisbit et al. 2002; Slotman et al. 2004). Otherwise, however, the generality of speciation phenomena characterised from *Drosophila* remains uncertain. For instance, the magnitude of sex chromosome differentiation necessary to cause the large-X effect is unknown, since

studies have previously only considered groups with highly differentiated sex chromosomes. Also, Vrana et al. (2000) have shown that disruption to imprinted genes regulating growth and development was largely responsible for hybrid inviability in rats. Similar effects have not been documented elsewhere. Because the phylogenetic distribution of imprinting mechanisms appears to be limited to seed plants, mammals and some insects excepting *Drosophila* (Reik & Walter 1998), the genetic architecture of intrinsic isolation feasibly differs in parallel with this distribution. Another example pertains to the influence of duplicated genes. Because the life-history of independent gene duplications exhibits some lineage specificity (Venkatesh 2003), the importance of contributions of 'divergent resolution' (Lynch & Force 2000; Chapter 1.3.2) to reproductive isolation may differ too. It is also possible that the genetic architecture varies in relation to life history traits such as lifespan (Rieseberg et al. 2000). In summary, there is considerable potential for variation amongst groups in the genetic architecture of intrinsic isolation - an important issue as lineage specific properties may determine differences in the mode and tempo of speciation.

The *Drosophila* experimental bias is mainly due to the large complement of genetic and molecular biological tools available for this taxon. However, genetic architectures can be productively investigated in the absence of such tools using the biometrical approach. This approach allows the importance of particular components of the genetic architecture to be evaluated, such as deviations from additivity and sex chromosome effects. And it is easily implemented, entailing, as it does, the simple examination of means and variances of trait values across parental and hybrid lines. But despite their potential worth, biometrics have only once been used in connection with intrinsic isolation (Edmands 1999), although they have seen extensive usage in studies of behavioural isolation (Ritchie & Phillips 1998; Coyne & Orr 1998).

Considerable uncertainty also surrounds whether differences in the rate of evolution of different reproductive barriers exist. In general, it is more probable that rapid speciation between allopatric populations is driven by behavioural rather than intrinsic isolation (Turelli et al. 2001). This is because intrinsic isolation generally results from the gradual accumulation of complex epistatic incompatibilities, regardless of whether selection promotes population divergence (Chapter 1.3.2). In contrast, behavioural isolation can theoretically evolve rapidly under sexual selection when, for example, environmental

perturbations displace equilibria between male trait means and female preferences (Turelli et al. 2001). Yet, this does not mean that behavioural isolation *generally* evolves more rapidly than intrinsic isolation, even amongst lineages characterised by strong sexual selection, despite occasional statements to the contrary (e.g., Mendelson 2003). This distinction is particularly apt because abundant evidence suggests the significance of intrinsic isolation in the wild. For instance, it often impedes gene flow between taxa in hybrid zones (Rieseberg et al. 1999; Machado et al. 2002; Presgraves 2002; see also Chapter 5). Moreover, several studies have documented the appearance of novel genetic factors that induced intrinsic isolation between laboratory stocks, which were not purposefully subjected to divergent selection (e.g., Slotman et al. 2004). It is also noteworthy that an instance of speciation caused by sexual selection remains to be categorically demonstrated (Panhuis et al. 2001). Finally, most hybridisation experiments have neglected post-F_1 hybrid generations, even though hybrid breakdown is common (Edmands 2002), suggesting that intrinsic isolation may be more important than is often thought.

What data exist to evaluate potential rate differences? Examining *Drosophila*, Coyne & Orr (1989, 1997) found that behavioural and intrinsic isolation evolved at comparable rates between allopatric populations, but that behavioural isolation evolved more quickly between sympatric populations. In contrast, using a much smaller *Etheostoma* fish dataset, Mendelson (2003) inferred that behavioural isolation evolved more rapidly than hybrid inviability. It is possible that the contrasting conclusions of these studies reflect methodological artefacts. For example, Mendelson's (2003) study suffered from pseudoreplication in the estimation of behavioural isolation, the quantification of only one intrinsic fitness variable (egg hatchability) and the generation of non-independent crosses. Regardless of the veracity of Mendelson's (2003) conclusions, though, it is obvious that very few relevant data are available. There is therefore an urgent need to compare the forms and magnitudes of multiple barriers between many more taxon pairs.

Intrinsic isolation between guppy populations has previously been treated only cursorily. Several studies have suggested that F_1 hybrids between Caroni and Oropuche Drainage populations are viable (these studies are alluded to in Endler 1995; Endler & Houde 1995; Houde 1997). Their results remain unpublished, but it is clear that they simply dealt with obvious, easily scored traits such as brood sizes. They neglected additional traits that also

influence reproductive isolation, notably fertility and behavioural sterility, and completely ignored post-F_1 generations.

I crossed Caroni and Oropuche guppies to systematically test for reductions in hybrid fitness, and to test for any underlying effects of the X chromosome. Multiple recombinant classes were assayed in case hybrid breakdown existed. A further rationale for this study was the identification of what reproductive barriers can act between intra-specific populations and which might therefore contribute to speciation.

2.3 Materials and Methods

2.3.1 Crossing design and stock maintenance

I used laboratory stocks descended from wild populations in the Tacarigua and Oropuche Rivers, which occur in the Caroni and Oropuche drainages respectively (see Fig. 1.4). Both source populations came from downstream habitats where they co-occurred with the pike cichlid *Crenicichla alta*, an important guppy predator. Both show high fecundity, perhaps as a result of the ensuing elevated predation risk, and so were selectively bred in the laboratory. The laboratory stocks had intermittently received supplements of wild fish to prevent inbreeding, and the last supplement occurred at least two generations (10 months) before the start of the experiment.

In each cross, single males were left with single virgin females in small 0.012 m^3 maternity tanks for seven days (Fig. 2.1). The allocation of individuals to particular crosses and tanks was randomized. Following this period, males were killed (see below) and females were isolated until they gave birth. Because the birth of fry from single broods may occasionally be staggered over 24 hrs (personal observation), and because females often cannibalize their young, tanks were checked at least daily for the presence of new-born fry, which were then separated from their mothers. Once 24 hrs had elapsed following the appearance of the first-born, females were also killed. Consequently, no individual was used in more than one cross. Although females can store sperm for up to six months, females that had failed to produce broods within three months were discarded from analysis. All parents were photographed with a digital camera after being killed.

Broods were distributed amongst and reared in 0.004m^3 plastic transparent bottles (Fig. 2.1). A maximum of six offspring were reared within any single bottle to minimize competition effects on growth. Broods from different females were reared separately and, upon sexual maturation, the numbers and sex ratios of surviving offspring were recorded. Adolescent males were identified by the presence of modifications to the anal fin rays, which eventually form the gonopodium (Clark & Aronson 1951), and adolescent females were identified by the presence of abdominal spots (Houde 1997). Progeny from different broods were then housed together in larger 0.035m^3 stock tanks (Fig. 2.1), with individuals being segregated according to sex and cross type.

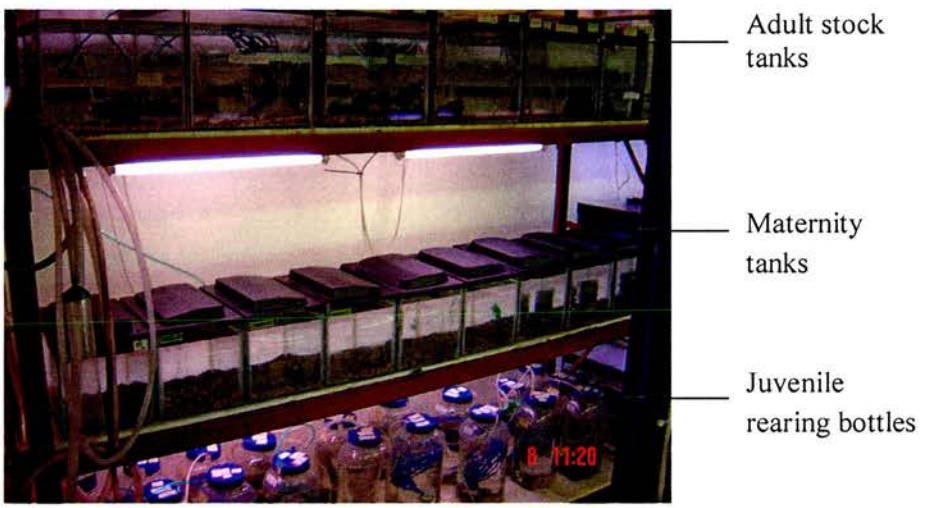


Fig. 2.1. Typical fish housing apparatus for the crossing experiment.

Fish were fed *ad libitum* daily with commercially prepared flake food and *Artemia* brine shrimp. All tanks and rearing bottles had charcoal filters, gravel bottoms and items to increase the architectural complexity of the fishes environment, namely either Java moss (*Vesicularia dubyana*) or artificial plants. Illumination was provided by 40-W overhead fluorescent strips set on a 12: 12 hr light: dark cycle, and temperature was maintained at 24 - 26°C. Fish stocks were periodically culled to prevent aged individuals being used in the experiment. All killed fish were humanely culled using an overdose of benzocaine anaesthetic (ethyl p-amino benzoate), in accordance with Home Office regulations.

In addition to the parental controls, eight recombinant lines were established: two reciprocal lines each for the F_1 and F_2 generations, and four different reciprocal

backcrosses (Table 2.1). The backcrosses were used to test for maternal effects on hybrid fitness and, along with the F_2s, were examined for hybrid breakdown. By convention, throughout this chapter, the initial sex reported in any cross is female (i.e. ♀ x ♂). Not all backcrosses were performed because of a deficit of Oropuche individuals.

Cross			# crosses	# successful crosses
♀	♂	Abbreviation	attempted	(% success)
T	T	P_1	32	24 (75)
O	O	P_2	26	21 (81)
T	O	F_1	29	24 (83)
O	T	F_1R	33	27 (82)
(O x T)	(O x T)	F_2	24	20 (83)
(T x O)	(T x O)	F_2R	25	20 (80)
(T x O)	T	BC_1	27	21 (78)
(O x T)	T	BC_2	25	18 (72)
T	(T x O)	BC_3	30	22 (73)
T	(O x T)	BC_4	26	19 (73)
Total			277	216 (78)

Table 2.1. Crosses performed in the experiment and their reproductive success. T= Taracigua, O = Oropuche.

2.3.2 Male behavioural sterility

Male behavioral sterility was assessed using an open aquarium design, which helps to replicate natural social environments and allows behaviours relevant to wild populations to be observed (Houde 1997). Each 0.065 m^3 observation tank had a charcoal filter, a gravel bottom and Java moss (*Vesicularia dubyana*). Water temperature was maintained at 25 - 26°C. Observations occurred randomly with respect to the time of day and to the particular tank used (one out of a pool of three). The behaviour of single focal males was observed in relation to five females from 'mixed' stock. Four 'mixed' stock males were also present to simulate a sexually competitive environment and a sex ratio of unity that typifies natural populations in Trinidad (Pettersson et al. 2004). These mixed stock individuals are largely

advanced backcrosses derived from multiple parental populations, although pure parental individuals and F_1 hybrids may also have occasionally been present. Behavioural observations used the same common pool of mixed stock fish (c. 75 individuals), and the exact composition used in each trial was randomized. Mixed stock fish were allowed to acclimatize to the observation tanks overnight, before focal males were added. Focal males were given 1 hr to acclimatize before observations began. To minimize asymmetries in male competitive success, males were of approximately the same size (standard length: mean ± SE = 22.42 ± 0.12 mm, $N = 40$). Females were size matched too (standard length: mean ± SE = 30.79 ± 0.28 mm, $N = 30$). All fish were fed to satiation before trials started.

Each focal male was observed for two 15 min periods, during which time the number of sneaky matings and sigmoid displays was recorded, in addition to the length of time he spent following females. If focal males showed no mating behaviour their trials were discarded from analysis. Behavioural sterility of females or of Bc or F_2 hybrid males was not appraised owing to time constraints.

Data from individual males were averaged over the two trials. Median tests were used to test for significant differences between groups. Non-parametric statistics were used because of unequal variances and data that could not be normalised.

2.3.3 Fertility and fecundity measurements

Mature progeny were killed and photographed with a digital camera prior to fertility measurements. Male fertility was assessed by measuring sperm counts and testes weights. To extract sperm, males were placed on a slide under a low-power dissection microscope. The gonopodium was swung forward and 20 µl of 100 mM NaCl was deposited onto it. Pressure was then applied to the side of the abdomen in a forward stroking motion to induce ejaculate release into the salt solution (Matthews et al. 1997). This process was repeated twice before the discharged sperm bundles were retrieved using a P100 Gilson pipette and added to 80 µl of 100 mM NaCl. NaCl stimulates sperm bundle disassociation (Morisawa & Suzuki 1980), and samples were left for 20 min to dissociate. Sperm counts were then performed under 400X using an 'improved Neubauer chamber' haemocytometer. Sperm counts were restricted to the parental, F_1 and F_2 lines.

In contrast, assays of testes weight used all available lines. They also used different individuals from those measured for sperm counts. Fish were dissected to remove the guts and testes, but not any associated fatty tissue. The testes and body carcass were dried at 60 °C for 24 hr.

The fecundity of virgin females was examined by measuring the number and total weight of fully developed eggs available for immediate fertilization. Such eggs occupy stage three in Haynes's (1995) classification of embryonic development in poeciliids (Table 2.2). They are characterized by being fully yolked and translucent golden-yellow in colour, and by having oil droplets evenly dispersed across their yolk surface (Haynes 1995). These ova were dried at 60 °C for 24 hr along with the body carcass, minus guts.

Developmental stage	Distinguishing features
1	Small immature ova
2	Large early-yolked ova
3	Mature ova
4	Blastodisc embryos
5	Embryonic shield / primitive streak embryos
6	Embryos with pronounced optic cups and little or no eye pigmentation
7	Early-limbed bud embryos. Caudal and pectoral fin buds present
8	Embryos with pigmentation over dorsal surface of head. Optic cups more pronounced. Caudal fin rays forming
9	Embryos with pigmentation along complete dorsal axis. Pectoral fin rays present
10	Embryos with full sized eyes. "Neck-strap" present
11	Mature embryo. "Neck-strap" absent. Pectoral fins elongate

Table 2.2. Summary of Haynes's (1995) classification of embryonic developmental stages in poeciliids.

Female fecundity and testes weight, in addition to brood sizes, may be influenced by body size. The computation of ratios or size specific indices such as the gonadosomatic index is often ineffectual in removing these effects (Packard & Boardman 1999; Tomkins & Simmons 2002). Because standard body length differed between the lines (e.g., for mothers: ANOVA: $F_{9,214} = 7.38$, $P < 0.01$), indicating that some adjustment was required, ANCOVAS were used instead to test for differences in fertility. ANCOVAs used log-transformed data (Tomkins & Simmons 2002), and the covariate was standard length estimated from digital photographs (using ImageJ v.1.30; Rasband 2003). The validity of the assumptions underlying these tests was explored using Lilliefor's-corrected Kolmogorov-Smirnov test for normality and Levene's test for homogeneity of variances, both on the original data and their residuals. The heterogeneity of regression coefficients was also checked by examining the interaction term between the factor and the covariate.

2.3.4 Survival

To examine embryonic inviability, mothers that had produced broods were dissected for the presence of embryos. If present, embryos were recorded as inviable if they occupied a developmental stage in Haynes's (1995) classification that was under ten. This scheme allowed inviable embryos to be clearly distinguished from viable, fully mature embryos that had not been born and which constitute stage 11 (Table 2.2). This protocol was adopted because fertilisation occurs over intervals in guppies, causing slight developmental asynchronies within broods (Haynes 1995). This variation amounts to about three days, or one developmental stage (C. Dreyer, personal communication), meaning that viable embryos at stage ten also had to be accounted for. Besides differential survival, an alternative explanation for the presence of embryos at different stages is superfetatation, where females simultaneously carry different broods. But this explanation is likely untenable for guppies, which are assumed to be non-superfetatating (Houde 1997).

Because of occasional difficulties in distinguishing early stage non-pigmented embryos from surrounding ovarian connective tissue, records of embryonic inviability may be biased towards later stages. This possibility, together with the fact that embryo reabsorption almost certainly occurs in guppies (A.E. Magurran, personal communication), meant that data were not analysed in relation to brood sizes as embryo mortality rates. Instead, embryo inviability was analysed as count data using the Kruskal-Wallis H test

following $\sqrt{(y + 0.5)}$ transformation. 10,000 Monte Carlo simulations and a confidence level of 99% were used to generate the probability statistic. For this analysis, reciprocal lines were pooled to form just three hybrid classes.

Juvenile mortality data were analysed as count data using the program BETABINO (Jiggins et al. 2001). The statistical approach implemented in this program is advantageous whenever biological factors underlie differences in survival between replicate broods, a situation that is quite likely (Reed & Markov 2004). The method assumes that the probability of survival varies amongst broods within a line according to a beta-binomial distribution. Two models fitted by the program were tested: (1) the lines have the same mean but different variances, and (2) the lines have different means and variances. These particular models were used since Levene's test indicated that variances were heterogenous. The log-likelihood values for these models were then compared using a likelihood ratio test, and the resultant statistic was compared with the χ^2 distribution to test for differences in means between lines, with degrees of freedom equal to the number of lines minus one. Parametric statistics were not used because transformations could not normalize the data.

2.3.5 Miscellaneous analyses

Body condition was examined using ANCOVAS on dry body weight (including gonads), with standard length as the covariate. The relevant statistical assumptions were tested as in Chapter 2.3.3.

Deviations in the sex ratios of hybrid generations from those of the parental lines may suggest the existence of Haldane's rule and were therefore tested for too. Proportional sex ratios were calculated by dividing the number of females by the total number of surviving adult progeny. Data were arcsine-square root transformed, and differences between groups were tested using a general linear model, where initial brood size was the WLS weight. Data were weighted because larger broods allow more reliable estimates of sex ratios and because brood sizes differed between lines [see section 2.4.2]).

2.4 Results

2.4.1 Male behavioural sterility

Median tests indicated that significant differences existed between lines in the number of displays ($\chi^2_3 = 21.132$, $P < 0.001$), the number of thrusts ($\chi^2_3 = 15.441$, $P < 0.005$) and the length of time that a male had spent following females ($\chi^2_3 = 13.291$, $P < 0.005$) (Fig. 2.2). Games-Howell post-hoc multiple comparisons were therefore performed on each variable and, upon applying sequential Bonferroni corrections (Rice 1989) to adjust α values conservatively for six comparsions ($\alpha = 0.0083$), significant comparisons remain between parental and hybrid lines (Table 2.3). Hybrid dysfunction is statistically more obvious for thrusting than other behaviours, although clear trends for reduced hybrid fitness always exist (Fig. 2.2).

Comparison	# displays	# thrusts	Time spent following females
T vs. O	.997	.951	.999
T vs. (T x O)	.000*	.024	.000*
T vs. (O x T)	.000*	.007*	.012
O vs. (T x O)	.004*	.129	.066
O vs. (O x T)	.009*	.065	.349
(T x O) vs. (O x T)	.877	.471	.197

Table 2.3. Probability values for Games-Howell post-hoc comparisons that tested differences between lines in mating behaviours. *, significant at the $P = 0.05$ level following sequential Bonferroni correction. T = Tacarigua, O = Oropuche.

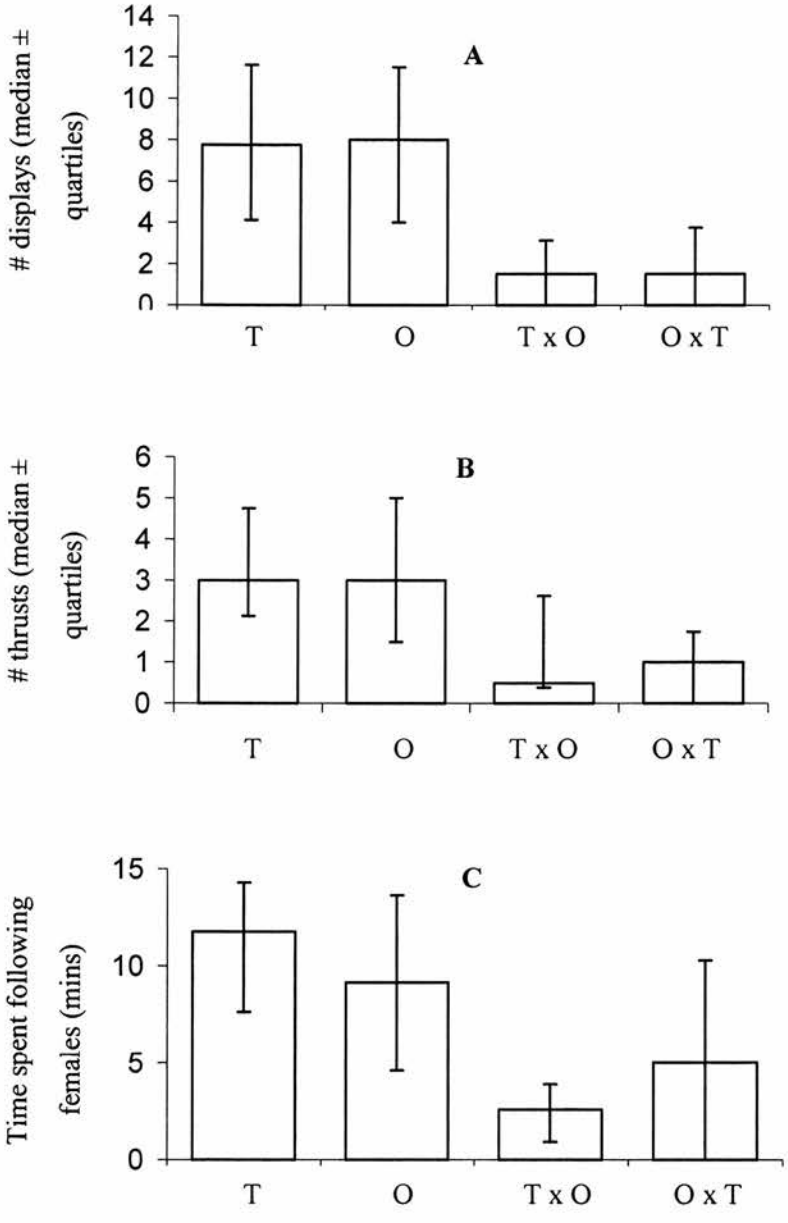


Fig. 2.2. Mating behaviours in the parental and reciprocal F_1 lines. O = Oropuche, T = Tacarigua. Sample sizes (*N*) for each line are: T = 28, O = 29, T x O = 31, O x T = 27.

2.4.2 Fertility

Female mortality contributed to roughly one half of the instances of cross failure (data not shown). Approximately 70 – 80 % of attempted crosses yielded progeny, and there were no marked differences in cross success between lines (Table 2.1). However, brood sizes differed significantly between lines (ANCOVA: $F_{9, 214} = 6.76$, $P < 0.001$) (Fig. 2.3). Post-hoc multiple comparisons, where α was Bonferroni corrected, showed that these differences were restricted to comparisons involving parental or F_1 and post-F_1 lines. For example, the Tacarigua control (P_1) differed significantly from F_2, BC_2 and BC_4. Similarly, F_1R differed from all post F_1 hybrid lines apart from F_2R. Planned comparisons were also conducted using both (T x O) backcrosses into Tacarigua to determine whether a maternal effect on brood size existed, which is quite likely in a vivparous fish. A significant contrast between the (T x O) x T backcross (ie. BC_1) and the Tacarigua control (P1), in tandem with a non-significant contrast for the reciprocal backcross (BC_3), would have demonstrated a maternal effect. However, both contrasts were significant ($P < 0.05$), rendering any maternal effect undetectable. An equivalent analysis using (O x T) backcrosses into Tacarigua (BC_2 and BC_4) was not attempted, since the statistical results had already indicated that Tacarigua differed significantly from each.

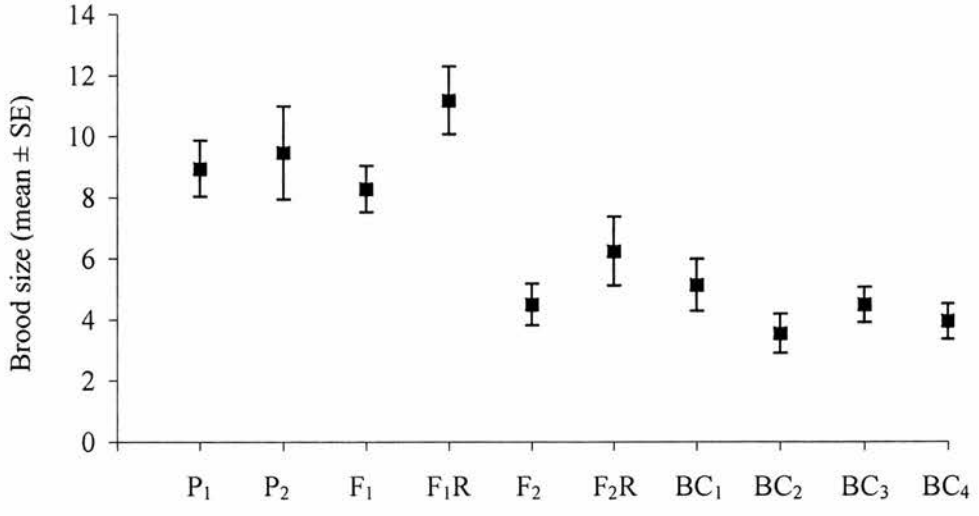


Fig. 2.3. Brood sizes for parental and hybrid lines. Calculations based on untransformed data only. See Table 2.1 (p. 41) for identities of crosses and sample sizes.

In relation to virgin female fecundity, ANCOVAs detected no significant differences between lines in the weight or number of mature eggs (weight: $F_{8,215} = 0.704$, $P >> 0.05$; number: $F_{8,259} = 0.831$, $P >> 0.05$) (Table 2.4). Similarly, male fertility did not differ between groups when testes weight was assayed (ANCOVA: $F_{8,242} = 0.58$, $P > 0.05$) (Table 2.4). To analyse the sperm count data, reciprocal crosses were pooled within hybrid lines to increase replicate sizes. An ANCOVA on the data generated a significant statistic ($F_{3,96} = 3.59$, $P < 0.05$), and Bonferroni post-hoc multiple comparisons showed that the F_2 line had significantly reduced sperm counts relative to both parental lines and to the F_1 population ($P < 0.05$, $\alpha = 0.0125$) (Fig. 2.4).

Line (♀ x ♂)	Female fecundity				Male fertility	
	# eggs		Dry weight of eggs (mg)		Testes weight (mg)	
	Mean (*N*)	SE	Mean (*N*)	SE	Mean (*N*)	SE
T	3.20 (20)	0.38	5.16 (16)	0.65	0.59 (31)	0.04
O	5.74 (34)	0.52	6.92 (29)	1.26	0.58 (43)	0.02
T x O	4.77 (34)	0.55	5.29 (32)	0.64	0.55 (24)	0.02
O x T	4.39 (23)	0.71	4.88 (22)	0.59	0.61 (23)	0.04
(O x T) x (O x T)	4.91 (46)	0.43	6.47 (35)	1.04	0.53 (28)	0.03
(T x O) x (T x O)	4.96 (24)	0.66	4.72 (20)	1.30	0.61 (30)	0.02
(T x O) x T	4.59 (27)	0.52	6.80 (20)	1.22	0.55 (21)	0.03
(O x T) x T	5.14 (29)	0.57	5.39 (19)	0.68	0.48 (19)	0.04
T x (T x O)	4.8 (24)	0.58	5.79 (24)	1.26	0.58 (25)	0.03
T x (O x T)	-	-	-	-	-	-

Table 2.4. Descriptive statistics for male and female fertility variables. Data for the T x (O x T) line (ie. BC_4) are not reported as its broods were culled following birth. T = Tacarigua, O = Oropuche.

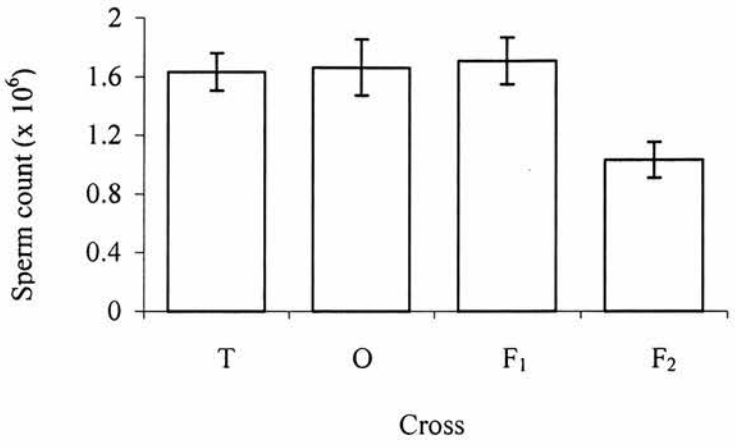


Fig. 2.4. Sperm count data. T = Tacarigua, O = Oropuche. Sample sizes (*N*) for each class are: T = 36, O = 21, F_1 = 23, F_2 = 18.

2.4.3 Survival

Juvenile survival did not differ between lines as judged by the likelihood ratio test implemented in Betabino (Jiggins et al. 2001) ($L_{9,\,197} = 11.929$, $P >> 0.05$) (Table 2.5). However, embryo viability differed significantly between lines (Kruskal-Wallis H test: $\chi^2_4 = 30.10$, $P < 0.001$) (Table 2.5). Games-Howell post-hoc tests showed that the pooled backcross class differed from the pooled P_1, P_2, F_1 and F_2 classes ($P < 0.01$). Planned comparisons involving (T x O) or (O x T) backcrosses into Tacarigua were again conducted (prior to pooling) to test for maternal effects on embryo viability. However, all contrasts were non-significant ($P > 0.05$), rendering any such effects undetectable.

Line (♀ x ♂)	Juvenile survival (proportion of offspring from a brood surviving to maturity)			Embryo inviability (# dead embryos per female)		
	N	median	Interquartile range	*N*	median	75 + 90 percentiles
T	24	1.00	0.88 - 1.00	20	0	0 + 1.0
O	21	1.00	0.91 - 1.00	20	0	0 + 1.0
T x O	24	1.00	0.93 - 1.00	40	0	0 + 1.8
O x T	27	0.96	0.84 - 1.00			
(O x T) x (O x T)	20	1.00	0.85 - 1.00	40	0	0 + 0
(T x O) x (T x O)	20	1.00	1.00 - 1.00			
(T x O) x T	21	0.86	0.65 - 1.00	121	0	3 + 7.9
(O x T) x T	18	0.94	0.65 - 1.00			
T x (T x O)	22	1.00	0.75 - 1.00			
T x (O x T)	-	-	-			

Table 2.5. Percentiles for untransformed juvenile survival and embryonic inviability data. Reciprocal hybrid lines have been pooled for embryo inviability.

2.4.4 Miscellaneous analyses

Adult brood sex ratios did not differ significantly between lines (GLM with brood size as WLS weight: $F_{8, 195} = 1.69$, $P > 0.05$) (Fig. 2.5). Male and female body condition also did not differ between lines (males: ANCOVA: $F_{8, 242} = 0.74$, $P >> 0.05$; females: ANCOVA: $F_{8, 215} = 1.39$, $P > 0.05$).

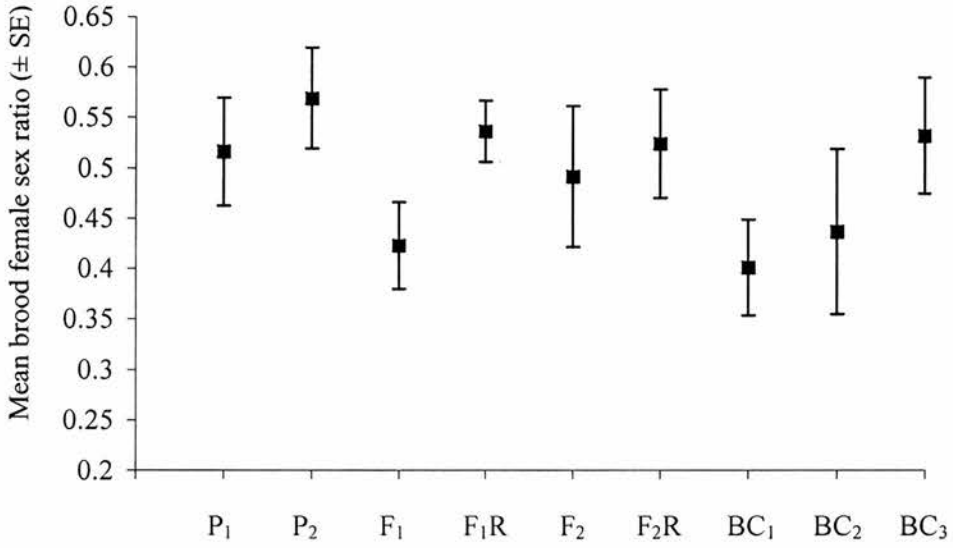


Fig. 2.5. Mean brood female sex ratios (untransformed data). See Table 2.1 for details of cross directions.

2.5 Discussion

2.5.1 Male behavioural sterility

Although male F_1s showed substantial behavioural sterility, the success of attempted matings did not obviously differ between parental crosses and those involving male F_1s (Table 2.1). This discrepancy may solely reflect the lack of mating discrimination characteristic of virgin female guppies (Houde 1997) or the confinement of mating pairs.

The observed behavioural dysfunction could reflect pleiotropic effects of divergence in other traits. For instance, divergence in physiological processes such as muscle performance could have resulted in a reduction in general vigour. This hypothesis was also invoked for *Anartia amathea* x *A. fatima* butterfly hybrid females, whose reduced mating propensities appeared to correlate with a general lassitude (Davies et al. 1997). Similarly, courtship dysfunction in *Drosophila pseudoobscura* x *D. persimilis* hybrid males seemed to reflect a decrease in overall locomotor activity, although additional causes were probably also responsible (Noor 1997). An alternative explanation for courtship dysfunction generally is that the complex of traits involved in courtship may be particularly sensitive to disruption in hybrids. Further examples of behavioural sterility

have been reported for male F_2 hybrids between *Nasonia* wasps (J.H. Werren personal communication; quoted in Coyne & Orr 2004) and for female hybrids between *Agapornis* parrots (Buckley 1969).

Demonstrating whether female guppy hybrids show comparable levels of dysfunction would be interesting as Noor (1997; see also Davies et al. 1997) has suggested that behavioural sterility is another trait that, like physiological sterility and viability, follows Haldane's Rule. Although very few examples of hybrid behavioural dysfunction have been catalogued, the available evidence is at least consistent with Noor's hypothesis (Coyne & Orr 2004). General observations of the behaviour of hybrid guppy females did not reveal any striking behavioural dysfunction, though experimental tests are required to verify this. Incidentally, if present, female dysfunction would complicate studies of the genetic architecture of female mating preferences (Noor 1997), which are currently being envisaged (C. Dreyer personal communication).

Finally, behavioural sterility may be a common but relatively neglected component of reproductive isolation. It may envelop a broad range of phenotypes such as poor parental care (e.g., the three-spined stickleback, *Gasterosteus aculeatus*; McKinnon & Rundle 2002) in addition to courtship dysfunction. It may also constitute a plausible alternative explanation for hypothesised cases of extrinsic isolation. For example, field studies of *G. aculeatus* hybrids have detected a reduced mating success (Vamosi & Schluter 1999) that could either reflect the inability of intermediate phenotypes to find mates (extrinsic isolation) or a reduced vigour caused by intrinsic factors. This possibility emphasises the fact that extrinsic and intrinsic processes are not wholly distinct (Turelli et al. 2001).

Genetic architecture of male behaviour

Population crosses can provide insights into the linkage relationships of sexual traits, which strongly influence the evolutionary dynamics of sexual selection. For example, sexually antagonistic traits that benefit the heterogametic sex are more likely to increase in frequency if linked to the sex-determining locus (Rice 1984). Also, genetic correlations between male secondary sexual traits and female preferences may aid trait coevolution (Fisher 1958), and thereby promote sexual isolation between populations.

Little is known about the linkage relationships of mating behaviours in guppies. Using backcrosses between inbred laboratory strains, Farr (1983) documented Y-linkage of display and thrusting behaviours. Also, Houde (1997) crossed two Northern Drainage populations, from the Paria and Yarra Rivers (Fig. 1.4), and her data are suggestive of Y-linkage for courtship behaviour in Paria only, indicating that there may be variation in linkage patterns amongst natural populations.

Unfortunately, because no significant differences in mating behaviour were observed between Tacarigua and Oropuche, my crosses are uninformative regarding the inheritance of these behaviours. Instead they have utility for the analysis of the genetic architecture of behavioural dysfunction, which has been studied only twice previously. Coyne et al. (2002) showed that dysfunction in *Drosophila yakuba* x *D. santomea* hybrid males might result from interactions involving the *santomea* X and the *yakuba* genome. And Noor (1997; Noor et al. 2001) has shown that dysfunction in *D. pseudoobscura* x *D. persimilis* hybrid males is due to an interaction involving the *persimilis* X and the *pseudoobscura* Y. In contrast, the absence of significant differences between the reciprocal F_1 guppy lines suggests that sex chromosomes do not contribute disproportionately to their behavioural dysfunction (de Belle & Sokolowski 1987; Reinhold 1998). This absence is consistent with the lack of marked sex chromosome differentiation in guppies (Traut & Winking 2001) (but see below).

2.5.2 Physiological viability and sterility

Hybrid breakdown was observed for brood sizes (F_2s and BCs), sperm counts (F_2s) and embryonic inviability (BCs). Such breakdown is commonly considered to reflect recessive gene actions (Edmands 2002).

Genetic studies indicate that incompatibilities causing sterility appear to accumulate more rapidly than those causing inviability (Singh & Kulathinal 2000). A likely explanation is the action of sexual selection (Wu & Davis 1993) or sexual conflict (Howard et al. 1998; Rice & Chippindale 2002) on sex-related genes, since either process is expected to rapidly drive adaptive genetic change at these loci. Regardless of the explanation, the phenotypic consequences of this differential molecular evolutionary rate are often seen in crosses. For instance, using comparative analysis of data from crossing experiments, Russell (2003)

showed that hybrid sterility generally evolved more rapidly than hybrid inviability in fish possessing a wide variety of sex-determination mechanisms (see also Chapter 1.3.3). However, looking solely at guppy hybrids, both inviability and sterility effects exist, so it is impossible to determine which trait evolved first.

Guppies obey Haldane's rule for sterility, since only males showed any reduction in hybrid fertility. It is unlikely that this observation stems from a methodological artefact. One reason is that the metrics used to assay female fertility have previously been shown to be quite powerful (e.g., Reznick & Endler 1982). Another is that, due to difficulties in catching all the sperm bundles released from a stripped male, which sometimes adhere to his body, the experimental error for measuring sperm counts probably exceeded that for measuring female fecundity. If anything, therefore, the sex bias in infertility may have been underestimated.

The most important explanation for Haldane's rule as a whole appears to be the Dominance theory; i.e. the recessivity of X-linked incompatibilities (see Chapter 1.3.3). Should it explain Haldane's rule for sterility in guppies, then some molecular differentiation of the (homorphic) sex chromosomes would already seem to have contributed to reproductive isolation in guppies. This possibility contrasts the absence of any large-X effects inferred for behavioural dysfunction. One reason for this difference might be that the genetic architecture of the two traits differs – incompatibilities inducing behavioural dysfunction may have a greater autosomal distribution. Alternatively, the X chromosomes of both Caroni and Oropuche lineages may have effects on hybrid unfitness that are roughly of the same magnitude, thus rendering large-X effects undetectable using biometrical analyses of reciprocal F_1 lines.

However, an alternative explanation for Haldane's rule for sterility in male heterogametic taxa such as guppies is the faster-male theory (Wu et al. 1996; see Chapter 1.3.3 and 1.4.4). This theory posits that sterility factors either accumulate more rapidly in males or are more likely to disrupt male reproduction. It does not predict a disproportionate effect of the X chromosome (Presgraves & Orr 1998). Ultimately, more detailed (genetic) analyses are required to differentiate between these possibilities, and should concentrate on evaluating the possible existence and dominance relations of X-linked incompatibilities, and on whether male sterility effects appear more commonly than female ones following

introgression of chromosomal blocks between populations (e.g., see True et al. 1996). These experiments, however, will have to wait until the development of appropriate molecular genetic tools.

In contrast to sterility, the 1: 1 sex ratios observed amongst adult hybrid progeny indicate that embryonic inviability did not obey Haldane's Rule. More generally, there is little evidence for Haldane's rule in fish as a whole (Russell 2003). This probably reflects the plethora of sex-determination systems that could be found in any given fish cross and the general difficulties in characterizing fish sex chromosomes, which are usually homorphic (Ohno 1974). For instance, although ~1700 fish species have been cytogenetically characterised, only ~ 10% possess distinct sex chromosomes, and only ~ 6% have simple male or female heterogamety (Arkhipchuk 1995; Devlin & Nagahama 2002).

2.5.3 Relative importance of different reproductive barriers

In behavioural experiments, female guppies occasionally show small but significant preferences for native males (e.g., Luyten & Liley 1991; Endler & Houde 1995). Males also show slight preferences for females from their own populations, although they are otherwise incapable of distinguishing females that come from native or foreign drainages (Magurran & Ramnarine 2004). Behavioural isolation in guppies is therefore slight, especially in comparison with the strength of intrinsic isolation.

Several hypotheses have been advanced to explain this difference. The motivation for each is the observation of intense sexual selection within guppy populations, which is generally assumed to be capable of rapidly driving the evolution of behavioural isolation, although empirical demonstrations of this effect are lacking (Panhuis et al. 2001). One hypothesis postulates a role for sneaky mating, since it may undermine female choice (Magurran 1996, 1998, 2001). Another stresses the similarity of environments experienced by different populations in Trinidad, which may select for similar female choice criteria (Magurran 2001). Both factors are expected to constrain the evolution of behavioural isolation between allopatric populations and both could act simultaneously. No data are currently available to appraise either hypothesis, although the potential importance of sneaky mating could be evaluated by examining whether post-copulatory processes

somehow differentially select against sperm from forced copulations. This possibility is discussed further in Chapter 5.2.

The effective magnitude of any barrier to gene flow depends on its absolute magnitude and when it acts in the reproductive cycle – early acting barriers have a greater effective magnitude than later ones, even when they have identical absolute sizes (Coyne & Orr 2004). Regarding behavioural and intrinsic isolation, this caveat is largely obsolete for the L. Tacarigua – Oropuche cross since behavioural isolation is non-existent (but see Chapter 5.1 for a discussion of gametic isolation). So, intrinsic isolation remains the much stronger barrier to gene flow. Although it can not be assumed that this pattern will hold throughout the remaining speciation process, this work has at least established that intrinsic isolation could contribute to speciation. Making comparable inferences from studies of inter-specific taxa is often more difficult because reproductive barriers can increase in strength after speciation (see Chapter 1.3). Further, the future importance of intrinsic isolation is actually suggested by several considerations. One is that complex intrinsic incompatibilities are more likely to persist following population admixture than other barriers such as behavioural isolation, which are based on phenotypic differences (Rieseberg et al. 2003). (Though selection does seem capable of removing intrinsic incompatibilities following plant hybrid speciation; Rieseberg et al. 1996; Carney et al. 2000; Lexer et al. 2003) This is an important point as population admixture must occur frequently during early phases of population divergence (Coyne & Orr 2004). A subsidiary consideration is that those factors postulated to retard the evolution of behavioural isolation are not expected to weaken over time (Magurran 2001).

An important caveat to this discussion is that only one representative population from each of the Caroni and Oropuche drainages was assayed in the present study. Therefore, conclusions regarding general patterns can only remain tentative.

This chapter has highlighted another advantage of conducting intra-specific crosses. It detected hybrid behavioural sterility, which has previously only been documented in inter-specific hybrids. It is therefore now apparent that behavioural dysfunction is feasibly involved in causing speciation.

Finally, additional insights into the relative importance of different barriers are available from the Cumana guppy, a phenotypically divergent guppy population from Venezuela (Alexander & Breden 2004). Males of the Cumana guppy are distinct in the colour quality of their spots, and in possessing an unusually high incidence of double sword colour patterns on their caudal fins. Alexander & Breden (2004) performed laboratory crosses between the Cumana guppy and populations representing the typical morph from Venezuela, Suriname and the Paria River in Trinidad, and found no detectable brood size differences between the parental and hybrid lines. In a separate experiment, they allowed individuals of the Cumana and typical (Venezuelan) morphs to breed freely in aquaria. Then, using colour patterns to determine paternity of the male offspring, they determined that native males sired a larger proportion of a female's brood. The authors attributed this finding to divergent mating preferences between populations, and thus concluded that behavioural isolation was stronger than intrinsic isolation. Unfortunately, the available evidence is presently insufficient to justify this conclusion. This is because gametic isolation or an alternative component of intrinsic isolation could have caused the difference in paternity success. So, the current importance of behavioural isolation is uncertain in the Cumana guppy, but seems negligible amongst Trinidadian populations.

CHAPTER THREE

Population genetic analysis of admixed populations

3.1 Summary

Artificial introductions can severely impact native populations. But they may also be useful in elucidating evolutionary mechanisms. Both aspects are illustrated by examining the genetic consequences of an introduction of Caroni fish into the Oropuche Drainage (Turure River) that occurred in 1957. One hundred and eighty-one individuals were sampled in 2001 from four sites along the Turure River. These were then typed at seven microsatellite loci and assayed using PCR-RFLP of mitochondrial *NADH2* sequences. Microsatellite data were analysed using Bayesian and maximum-likelihood procedures to estimate nuclear introgression statistics. Introduced nuclear alleles have introgressed asymmetrically into the native populations, and may now comprise $\geq$ 83% of the nuclear genome throughout the river. Displacement of the native mitochondrial haplotype is also considerable, but appears to have declined with distance from the point of admixture. Although dispersal rates between the sampled populations were generally negligible, ~12% of one population was composed of migrants from another population 0.9 km upstream. Thus, the scale of dispersal is potentially large in relation to ecological gradients (particularly those of predation regime), a scenario that has been hypothesised to impede adaptive population differentiation in the Trinidadian guppy. Patterns of introgression within individuals could not be clearly determined, so the effective strength of intrinsic barriers to geneflow between sympatric populations could not be resolved.

3.2 Introduction

Biological introductions are an important element of human-induced environmental change. This is because introduced taxa can erode the genetic integrity and biodiversity of recipient communities, and concomitantly alter ecosystem functioning. These effects may be realised by displacement of indigenous taxa through ecological or demographic factors alone (Leary et al. 1995), or through the additional influence of introgressive hybridisation (e.g., Lamb & Avise 1986; see reviews by Leary et al. 1995; Rhymer & Simberloff 1996; Allendorf et al. 2001; Utter 2001). The considerable latent capacity of freshwater fish to

hybridise with inter-specifics means that introgressive displacement is often particularly problematic in their conservation (Hubbs 1955; Utter 2001; Rahel 2002).

Nonetheless, artificial introductions can be a useful tool in evolutionary biology. For instance, introductions involving the guppy have been important in examining various life history, behavioural and colour traits in relation to predation regime (e.g., Endler 1980; Reznick & Bryga 1987). Artificial hybrid zones may also have use in speciation research, since it is only between sympatric populations that the true efficacy of barriers to gene flow can be assessed. Here, the magnitude and form of introgression should change with increasing strength of these barriers (e.g., Avise & Saunders 1984). Finally, introduction experiments offer some potential for examining patterns of dispersal and gene flow, parameters that have extremely important implications for an organism's evolutionary biology. In particular, dispersal may constrain adaptive population divergence, and thereby prevent populations from reaching local adaptive peaks (Garcia-Ramos & Kirkpatrick 1997). But dispersal also has beneficial consequences, including the spread of advantageous mutations and the prevention of population extinction (Hanski & Gaggiotti 2004).

Artificial introductions seriously threaten the genetic integrity and viability of native guppy populations (Magurran et al. 1995). A possible example is an introduction that brought into contact Caroni and Oropuche Drainage populations. In 1957, C. P. Haskins moved 200 guppies, with a sex-ratio approximating unity, from a downstream high-predation site in the Caroni Drainage into a low-predation site in the Upper Turure River (Oropuche Drainage) (Figs. 1.4 and 3.1). Before the introduction, the Lower but not the Upper Turure R. contained guppies, probably because a waterfall barrier had prevented their upstream dispersal (C.P. Haskins, personal communication; A. E. Magurran, personal communication). The translocation was not described in the literature, and only came to light following a broad allozyme survey of genetic variation in the Northern Range (Shaw et al. 1991). The identity of the source population initially remained ambiguous, since the one suggested in Haskin's correspondence (Lower Arima; C. P. Haskins, personal communication) is not supported by genetic analysis. Rather, both allozyme (Shaw et al. 1992) and mitochondrial DNA sequence (Fig. 1.7; Appendix I) data indicate that Guanapo R. individuals are more closely related to Upper Turure fish than those from Arima R. Preliminary molecular genetic analyses indicated that some displacement of native

mitochondrial (Becher & Magurran 2000) and nuclear allozyme genotypes (Shaw et al. 1992) occurred just downstream of the waterfall. No introduced allozyme alleles were detected further downstream (Shaw et al. 1992) near the Turure's confluence with the Quare R (Fig. 3.1), where Becher & Magurran (2000) did not sample. Because limited numbers of individuals were sampled in either study, the spatial scale and magnitude of introgression remains uncertain. Moreover, the inferences of Shaw et al. (1992) may not be robust given that their results largely depended on a single diagnostic marker.

Here, I use microsatellite and mitochondrial markers to more concretely describe patterns of population admixture throughout the Turure R. I also wished to determine whether the hybrid zone has implications for evolutionary biology that can be uncovered using currently available molecular genetic methodologies (Becher et al. 2002; Becher & Magurran 2004). More specifically, I wanted to test Endler's (1977, 1995) hypothesis that scales of dispersal are large relative to those of habitat change, and so whether dispersal is likely to retard adaptive differentiation between upstream and downstream populations. I was also interested in determining if the reproductive isolation observed between Caroni and Oropuche guppies (Chapter 2) was sufficient to prevent (introgressive) hybridisation.

3.3 Materials and Methods

3.3.1 Sampling

Four populations (T1-T4) were sampled along the Turure R. (Fig. 3.1; Table 3.1). Samples T1, from the introduction site, and T2 both come from the originally unpopulated upstream stretch. T3 and T4 come from the originally populated, now admixed, downstream section. Lower Guanapo R. (GU), the source population inferred from genetic data, was sampled to appraise possible founder effects. An additional sample (Lower Oropuche R. (OR)) was used with GU to test the mtDNA markers' diagnostic capabilities (Fig. 3.1; Table 3.1).

Accessibility determined what sites could be sampled, such that most sampled sites were directly accessible by road. Variance in sample size exists because of concerns that more exhaustive sampling would have detrimentally impacted populations. All samples, except

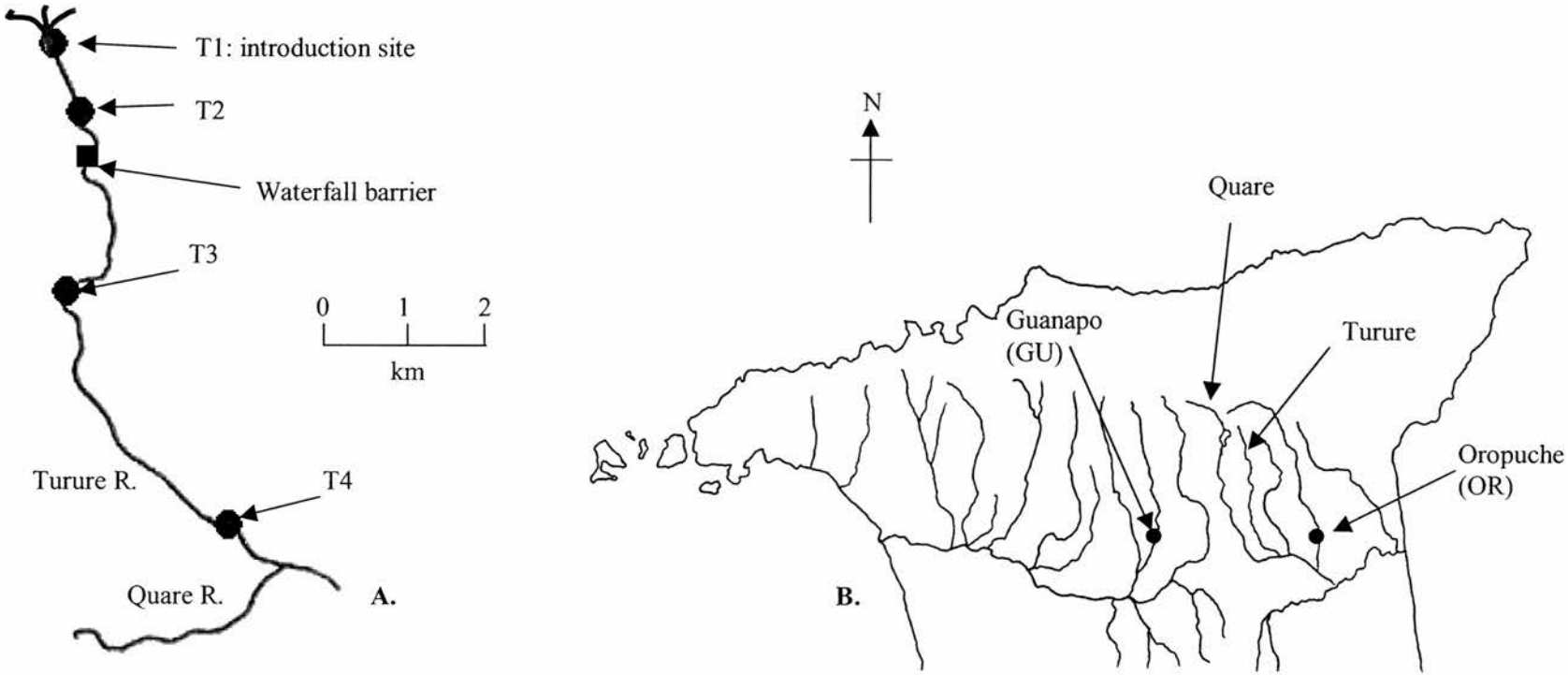


Fig. 3.1. Location of sampling sites. **A**, Turure River samples: T1, introduction site in upper Turure R.; T2, intersection of Turure R. and Cumaca Road; T3, intersection of Turure R. and Valencia Rd.; T4, just upstream of the confluence of the Turure and Quare Rivers. **B**, Location of the Turure R. in relation to the Lower Guanapo R. and Lower Oropuche R. sampling sites. Guanapo is the putative source population

OR, were collected on 29 March 2001. OR was instead derived from two collections made in December 1998 ($N = 17$) and March 2001 ($N = 32$). Assuming a generation time of 1.74 – 4.0 generations per year (Chapter 1.4.5), sampling therefore occurred some 77 – 176 generations following the introduction. Whole fish specimens were stored in 96% ethanol at -20°C until DNA extraction.

Population	Geographic coordinates	*N*
T1	PS 998 816	40
T2	QS 004 810	56
T3	QS 004 785	61
T4	QS 019 740	24
GU	PS 915 766	57
OR	QS 043 787	49

Table 3.1. Sample sizes and geographic locations. Geographic coordinates derived from Series E 804 (D.O.S.) maps published by the Trinidad and Tobago Government, 1978.

3.3.2 Laboratory protocols

Isolation and characterisation of microsatellite loci

Appendix II describes the enrichment and cloning procedures used to isolate the microsatellites used here. These procedures yielded 9 microsatellite loci that are described in Becher et al. (2002; Appendix II) and Becher & Magurran (2004).

Microsatellite amplification and PCR-RFLP of mtDNA

Genomic DNA was extracted from tail tissue using standard phenol-chloroform procedures (Sambrook et al. 1989). Polymerase chain reaction (PCR) amplification occurred at seven microsatellite loci (Table 3.2). Forward and reverse sequences for TAA are (5' – 3'): GTC ACC GAA CGA AAG GAT A and CCC CAA AGG AAC ACT GTA respectively (F. Breden, personal communication). Amplifications occurred in 15 µl reaction volumes containing 2.0 mM of each dNTP, 1 X Taq buffer, 0.2 pmol of each primer, 2mM $MgCl_2$, 1 unit Taq (Bioline) and 5 µl DNA template (10-20 ng / µl). All PCR amplifications occurred in PTC-100 thermocyclers (MJ Research) under the following

conditions: initial denaturation at 92 °C for 3 min, 30 cycles of amplification (92°C for 30 s, x °C (Table 3.2) for 30 s, 72°C for 30 s), final extension at 72°C for 5 min. Amplification products (3 µl) were electrophorised on 6% denaturing polyacrylamide gels for 3 – 6 h, and visualised using silver staining.

Mitochondrial *NADH2* sequences were amplified and restricted with two enzymes (*Bgl*II and *Dde*I) following Becher & Magurran (2000). Both enzymes generated restriction digests that accurately assigned the control population (GU, OR) individuals to either the Caroni (GU) or Oropuche (OR) drainage. Moreover, the ability of these enzymes to reciprocally diagnose Caroni from Oropuche Drainage guppies was further demonstrated by Becher & Magurran (2000), who used fish from the Aripo (Caroni Drainage) and Oropuche Rivers. So, samples T1 – T4 were restricted with both enzymes.

3.3.3 Statistical Analyses

Calculation of basic statistical properties

POPGENE v.1.31 (Yeh et al. 2001) was used to calculate H_O and H_E per locus. H_O is the observed heterozygosity, and H_E is the expected heterozygosity calculated under random mating after Nei (1978). Estimation of null allele frequencies used these values following Brookfield (1996). To test for possible effects of bottlenecks and admixture on genetic diversity, additional diversity statistics were obtained: mean allele number [$\bar{A}$] and H_E per locus per population. To correct for unequal sample size, these latter measures were obtained with the "cumulative heterozygosity and allele count" subroutine of DOH (Brzustowski 2002). 1000 subsampling randomisations were used to standardise N to the lowest sample size (24) (see also Leberg 2002). Sign tests were performed on both measures across all loci and probability values were Bonferroni corrected.

The distribution of genetic diversity was analysed in various ways: (1) Tests for departure within samples from Hardy-Weinberg equilibrium (HWE) used F_{IS}, which measures the heterozygote deficit within populations. F_{IS} was calculated with FSTAT v.2.9.3 (Goudet 2001), having permuted alleles among individuals (within samples) 1000 times. (2) Pairwise population differentiation was tested using the multilocus G-statistic of FSTAT by not assuming HWE within samples. Multilocus genotypes were randomised 1000 times between samples and probabilities were Bonferroni corrected. (3) Pairwise population

differentiation was measured with R_{ST} (Michalakis & Excoffier 1996) and F_{ST} (Weir & Cockerham 1984) using GENEPOP. Both differentiation estimators were used because neither of their underlying mutation models was likely to perfectly fit all the microsatellite loci (Estoup & Angers 1998; Ellegren 2000; Balloux & Lugon-Moulin 2002). R_{ST} calculation ignored G49 because it showed large differences in allele size between drainages, and so may in particular have deviated from the assumption of stepwise mutation. F_{ST} calculation used all seven loci. (4) Finally, inter-population genetic structure was also examined with an AMOVA (Analysis of Molecular Variance) on samples T1 – T4 using Arlequin v.2.000 (Schneider et al. 2000).

Estimation of dispersal rates

Migration rates between pairs of geographically adjacent Turure R. samples were estimated with BayesAss (Wilson & Rannala 2003). BayesAss analyses multilocus genotypes using Bayesian statistics to estimate m, the proportion of individuals in one population that emigrated from another population in the present generation. m is therefore not a dispersal measure estimated over multiple generations, in contrast to allele frequency dependent measures, which are based on F_{ST} (Wilson & Rannala 2003). BayesAss does not assume HWE, constant population sizes, symmetrical migration or population stability, properties that are required by other dispersal estimation methods, and which are together probably lacking amongst Turure R. populations (see Results) (Wilson & Rannala 2003). So, only BayesAss was used to examine migration patterns. The posterior probability distributions of m were calculated using 2×10^6 Markov chain Monte Carlo (MCMC) iterations following a burn-in period of 1×10^6 iterations. Samples were collected every 2000 iterations.

Unfortunately, no non-genetic means of estimating dispersal rates, such as mark-recapture experiments, were conducted during the sampling period. Hence, an independent appraisal of the accuracy of BayesAss in estimating current migration rates is unavailable, though indirect and direct approaches do routinely generate markedly divergent estimates (Whitlock & McCauley 1999).

Calculation of nuclear introgression statistics and genotypic disequilibria

Because no diagnostic Caroni Drainage allozyme alleles (Shaw et al. 1992) or mtDNA haplotypes (Fig. 1.7; Appendix I) have been recovered from sites T1 or T2 respectively in the Upper Turure R., nuclear introgression analysis concentrated on downstream samples T3 and T4 only. The Guanapo R. was assumed to be the source for the introduction, since its fish are more closely related to those of the upper Turure R. than those from the Arima R. (Appendix I; Shaw et al. 1992). Both Bayesian and maximum-likelihood analyses were used to calculate introgression statistics.

The Bayesian analysis used Structure v.2 (Pritchard et al. 2000), whose efficacy in elucidating cryptic population structure at the individual level is greater than more traditional approaches such as assignment tests and distance-based clustering methods (Hansen et al. 2001a). Structure estimates the posterior distribution of an individual's admixture coefficient (q); i.e., the proportion of an individual's genome that is derived from one or the other population. This analysis can be effective when incomplete baseline data are available (e.g., Beaumont et al. 2001; Hansen et al. 2001b; Vernesi et al. 2003), and was chosen because allele frequencies for T3 and T4 prior to introgression are unavailable. Structure also estimates the probability that a sample comprises a certain number of populations (k). When individuals are grouped into a number of populations causing the least within-sample Hardy-Weinberg disequilibria, the most probable k has been identified.

First, the most probable k for each downstream sample (T3 and T4) was determined. Multiple scenarios (k = 1-5) were tested because of the possible existence of multiple distinct hybrid classes, for which introgression statistics would have to be estimated separately. As k was most probably one within each sample ($P = \sim 1.0$), single populations were assumed. q values and their probability intervals were then estimated for every individual within T3 and T4. T1 was used as the training sample to provide baseline data for this analysis; i.e., the non-hybridised sample T1 was used to determine the hybrid status of each individual within T3 and T4. All analyses used uncorrelated allele frequencies because Caroni and Oropuche drainage populations are genetically highly differentiated; otherwise, default parameter settings were used. All analyses involved burn-in periods of 1×10^4 steps followed by 1×10^6 MCMC replicates.

Population-level admixture proportions were calculated for T3 and T4 by averaging individual q values. 95% confidence intervals of these means took two sources of variation into account: inter-individual variation in q, and variation in q amongst MCMC replicates (Hansen et al. 2001b). Individual q values from 40 single MCMC steps (each after a 100,000 step burn-in) were pooled, and PopTools (Hood 2002) was used to generate 1000 bootstrapped replicates from these. Mean q and confidence intervals were then determined from the bootstrapped samples.

A maximum-likelihood method (Leadmix v.1.0; Wang 2003) was also used to calculate population-level admixture proportions and associated 95% confidence intervals. Leadmix allows for incomplete baseline data too. Here, genetic differentiation between the parental populations was explicitly allowed in the modelling process, and 1000 integration points were used to calculate the likelihood function; otherwise, default parameter settings were applied.

Genotypic disequilibria were calculated using a likelihood ratio test in FSTAT v.2.9.3 (Goudet 2001). Because genotypic disequilibria can exist in single samples due to sampling artefacts or drift, and because power to detect disequilibria can be increased by pooling populations, the test was applied to the admixed populations together and separately. In both cases, the maximum permissible numbers of permutations (840) were used, and probabilities were Bonferroni corrected.

3.4 Results

3.4.1 Diversity measures

Null allele frequencies were reasonably low (< 0.07; Table 3.2); hence, allele non-amplification due to the large inter-drainage genetic differentiation should not have markedly affected introgression statistics. These microsatellites are not linked (S. A. Becher, personal communication), and are assumed to be selectively neutral. All microsatellites generated moderate (Pr36, G10 and TAA) to large numbers of alleles (Pr67, Pr171, Pr172, G49) (Table 3.3).

Locus	Annealing temperature (°C)	H_O	H_E	N	Null allele frequency
Pr36*	52	0.289	0.300	238	0.008
Pr67[¶]	53	0.806	0.911	238	0.055
Pr171*	53	0.776	0.900	238	0.065
Pr172*	53	0.774	0.872	238	0.068
G10[†]	55	0.838	0.779	238	0.033
G49[†]	63	0.846	0.881	238	0.019
TAA[§]	52	0.798	0.871	238	0.039

Table 3.2. General per locus characteristics. *, Becher et al. 2002; [¶], Becher & Magurran 2004; [†], Parker et al. 1998; [§], F. Breden, personal communication.

		Population									
		GU		T1		T2		T3		T4	
		A	H_E	A	H_E	A	H_E	A	H_E	A	H_E
Locus	Pr36	4.86	0.36	3.08	0.48	2.67	0.23	4.19	0.31	6.00	0.30
	Pr67	15.14	0.82	11.60	0.91	13.11	0.84	13.09	0.85	19.00	0.88
	Pr171	14.91	0.85	10.78	0.90	8.57	0.90	14.28	0.84	16.00	0.90
	Pr172	17.10	0.83	9.79	0.81	12.63	0.88	12.26	0.76	13.00	0.89
	G10	7.89	0.76	6.60	0.85	6.45	0.76	5.58	0.78	9.00	0.74
	G49	17.32	0.83	10.35	0.87	8.28	0.85	13.47	0.85	17.00	0.86
	TAA	10.54	0.83	9.30	0.89	8.92	0.83	10.59	0.78	13.00	0.82
Total (uncorrected)		105	0.73	69	0.67	62	0.70	82	0.70	93	0.81

Table 3.3. Mean number of alleles (A) and expected heterozygosity (H_E) per population per locus. Per locus diversity measures have been corrected for a minimum sample size of 24. Per sample total values have not been corrected. Turure River samples T1 and T2 are upstream, whilst T3 and T4 are downstream. GU (Guanapo R.) is the putative source population.

Estimates of mean allele number (A) per locus were significantly greater for the source population (GU) than for the introduction site sample (T1) (sign test: $N = 7$, $P < 0.05$). A was also larger in sample T4 (downstream) than in sample T1 (upstream) (sign test: $N = 7$, $P < 0.05$). No equivalent differences in H_E were detected (for both sign tests: $N = 7$, $P > 0.05$). T2 and T3 possess intermediate diversity levels (Table 3.3).

3.4.2 *F*-statistics and population differentiation

Four out of five samples had significantly positive F_{IS} values (0.071 – 0.105; Table 3.4), indicating heterozygote deficiencies. T4 did not depart from HWE.

Pairwise differentiation estimates were always low (< 0.05; Table 3.5), although such low levels are not necessarily negligible biologically (Balloux & Lugon-Moulin 2002). Generally, R_{ST} values were smaller (70% on average) than their F_{ST} analogues. This difference is not due to the exclusion of G49 in R_{ST} calculation, since R_{ST} estimates based on all seven loci are similar (data not shown). This difference may be due to the higher sampling variance of R_{ST}, because the differences in mutation model underlying the two estimators are probably small relative to gene flow (Balloux & Goudet 2002; Balloux & Lugon-Moulin 2002). True differentiation values are probably intermediate between F_{ST} and R_{ST} (Balloux & Lugon-Moulin 2002). Differentiation was greatest between GU and T1 ($F_{ST} = 0.042$; $R_{ST} = 0.035$), but was smaller between populations along the Turure ($F_{ST} < 0.024$; $R_{ST} < 0.034$) (Table 3.5). Nearly all genetic variation (AMOVA: 98.31%; $P < 0.001$) occured within populations. Nevertheless, all tests of pairwise population differentiation were significant ($P < 0.05$).

Population	F_{IS}
T1	0.105*
T2	0.083*
T3	0.085*
T4	0.029
GU	0.071*

Table 3.4. Hardy-Weinberg relationships per population. *, $P < 0.05$.

Population	T1	T2	T3	T4	GU
T1	-	0.019	0.022	0.024	0.042
T2	0.006	-	0.011	0.018	0.024
T3	-0.006	0.034	-	0.011	0.018
T4	-0.013	0.008	-0.008	-	0.010
GU	0.035	-0.008	0.029	0.006	-

Table 3.5. Pairwise population F_{ST} (upper diagonal) and R_{ST} (lower diagonal). F_{ST} calculated following Weir & Cockerham (1984); R_{ST} calculated following Michalakis & Excoffier (1996).

3.4.3 Dispersal rates

Standard deviations of the posterior distributions of the dispersal rate, m, are all small (< 0.05), indicating that estimates of m may be reasonably accurate. Migration rates between populations are generally negligible (Table 3.6); the principal exception is a rate of 0.118 (S.D. = 0.041) from T1 into T2 (both upstream). Most individuals are derived from the location at which they were sampled, further demonstrating the existence of population substructure. Dispersal is highly asymmetrical, almost always occurring downstream: upstream m varies 0.001 – 0.003; downstream m varies 0.018-0.118.

		Donor population			
		T1	T2	T3	T4
Recipient population	T1	0.999 (0.003)	0.001 (0.003)	-	-
	T2	0.118 (0.041)	0.882 (0.041)	0.003 (0.004)	-
	T3	-	0.018 (0.013)	0.982 (0.013)	0.001 (0.004)
	T4	-	-	0.027 (0.030)	0.973 (0.030)

Table 3.6. Estimates of m (and S.D.) between adjacent Turure River populations. m is the proportion of individuals in one (recipient) population that is derived from another (donor) population in the current generation.

3.4.4 nDNA introgression and genotypic disequilibria

In the Lower Turure R. sample just downstream of the waterfall barrier (i.e., T3), 56 out of 61 individuals had admixture coefficients (q) ≥ 0.95 (Fig. 3.2). Their posterior probability intervals were tightly constrained between $q = 0.67$ and 1.00; most individuals thus had a nuclear genome largely derived from the introduced population. Indeed, 41 individuals had a lower probability interval above 0.90. The population-level admixture proportion was $0.97 \pm 0.02\%$.

By contrast, within T4, which occurs ~ seven km downstream from T3, only 11 out of 24 individuals had $q > 0.94$. Probability intervals were generally much larger, and only one individual had a lower probability interval above 0.90 (Fig. 3.3). The population-level admixture proportion ($0.90 \pm 0.04\%$) is significantly smaller than that for T3, as adjudged by comparing probability intervals. The wide probability intervals for individuals with intermediate q values (ie. putatively introgressed individuals) make it difficult to identify single admixed individuals.

The maximum-likelihood analysis of population admixture proportions indicated that the upstream source population's (T1) genetic contribution to T3 and T4 was 94.9 (± 24.6%) and 83.0 (± 15.1%) respectively.

No significant genotypic disequilibria were detected either within or across both admixed populations (Table 3.7).

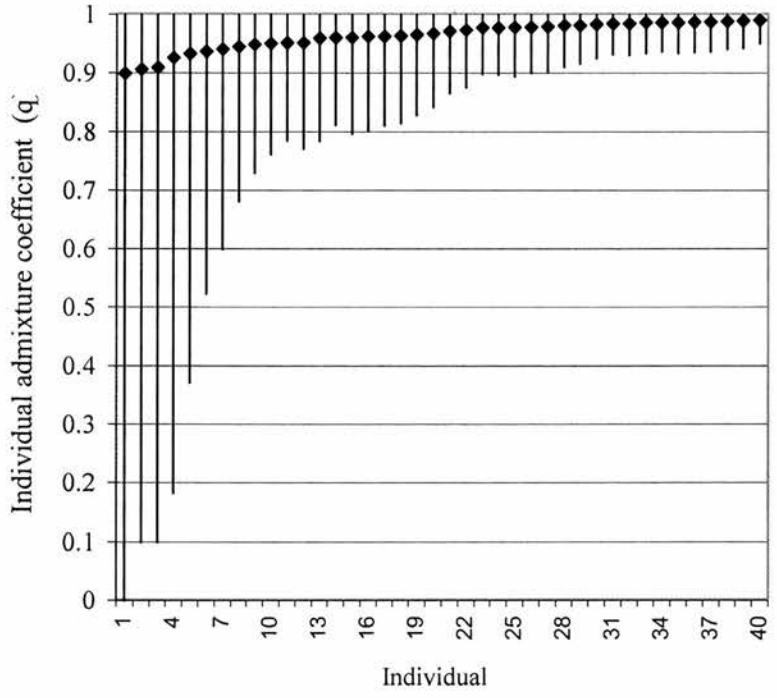


Fig. 3.2. Estimates of individual admixture coefficients (q) and their posterior probability intervals for the downstream Turure River sample T3. q values of 1.0 and 0.0 indicate genomes comprised entirely of introduced and native alleles respectively.

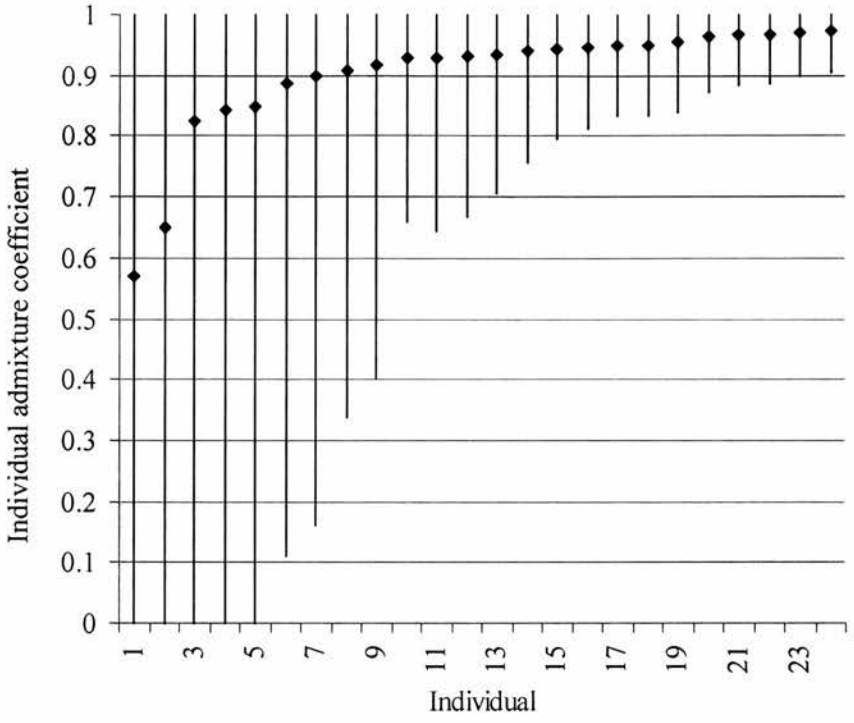


Fig. 3.3. Estimates of individual admixture coefficients (q) and their posterior probability intervals for the downstream Turure R. sample T4. q values of 1.0 and 0.0 indicate genomes comprised entirely of introduced and native alleles respectively.

3.4.5 mtDNA introgression

Only the introduced Caroni-type haplotype was found in the upstream samples T1 and T2. In T3, the introduced haplotype was detected in 51 out of 61 (83.6%) individuals. Becher and Magurran (2000) detected similar levels of mitochondrial introgression (88%) at this site from samples collected in 1998. In T4, only ten out of 24 (41.67%) individuals had the introduced haplotype.

Locus pair	T3	T4	T3 and T4 pooled
Pr171 x Pr172	0.605	1.000	0.657
Pr171 x G10	0.017	0.158	0.007
Pr171 x G49	0.007	1.000	0.010
Pr171 x TAA	0.549	1.000	0.564
Pr171 x Pr67	0.395	1.000	0.452
Pr171 x Pr36	0.636	0.713	0.648
Pr172 x G10	0.904	0.336	0.825
Pr172 x G49	0.380	1.000	0.457
Pr172 x TAA	1.000	1.000	1.000
Pr172 x Pr67	0.790	1.000	0.839
Pr172 x Pr36	0.229	0.938	0.397
G10 x G49	0.802	1.000	0.837
G10 x TAA	0.587	1.000	0.609
G10 x Pr67	1.000	1.000	1.000
G10 x Pr36	0.200	0.900	0.340
G49 x TAA	0.868	1.000	0.871
G49 x Pr67	0.730	1.000	0.770
G49 x Pr36	0.837	0.593	0.802
TAA x Pr67	0.683	1.000	0.698
TAA x Pr36	0.885	1.000	0.938
Pr67 x Pr36	0.976	0.479	0.920

Table 3.7. *P*-values for tests of genotypic disequilibria. Adjusted *P* value for 5% nominal level is 0.001. Samples T3 and T4 are in the admixed downstream section of Turure R.

3.5 Discussion

3.5.1 General comments

Because the available microsatellites were not diagnostic, they did not afford sufficient resolution to determine the hybrid or parental class that any one individual belonged to. So, it is currently impossible to say whether introgressive hybridisation has accompanied

population admixture, though it would seem likely, or to make inferences regarding the efficacy of barriers to gene flow. This is unfortunate given that the strength of intrinsic post-zygotic isolation assayed in the laboratory is considerable (Chapter 2), suggesting that a reduction in the frequency of hybrids may well have been present.

However, it is apparent that the magnitude of asymmetrical introgression by introduced nuclear alleles is very high in the lower Turure (mean admixture proportions $\geq$ 83%), even at the river's end (site T4), some 8.8 km from the introduction site. Broad agreement between the results of the Bayesian and maximum-likelihood analyses suggests that this conclusion is fairly reliable. Turure guppies thus illustrate the potentially damaging consequences of artificial introductions. Previously, Shaw et al. (1992) did not detect introduced diagnostic allozyme alleles at T4. But their results are not directly comparable with mine, so it is uncertain if the invasion front has moved between the years separating our two studies. Lastly, it is not known how far such displacement has progressed, since sampling of sites downstream of T4 has not occurred.

In contrast to nuclear alleles, and although initially high (84%), the frequency of the introduced mitochondrial haplotype declines down the river (to 42%). Differences in the distribution of nuclear and mitochondrial alleles have many possible explanations, including stochastic effects, differential fitness amongst different cytonuclear combinations, male-biased dispersal and greater survival or mating success of migrant males over migrant females. Although evidence for male-biased mobility exists for the guppy (Magurran & Seghers 1994; Croft et al. 2003a), insufficient information exists to discriminate amongst these hypotheses here. Diagnostic nuclear markers would allow more detailed studies of possible cytonuclear disequilibria (Avise 2001).

Other signatures of the introduction and subsequent admixture exist. The differential effects of the introduction on the diversity measures A and H_E is characteristic of bottlenecks, during which A is typically reduced faster than H_E (Maruyama & Fuerst 1985). A bottleneck effect is also suggested by the fact that the greatest differentiation exists between samples T1 and GU (the source population), since bottlenecks tend to increase genetic distances (Hedrick 1999). (note that the effective size of the introduced population, numbering ~ 200 individuals with a sex ratio approximating unity, can not be estimated because of strong female mate choice and the capacity of females to store sperm

over several months (Winge 1937)) Also, increases in genetic diversity with population admixture are evident from both *A* and H_E. Finally, although genotypic disequilibria can arise from population admixture, their absence suggests that recombination has been sufficient to erode any that may have arisen.

Estimates of migration rates in the current generation *(m)* were not obviously related to those of population differentiation. This may be because recent migration rates differed from historical rates, whose cumulative effects influence differentiation statistics. The low levels of population differentiation, and the concomitant distribution of nearly all variation within populations, suggest that gene flow is historically appreciable. However, the extent to which the differentiation estimates reflect expansion of the introduced population remains uncertain.

3.5.2 Asymmetrical introgression at nuclear loci

What may be the cause of the displacement of native nuclear alleles? Perhaps the most parsimonious explanation is the dispersal of introduced genotypes into native downstream populations, in the absence of any differential selection regimes acting on immigrants and residents (i.e., 'genetic swamping'; see Kondrashov 1992 and Huxel 1999). The resultant displacement becomes more probable as immigration rates increase (Huxel 1999). Some consider this scenario an unlikely consequence of most introductions, given the initial relative rarity of an introduced taxon (e.g., Avise et al. 1997). But it is feasible here because of the sustained asymmetrical dispersal the native populations would have been subjected to from introduced populations located upstream.

Alternatively, the displacement could reflect fitness differentials between the introduced and resident individuals. Such differentials can result from differences in life-history traits, particularly growth rate (e.g., Scribner 1993; Scribner & Avise 1994a,b; Crespin & Berrebi 1999; Perry et al. 2001), or in sexual behaviour. Magurran et al. (1996) identified large differences in male mating success between Lower Tacarigua R. (Caroni) and Oropuche R. (Oropuche drainage) guppies in the absence of discriminatory female choice, due possibly to differences in male dominance and aggression. However, this hypothesis cannot be investigated here because of the lack of putative F_1s. Moreover, sexual

behaviour alone is insufficient to explain asymmetrical introgression at nuclear loci (e.g., Sperling & Spence 1991).

Finally, hybrid unfitness may facilitate species displacement (e.g., Crespin & Berrebi 1999). In guppies, intrinsic (environment-independent) isolation is strong (Chapter 2) and extrinsic (environment-dependent) isolation may be substantial (Schluter 1998). Generally, however, the capacity of even strong post-zygotic isolation to cause displacement appears weak, unless other factors are involved (e.g., Avise & Saunders 1984; Baker et al. 1989; Allendorf et al. 2001).

3.4.3 Dispersal rates

Adaptive differentiation of guppy populations inhabiting downstream and upstream sites is promoted by many factors, including differences in predation regime and in abiotic factors such as temperature and water turbidity (see Chapter 1.4.1 and 1.4.2). Yet, despite the presence of female site fidelity (Haskins et al. 1961), adaptive population differentiation is feasibly being constrained by inter-population dispersal. This is because the magnitude of such dispersal remains unknown and because males do not show site fidelity (Croft et al. 2003a,b). So, any existing dispersal could readily be translated into gene flow by the sneaky mating of immigrant males, rare male advantage in sexual selection (Hughes et al. 1999) and incomplete behavioural isolation based on female mating preferences (Houde 1994). Moreover, gene flow-induced constraints on adaptive differentiation are more likely when the spatial scale of gene flow is large relative to that of habitat change; a situation Endler (1977, 1995) has suggested exists for Trinidadian guppy populations.

The little information that exists on guppy dispersal comes from mark-recapture experiments. Reznick et al. (1996) suggested that dispersal was not appreciable by estimating emigration rates between natural pools as 4-5% over 12 days. More notably, Haskins et al. (1961) introduced a novel colour marker into a natural population (Arima River), which subsequently dispersed ≥ 6.1 miles over 18 – 36 generations. In the current study, ~ 12% of individuals in T2 were migrants from T1, 0.9 km upstream. This value suggests that inter-population dispersal is appreciable. It is unlikely to be an artefact of the introduction, which preceded sampling by 88-176 generations, or of interactions between native and introduced genotypes because T1 and T2 exist in the upstream section, where

native genotypes have never been recorded. However, the caveat that flash flooding may explain much of this migration can not be discounted, though there are no records or observations to suggest that they were responsible. m was negligible between samples T2 and T3, and between T3 and T4, which are separated by 3.3 and 5.6 km respectively, indicating that greater distances impede dispersal.

From Haskins's et al.'s (1961) experiment, Endler (1977) inferred the scale of male gene flow (l) as ~ 0.75 km. Because stream drainages are small (Endler 1980), populations inhabiting different selection regimes are thus not distantly separated (in l units), indicating that gene flow could constrain adaptive differentiation (Endler 1995). Our results are consistent with Endler's (1977, 1995) hypothesis. The Upper Turure R. is short ($\geq$ 1.0 km; discounting tributaries), whereas m over 0.9 km was high (12%), suggesting that a non-trivial proportion of dispersal events in this section may result in downstream gene flow. This scenario assumes the successful navigation of waterfalls, which is reasonable because downstream populations possess greater genetic diversity consistent with frequent downstream (and little upstream) dispersal (Shaw et al. 1991), and because allozyme studies have not detected marked genetic differentiation coincident with dispersal barriers (Shaw et al. 1994). This scenario also assumes that dispersal translates into gene flow, which is likely as females show non-exclusive mate preferences (Brooks & Endler 2001), and because sneaky mating is highly prevalent (Matthews & Magurran 2000).

The consequences of inter-population dispersal for adaptive differentiation remain to be more thoroughly explored using introduction experiments. Such experiments would have to avail themselves of more detailed information regarding phenotypic differentiation and differences in selection regime than is available for the current study.

CHAPTER FOUR

Assessing the prevalence of inter-specific inseminations

4.1 Summary

The reproductive success of sneaky mating in internally fertilising fish is difficult to determine. This problem is particularly acute in the guppy, where sneaky mating may have significant antagonistic effects on key evolutionary processes. This problem was first approached by testing the efficacy of the tactic in securing inseminations. To facilitate this test, a novel assay was developed that allows recovery of sperm DNA from the reproductive tract of females. This assay was used to test whether sperm transfer occurs between sympatric populations of the guppy and its putative sister species, *P. picta*, which could be due only to sneaky matings. Having amplified tract extracts from 600 females of both species from two sites, conspecifc sperm was detected in approximately 86% of females. Heterospecific inseminations were far less common, at less than 4%. Nevertheless, because strong behavioural isolation exists between guppies and *P. picta*, these results provide conservative proof for the efficacy of sneaky mating in transferring sperm. Also, the results are consistent with the hypothesis that sexual conflict can retard population divergence, and suggest that behavioural isolation alone is unlikely to have driven speciation between these taxa.

4.2 Introduction

Alternative male mating tactics are taxonomically widespread, occurring in mammals, birds, crustaceans, anurans and insects (Schuster & Wade 2003). They are particularly common in fish (Avise et al. 2002), where male tactics include resource monopolisation, reproductive parasitism (e.g., sneaking and female mimicry) and co-operative breeding (Taborsky 2001).

Alternative mating tactics (AMTs) exist for several reasons. Although genetic polymorphisms are occasionally responsible (e.g., the cichlid *Lamprologus callipterus*; Taborsky 2001), AMTs mostly occur in genetically monomorphic systems, due to ontogenetic shifts in development (e.g., the peacock blenny, *Salaria pavo*; Oliveira et al.

2001) or to phenotypic plasticity. Phenotypic plasticity in mating tactics entails the facultative use of tactics depending on environmental and social factors. For example, the incidence of sneaking may increase with male bias in population sex ratio (e.g., the common goby, *Pomatoschistus microps*; Magnhagen 1998), and population density (e.g., rose bitterlings, *Rhodeus ocellatus*; Kanoh 2000). It also reflects the distribution of relative body size amongst competitors (e.g., *Gambusia holbrooki*; Pilastro et al. 1997; Magnhagen 1998; Kanoh 2000), and concomitantly whether or not an individual is in possession of a territory (e.g., European bitterlings, *R. sericeus*; Candolin & Reynolds 2002).

Whatever the mechanistic basis of AMTs, few population genetic data are available to evaluate their reproductive success (Avise et al. 2002). Outside of the Salmonidae, all genetic studies have examined mating systems involving external fertilisation and territorial males that provide brood care. Their results indicate that fertilisations derived from sneaky matings can be very common (examples that I have compiled from the literature are given in Table 4.1). Similarly, in salmonids, where males do not build nests but compete for proximity to spawning females, the reproductive success of sneaking males is often considerable (see Blanchfield et al. 2003 for review). The success of sneaking males in internally fertilising fish has not yet been established for any species.

Male guppies are phenotypically plastic in their use of two mating tactics: sigmoid displays that may result in consensual copulations and sneaky matings towards unreceptive females (Chapter 1.4.1). Although there has been extensive research on consensual copulations in relation to sexual selection (Chapter 1.4.1), the evolutionary consequences of sneaky mating have been relatively neglected. And these effects may be substantial; for example, sneaky mating may impede adaptive differentiation between populations connected by dispersing males (Magurran 1998). It may also diminish the opportunity for sexual selection within populations by reducing the variance in male mating success (Jones et al. 2001b), and thereby retard the evolution of behavioural isolation between populations (Magurran 1996, 1998, 2001).

Species	Reproductive success of sneaky mating		Reference	Notes
	# nests with sneaked fertilisations (total # nests assayed)	Average % of fertilisations within mixed-parentage broods derived from sneaky mating		
Bluegill sunfish (*Lepomis macrochirus*)[¶]	N/A	N/A	7	Of 1677 young from 38 nests, ~ 20% derived from sneaked matings.
Dollar sunfish (*L. marginatus*)	2 (23)	45	6	
Redbreast sunfish (*L. auritus*)	14 (36)	<10	1	
Sand goby (*Pomatoschistus minutes*)	12 - 20 (41)	10	3,4	
15-spined stickleback (*Spinachia spinachia*)	5 (28)	63	2	
Rose bitterling (*Rhodeus ocellatus*)	N/A	N/A	5	Experimental competition trials testing sneakers against territory holders; sneakers obtained 30% of all fertilisations.

Key: 1, DeWoody et al. 1998; 2, Jones et al. 1998; 3, Jones et al. 2001a; 4, Jones et al. 2001b; 5, Kanoh 2000; 6, MacKiewicz et al. 2002; 7, Neff 2001.

Table 4.1. Some non-salmonid studies that have examined the reproductive success of sneaky mating in fishes. In these systems, dominant males construct and defend nests. All systems bar *L. macrochirus* are phenotypically plastic.[¶], study could not discriminate between sneaky and female mimicky phenotypes as causes of cuckholdry.

In fact, several considerations suggest that sneaky mating in guppies is important. First, it is extremely common, occurring at rates of up to one per minute per female (Magurran & Seghers 1994; Magurran et al. 1995), implying that it must have some adaptive value. Second, sneaky mating in systems where AMTs are conditional usually result in fertilisations (Table 4.1), and there is no reason to assume that guppies are atypical in this respect. Finally, there appears to be a correlation between the incidence of sneaky mating, which is determined by predation regime, and the strength of behavioural isolation (see Magurran 2001 and Chapter 5.2). So, sneaky mating should be regarded as a potentially important force in the evolutionary dynamics of guppy populations.

The neglect of sneaky mating is partly due to difficulties in its study, which stem from the highly promiscuous nature of guppy sexual behaviour, the very rapid transitions between mating tactics used by individual males and the presence of internal fertilisation that allows differential selection of sperm at the post-insemination stage (e.g., Evans et al. 2003a). As a result of these problems, research into sneaky mating has concentrated on its efficacy in transferring sperm to the female reproductive tract. The approach adopted in these studies has been to microscopically examine extracts from the female tract, though it has met with some criticism (see Discussion).

An alternative means of studying sperm transfer is the amplification of male DNA that has been isolated from the female tract. Because the polymerase chain reaction is involved, this approach offers a much more sensitive assay than microscopic procedures.

Here, I describe a novel protocol for the genetic detection of inseminated sperm in vertebrates, and illustrate it by examining the ability of sneaky mating by male guppies to inseminate females. Specifically, I tested whether inter-specific inseminations occur between sympatric populations of guppies and their putative sister species, *P. picta* (Breden et al. 1999). Laboratory experiments have shown that females of both species will not mate consensually with heterospecific males (Haskins et al. 1961; Liley 1966). In contrast, males mate assortatively but incompletely in the lab (Liley 1966; Magurran & Ramnarine 2004) and in the field (personal observation). Therefore, forced copulations only can drive inter-specific inseminations, and guppy – *P. picta* matings provide a conservative test of their efficacy in transferring sperm. The described protocol also has utility for speciation research, since it helps to illuminate the current relative importance of

different barriers contributing to reproductive isolation. This utility is explored in connection to guppy – *P. picta* matings too.

4.3 Materials and Methods

4.3.1 Sampling and characterisation of school composition

Although normally found in brackish or saltwater environments (Kenny 1995), *P. picta* occasionally occurs in lowland freshwater habitats in Trinidad, where it may then co-exist with guppies. We used two such two sympatric sites (Fig. 4.1): Sumaria Trace and Beharrylal Trace. Respective map coordinates are PS 730 674 and PS 731 677 for Series E 804 (D.O.S.) maps published by the Trinidad and Tobago Government (1978). Both sites are adjacent to the saltwater Caroni Swamp in western Trinidad.

Because rates of inter-specific insemination are likely to depend on species composition dynamics within schools, baseline information on school structure was obtained twice, on 1 day each in August 2003 and January 2004. On these occasions, whole schools were collected using a one-man seine net, and the sex and species identity of their constituent adult fish was recorded. These fish were then immediately released back to their capture points. During the second sampling period, 150 females of each species from each site were also sampled, but instead moved to aquaria at the University of West Indies for sperm extraction.

4.3.2 Laboratory procedures

Sperm retrieval

The rate of sperm loss from the female guppy reproductive tract is not known, though it is possible that this rate is greater for heterospecific than conspecific sperm (e.g., see Price et al. 2001). Thus, although sperm do remain in the tract for up to seven days following conspecific copulations (Matthews & Magurran 2000), sperm extractions took place within 36 hrs of field sampling to minimise potential sperm loss. Females were anaethised in a water bath containing a non-lethal dose of Benzocaine (0.4 g L^{-1} ethyl p-amino benzoate) and then held on a polystyrene platform to expose their genital pores. Fine plastic tubing

A. Beharrylal Trace

B. Sumaria Trace

Fig. 4.1. Sampling sites.

that contained 10 µL physiological solution (0.9% saline) was inserted into the reproductive tract and compressed to expel its contents. The resultant sperm suspension was transferred to an eppendorf, and this washing step was repeated twice more to ensure that all recoverable sperm were retrieved; see Pilastro & Bisazza (1999) and Matthews & Magurran (2000) for experimental validation of this procedure. Between samples, the tubing was rinsed three times in distilled water to prevent cross-contamination. Because microscopic examination of the third rinse's wastewater failed to detect any cellular material over several trial extractions, it was assumed that cross-contamination was negligible. Tract extracts were stored in 95% ethanol for roughly eight weeks until genetic analysis. Once females had been assayed they were returned to their sampling sites.

Genetic analysis

Sperm samples were dried before analysis. To allow recovery of sperm DNA that was uncontaminated by the female cell fraction, extracts were initially lysed with SDS/proteinase K, to which sperm but not female cells are resistant (Gill et al. 1985; Yoshida et al. 1995). 100 ml 1 X SSC (plus 1% SDS) was added to each extract and samples were lysed at 60 °C for 1 hr. Samples were spun down at 12,500 rpm for 5 min and then resuspended in 0.5 ml 1 X SSC (plus 1% SDS). This step was repeated twice, though following the third centrifugation the sample was resuspended in 0.5 ml 1 X SSC only. The resultant suspension was again centrifuged at 12,500 rpm for 5 min, and the pellet finally resuspended in 0.1 ml TNE buffer. This process yielded the male only fraction. Supernatants from each centrifugation were combined to form the female fractions of the extracts and were retained for later analysis (see Results).

Male fractions were lysed using dithiothreitol (DTT)/proteinase K. To each fraction, the following was added: 6.25 µl TNE buffer, 12.5 µl 10% SDS, 2.5 µl 1M DTT, 1.25 µl proteinase K (20 mg /ml) and 77.5 µl H_2O. Samples were incubated at 60°C for 1 hr and then cooled in ice for 5 min. Finally, samples were subjected to one round of standard phenol-chloroform extraction (Sambrook et al. 1989).

Polymerase chain reaction (PCR) amplification of tract extracts occurred at two microsatellite loci: Pp125 (this chapter) and Pr172 (Becher et al. 2002). Pp125 was cloned from a Tobagan *P. picta* sample using the same methods as Becher et al. (2002; Appendix

II). It has the following characteristics: forward primer (5' to 3'): CAA CTT TAG CTG GGA ATA GCT GAC; reverse primer (5' – 3'): TGT AGA GAG TGW ACA GAA ACG ATG. The Pp125 primers amplify a $(TG)_{17}(CG)_2(TG)_8(CG)_4(TG)_{27}AC(AT)_2$ repeat motif with an original clone length of 211 bp. Both loci are reciprocally diagnostic for *P. picta* and Caroni Drainage guppies, allowing insemination frequencies to be examined in both directions of the cross. Amplifications occurred in 15 µl reaction volumes containing 2.0 mM of each dNTP, 1 X Taq buffer, 0.2 pmol of each primer, 2mM $MgCl_2$, 1 unit Taq (Bioline) and 5 µl DNA template (10-20 ng / µl). All PCR amplifications occurred in PTC-100 thermocyclers (MJ Research) under the following conditions: initial denaturation at 92°C for 3 min, 30 cycles of amplification (92°C for 30 s, x°C for 30 s and 72°C for 30 s) and a final extension at 72°C for 5 min. The annealing temperatures for Pr172 and Pp125 were 53°C and 58°C respectively. Amplification products (3 µl) were electrophoresed on 3% agarose gels for 30 min and visualised using ethidium bromide.

4.4 Results

Field observations indicated that schools occurring in the zone of admixture generally contained both species and that their compositions were extremely dynamic (data not shown). Species admixture proportions were therefore averaged over schools (Table 4.2). These data show that between-year variation in admixture proportions exists. Yet, admixture appears to have persisted for at least two years (A.E. Magurran, personal communication), so it is likely that admixture is neither infrequent nor transient. Sex ratios are largely male-biased, but this may be an artefact of the sampling techniques, with which females were harder to catch.

Heterospecific inseminations occurred at both sites in both directions of the cross (Table 4.3), though at low frequencies (< 4 %). Conspecific inseminations were detected for 86.3% of all females and so were considerably more common (> 20 times). One potential caveat regarding the interpretation of these data is that female DNA may occasionally have contaminated the male sperm fraction, despite differential lysing of male and female cells. This possibility could be evaluated using sex-specific markers, but these are currently unavailable for the guppy. Nevertheless, the separation process has proved effective in a similar study (Tripet et al. 2001), and this issue does not affect estimates of heterospecific

matings. What may have affected the power to detect heterospecific matings, however, is the loss of sperm DNA to the female fraction. This latter possibility was checked using standard chloroform-phenol procedures (Sambrook et al. 1999) to complete the DNA extraction from a random subsample of female cell fractions ($N = 30$ for both species). These extractions were then amplified with both diagnostic primers. All attempted heterospecific amplifications failed, though most (50 / 60) conspecific amplifications yielded products, suggesting that loss of sperm DNA is not detrimental to detection power.

Population	Sampling period	% guppy males	% guppy females	% *P. picta* males	% *P. picta* females	Total # fish
Sumaria Trace	August 2003	35.4	30.3	16.2	18.2	99
	January 2004	31.4	12.8	33.1	22.9	343
Beharrylal Trace	August 2003	25.6	0.0	38.3	36.2	47
	January 2004	36.1	29.6	26.9	7.6	216

Table 4.2. Characteristics of the admixed populations.

♀	Inseminations detected from ♂s of:	Site	
		Sumaria Trace	Beharrylal Trace
Guppy	Guppies only	134 (89.3)	129 (86.0)
	Heterospecifics only	0 (0)	0 (0)
	Both species	5 (3.3)	2 (1.3)
P. picta	*P. picta* only	128 (85.3)	117 (78)
	Heterospecifics only	0 (0)	0 (0)
	Both species	3 (2)	0 (0)

Table 4.3. Numbers (and percentages) of females inseminated by heterospecific and homospecific males. Data come from a total of 150 females of either species at each of two sites.

4.5 Discussion

4.5.1 Efficacy of sneaky mating in transferring sperm

The issue of whether sneaky matings result in sperm transfer has seen extensive investigation, but has been unsatisfactorily resolved. Initial studies by Clarke & Aronson (1951) and Baerends et al. (1955) failed to detect sperm inseminated after 1000 and 197 sneaky mating attempts respectively. In contrast, Kadow (1954) did record sperm transfer, but only at low levels (~ 17%). Because the proportion of consensual copulations that resulted in insemination appeared to be much greater (90%; Baerends et al. 1955), it commonly became assumed that sneaky mating is fairly unimportant (e.g., Endler 1987; Kodric-Brown 1993; Godin 1995; Houde 1997). However, these early studies probably underestimated sperm transfer rates due to crudeness in the methodology for retrieving sperm. Moreover, they were laboratory based and so may have failed to replicate the environmental conditions that drive successful sneaky mating. Indeed, recent studies using more sophisticated methods on field caught fish have suggested that sneaky mating actually results in high rates of insemination. For example, Evans et al. (2003b) retrieved sperm from ~ 45% of 376 field caught females. These females carried late-stage broods and were therefore unreceptive and, as sperm can not be recovered from the reproductive tract eight days after mating (Matthews & Magurran 2000), the retrieved sperm is likely to have come only from sneaky matings.

One objection that has been raised against all these studies, however, is that the retrieved sperm may have been washed from the female's sperm storage sites, which are located in cavities surrounding the ovary (Constantz 1984; Kobayashi & Iwamatsu 2002). Because sperm can be stored for up to six months (Constanz 1984), the extracted sperm may thus have resulted from accepted rather than sneak copulations. The present study obviates this problem since it considered heterospecific inseminations that could only have come from sneaky matings. This study constitutes a conservative test of sneaky mating because male mating preferences are incompletely assortative in the wild. Yet, heterospecific inseminations were recorded, indicating that sneaky matings may commonly drive sperm transfer within guppy populations. To determine the evolutionary consequences of this finding now requires ascertaining whether sneaking males can sire progeny and whether

sperm from sneaky mating suffers in competition with accepted sperm during post-copulatory selection (see Chapter 5.2)

4.5.2 Evolution of reproductive isolation between guppies and *P. picta*

Despite being relatively neglected, the consequences of sneaky mating for population divergence may be profound. For instance, hybridization between brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*) appears to be driven by sneaky mating (Garcia-Vazquez et al. 2002), whilst it may also be responsible for the considerable amount of hybridization that occurs between *Lepomis* sunfish species (Jennings & Phillip 2002). In the case of guppies and *P. picta*, however, laboratory crosses do not result in offspring (Liley 1966) or fertilization (A.M. Ludlow, personal communication), indicating that complete gametic isolation exists. Gene flow driven by sneaky mating between these species is therefore impossible.

Behavioural isolation between guppies and *P. picta* is also strong, with females of both species mating completely assortatively (Liley 1966). But when guppy males are given a choice of heterospecific and conspecific females that occur in allopatry, they initially mate at random (Magurran & Ramnarine 2004). Interestingly, when these males are allowed to associate with allopatric *P. picta* females, they rapidly develop strong assortative mating preferences within five days (Magurran & Ramnarine 2004). Moreover, laboratory experiments probably underestimate the strength of behavioural isolation in the field (Coyne & Orr 2004). So, as long as population admixture has persisted for longer than several days, wild guppy males may reasonably be expected to show some behavioural isolation. The populations used in this study have been sympatric for at least two years (A.E. Magurran, personal communication). Yet, our work has shown that this length of time, or the selective forces driving reproductive character displacement, has been insufficient for the evolution of complete behavioural isolation.

Why should assortative mating preferences in males evolve more slowly than those in females? The answer may lie in sexual conflict, where the evolutionary interests of males and females differ, ultimately because of anisogamy (Chapman et al. 2003). In guppies, female fecundity is a product of longevity and foraging efficiency (Magurran & Seghers

1994), whereas male reproductive success should be more influenced by finding mates (Magurran 2001). So, as in most species, the optimal number of matings for males probably exceeds that for females. Sneaky mating may therefore have evolved in guppies and Poeciliids generally to enhance male reproductive success, at the expense of female fitness (Pilastro et al. 2003). One factor determining reproductive fitness is genetic compatibility amongst mates, either at the gametic or post-zygotic stage, and females may be expected to constrain their mating preferences more rapidly during population genetic divergence than males. The existence of sneaky matings between *P. picta* and guppies may be an artefact of this process; an artefact that has persisted despite the complete reproductive isolation that otherwise exists and despite, therefore, the associated gamete wastage.

Theoretical studies suggest that sexual conflict can retard speciation when males are in control of fertilisations, but that speciation may be promoted under female control (Parker & Partridge 1998; Gavrilets 2000). Cumulatively, the research on inter-specific copulations driven by sneaky matings provides partial empirical support for this theory.

CHAPTER FIVE

OVERVIEW: Speciation and the guppy

The guppy, *Poecilia reticulata*, is beginning to provide a badly required perspective on speciation. Because speciation takes a long time, typically millions of years (Coyne & Orr 2004), researchers are obliged to infer speciation processes from studies of inter-specific taxa. Yet, these inferences may bear little relation to what actually happens during speciation, and many issues have thus proved intractable. Perhaps the largest uncertainty concerns the relative importance of different barriers in causing speciation. This thesis has shown that guppy populations from the Caroni and Oropuche Drainages of Trinidad constitute an ideal intra-specific model to help counter this problem.

5.1 Relative importance of different reproductive barriers in the guppy

If speciation is to be interpreted in terms of reproductive isolation (RI), some investigation of the reproductive barriers causing speciation must take place. This suggests that analysis of the efficacy of barriers between sympatric populations is necessary, even though allopatric speciation is probably the dominant geographical mode of speciation (Lynch 1989). Thus, a philosophical quandary may seem to exist. In reality, several practical solutions are available. One is to assay barriers between allopatric populations belonging to the same species. Such analyses indicate what barriers are likely to act following population admixture. They may also offer advantages over equivalent inter-specific studies, since they can not confound those barriers evolving after speciation with those evolving beforehand.

Amongst the best material for performing such intra-specific work is the guppy. Phylogenetic analyses indicate that allopatric populations from the Caroni and Oropuche Drainages in Northern Trinidad are highly divergent (see Appendix I), suggesting they may already be in an early phase of speciation. Moreover, a considerable literature has accumulated over the last ~ 60 yrs that details the evolutionary biology and behavioural ecology of Trinidadian guppies (and of poeciliids generally) (see Houde 1997 for review). This literature greatly facilitates the analysis of reproductive barriers, and their interpretation within specific environmental or genetic contexts.

Laboratory experiments using Caroni and Oropuche guppy populations have indicated that behavioural isolation is currently slight (Luyten & Liley 1991; Endler & Houde 1995; Magurran et al. 1996). In contrast, this thesis (Chapter 2) has shown that intrinsic post-zygotic isolation is substantial. For instance, marked male behavioural sterility was observed in the F_1 generation. This is the first demonstration that behavioural sterility can act within species, and that it may therefore be involved in causing speciation. The crossing work also detected hybrid breakdown in embryo viability, brood size and sperm counts in the BC_1 or F_2 generations.

Recent work has also shown that gametic isolation acts between Caroni and Oropuche drainage populations (A.M. Ludlow, personal communication). Its operation was ascertained by simultaneously inseminating females with sperm from a native male (from the female's own population) and from a foreign male (from a different drainage), and then by determining the proportion of offspring sired by the native male. The preliminary data indicate that native males sire a disproportionate number of offspring within single broods (mean = 75%), although it is currently not known whether this pattern reflects competitive or non-competitive effects.

Together, these data allow the current relative strengths of different reproductive barriers to be calculated. The requisite analysis emphasises a distinction between the absolute and effective magnitudes of a barrier. The effective magnitude of any barrier depends on its absolute magnitude and when it occurs in the reproductive cycle – early acting barriers have a greater effective magnitude than later acting ones, even when they have identical absolute sizes (Coyne & Orr 2004). Given the absolute magnitudes of two barriers I_1 and I_2, it is possible to calculate their effective strengths (p_i) as:

$p_1 = I_1/[I_1 + I_2 (1 - I_1)]$, and $p_2 = [I_2 (1 - I_1)] /[I_1 + I_2 (1 - I_1)]$ (Coyne & Orr 2004; p. 59)

Table 5.1 lists these values for behavioural and gametic isolation, and for the strongest component of intrinsic isolation, male dysfunction in courtship display. Their calculation has assumed that this behavioural dysfunction directly affects the reproductive success of hybrid males. Because this dysfunction has an absolute magnitude greater than that of other components of hybrid unfitness, and because it acts earlier than them in the sexual cycle, it serves as a useful litmus test for the potential effective strength of intrinsic

isolation. From Table 5.1 it is evident that the proportional contribution to total isolation from gametic isolation is much greater than that from intrinsic isolation, even though the absolute strength of the latter is greater. Nonetheless, intrinsic isolation currently appears to be an important component of reproductive isolation. The guppy therefore supports results from inter-specific studies that have implicated the role of multiple barriers in causing speciation (see Chapter 1.3.1).

Reproductive barrier	Absolute strength	Effective strength
Behavioural isolation	0	0
Gametic isolation	0.75	0.75
Intrinsic isolation (male behavioural sterility)	0.82	0.24

Table 5.1. Strengths of reproductive barriers that have been assayed between Caroni and Oropuche drainage populations of the Trinidadian guppy. The effective strength represents a correction of the absolute strength to take into account its position in the sexual cycle.

Results from Trinidadian guppies strongly contrast with the prevalent notion that behavioural isolation evolves more rapidly than other barriers amongst allopatric populations, which experience strong sexual selection (e.g., Mendelson 2003). In fact, there is little theoretical or empirical justification for this opinion (Turelli et al. 2001; Coyne & Orr 2004), especially as sexual selection is expected to drive the accumulation of genetic factors inducing sterility and gametic isolation too (Turelli et al. 2001). Nonetheless, there may be particular reasons for the general lack of behavioural isolation between Trinidadian guppy populations. One is the action of sexual conflict (see Chapter 5.2). Another is that different populations inhabiting the same predation regime often experience comparable physical and ecological environments, which may then select for similar sexual choice criteria and thereby preclude the evolution of behavioural isolation (Magurran 2001). Currently, it is impossible to distinguish between these hypotheses, although it may be possible to test the potential importance of sexual conflict by examining post-copulatory selection processes (see Chapter 5.2).

Importantly, as the strength of all reproductive barriers increases with time following speciation, the estimated effective strength of intrinsic isolation should decrease. So, inter-specific studies may underestimate the importance of intrinsic isolation. This thesis has avoided this problem by concentrating on intra-specific populations, although there is obviously no guarantee that the patterns documented here will hold throughout the remaining speciation process. The latter uncertainty can only be approached generally by using comparative analyses to look at the evolution of different reproductive barriers, including gametic isolation, across multiple species pairs. But all such studies have simply considered intrinsic isolation alone (e.g., Presgraves 2002; Lijtmaer et al. 2003), or in addition to behavioural isolation (Coyne & Orr 1989, 1997). Therefore, conclusions regarding the larger importance of individual barriers in speciation can only remain tentative.

Finally, laboratory assays of the strength of individual reproductive barriers can not determine whether these barriers are actually effective in restricting gene flow between sympatric populations. This problem can be resolved by the genetic analysis of hybrid zones. The approach entails examining clines using genetic markers or quantitative traits, and using the observed patterns of linkage disequilibria to infer dispersal rates, the strength of reproductive barriers and the number of loci under selection (see Fig. 5.1; Harrison 1990, Barton & Gale 1993).

To date, inferences on speciation biology have not been drawn from guppy hybrid zones. Though artificial introductions pose an increasingly large threat to the genetic integrity of Trinidadian populations (Magurran et al. 1995), only one hybrid zone has been identified. This exists between Caroni and Oropuche guppies, and arose from an introduction into the Turure River in 1957 (see Fig. 3.1). The native nuclear genotype appears to have been largely displaced throughout the length of the Turure R. (see Chapter 3 for details), although the structure and location of the current hybrid zone remain to be characterised. It is likely that the hybrid zone is currently located in a population density trough, where the introduced population is numerically smaller than native Oropuche drainage populations. Such a density trough may exist just below the confluence of the Quarte and Turure Rivers. Once characterised, the hybrid zone may prove appropriate for clinal analyses, which would require the additional development of modest numbers of reciprocally diagnostic markers.

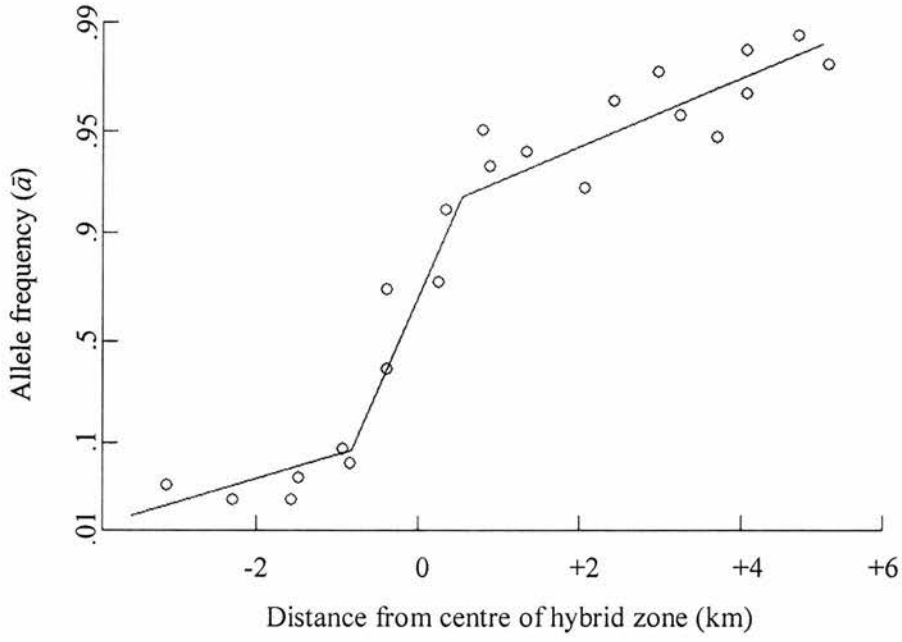


Fig. 5.1. A stepped cline between distance and marker frequency. The sharp step that is flanked by shallow tails of introgression reflects a barrier to gene flow, and is caused by linkage disequilibrium that results from selection against particular gene combinations. The strength of the reproductive barrier is measured by the ratio between the step in allele frequency and the gradient at the edge. This ratio has the dimensions of a distance, and corresponds to the distance that would pose the same obstacle to the flow of a neutral allele. (Figure adapted from Barton & Gale 1993)

A perspective from guppy – P. picta *crosses*

Complementary insights into the relative importance of different RI barriers were obtained by the genetic analysis of inter-specific sperm transfer (Chapter 4). Here, reproductive tract extracts were obtained from females living in sympatry with heterospecific (guppy or *P. picta*) males (*P. picta* is the putative sister species to the guppy; Breden et al. 1999). Heterospecific inseminations were detected in females of either species and indicated that mating preferences were incompletely assortative. However, inter-specifc crosses do not result in progeny because of gametic isolation (Liley 1966; A.M. Ludlow, personal communication). Thus, as with intra-specific guppy populations, post-copulatory barriers

seem to have evolved more rapidly than behavioural isolation. Again, sexual conflict may explain this observation (see Chapter 4.5.2).

A broader perspective from the Poeciliidae

Extrapolations from single crossing studies can often be imprudent given the stochastic nature of the evolution of speciation (see Chapter 1.3.3). However, speciation processes in the Poeciliidae have been well documented, relative to most other groups, such that meaningful generalisations may now be made from this family. Relevant studies are summarised in Table 5.2. The table shows that varied reproductive barriers are currently important in isolating related species. In most systems, excepting *Poecilia reticulata – P. picta* crosses, no reproductive barrier is complete, suggesting that some combination of barriers is necessary for total reproductive isolation. Hence, it is feasible that multiple barriers were involved in the speciation of these taxa.

Of particular interest is the cross between *Xiphophorus helleri* and *X. maculatus*. Progeny from backcrosses of F_1 hybrids into *X. helleri* are often inviable, due to the presence of invasive malignant melanomas. Genetic studies have ascertained that a two-locus system, corresponding to a simple Dobzhansky-Muller incompatibility, is responsible (see Orr & Presgraves 2000 for review). Preliminary evidence also suggests that Dobzhansky-Muller incompatibilities constitute a reasonable default explanation for intrinsic isolation in the guppy. Caroni and Oropuche guppy populations lack chromosomal rearrangements or differences in ploidy levels, indicating that alternative chromosomal explanations are untenable. Also, cytoplasmically inherited endosymbionts that can induce intrinsic isolation do not appear to occur in fish (Coyne & Orr 2004). So, the Dobzhansky – Muller model seems the most plausible explanation for guppies too.

Cross (♀ x ♂)	Behavioural isolation	Intrinsic isolation	Notes	Reference
Poecilia latipinna x *P. orri*	+0.6 (♀mating behaviour)	All crosses interfertile, but detailed data unavailable.		6
P. latipinna x *P. mexicana*	+0.89 (♀mating behaviour)			
P. orri x *P. latipinna*	N.S.			
P. mexicana x *P. latipinna*	N.S.			
Poecilia reticulata x. *P. picta*	+1.0 (♀mating behaviour); +0.9 (♂ mating behaviour between allopatric populations)	Not known	Strength of gametic isolation = +1.0	3, 4
Poeciliopsis occidentalis x *P. sonoriensis*	+0.49 (♂ mating behaviour)	+0.33 (brood size averaged across all BC lines)		1, 2
Xiphophorus helleri x *X. maculates*	Not known	BC to X. helleri often yield inviable progeny		5
X. pygmaeus x *X. nigrensis*	-0.58 (but +0.42 for the reciprocal)	Not known		7

Key: 1, Hurt & Hedrick 2003; 2, Hurt et al. 2004; 3, A.E. Magurran, personal communication; 4, Magurran & Ramnarine 2004; 5, Orr & Presgraves 2000; 6, Ptacek 1998; 7, Ryan & Wagner 1987.

Table 5.2. Studies that have examined reproductive isolation between poeciliid species. The strength of individual reproductive barriers are reported as absolute values. N.S. = non significant.

The Poeciliidae is also noteworthy for the existence of taxa with reticulate origins. For instance, *Poecilia formosa* probably arose from a hybridisation event between *P. mexicana* and a lineage belonging to the *P. latipinna/ P. velifera* complex (Schartl et al. 1995). This hybridisation perhaps occurred only 100,000 years ago (Schartl et al. 1995). *P. formosa* is hybridogenetic where, although sperm from *P. mexicana* or *P. latipinna* males is required to fertilise its eggs, the paternal genome is not transmitted into its gametes. Another example relates to unisexual lineages of the topminnow, *Poeciliopsis*. This complex includes diploid hybridogenetic and triploid gynogenetic groups of clones produced by a variety of hybridisation events involving *P. monacha* and some combination of four other species: *P. latidens*, *P. lucida*, *P. occidentalis* and *P. viriosa*. (Gynogenesis is where unisexual individuals require sperm from males of sexual species solely to induce embryogenesis.) These clonal lineages have typically been generated in the very recent past, although a comparatively ancient lineage, some 150,000 years old, has been identified (Quattro et al. 1992). Cumulatively, these parthenogens indicate that speciation can occur rapidly following hybridisation. This observation strongly contrasts with the gradual evolution of reproductive barriers between allopatric populations that has been inferred from comparative studies (see Chapter 1.3.3). Moreover, only ~ 70 unisexual vertebrate species are currently known (Vrijenhoek et al. 1989). So, although the incidence of hybrid species in the Poeciliidae seems appreciable, non-hybrid speciation is likely to be much more important across vertebrates as a whole.

5.2 Sexual conflict and speciation

What factors might influence the relative rate of evolution of different barriers? This question is important because lineage specificity in the distribution of these factors could vary the mode and tempo of speciation across taxa. In guppies, much evidence has accumulated suggesting that sexual conflict is one of these factors. (Sexual conflict can be defined broadly as "differences in the evolutionary interests between males and females (Chapman et al. 2003).") In particular, it is the combination of effects of predation regime and productivity that has been hypothesised to affect which sex is ahead in 'the battle of the sexes' within specific guppy populations (see Magurran 2001 for review). According to this hypothesis, the greater predation risk in downstream habitats simultaneously increases the incidence of sneaky mating whilst reducing the opportunity for females to express their mating preferences. The effect of productivity is to amplify these changes.

The result is that males and females are ahead in the battle of the sexes in downstream and upstream habitats respectively. Ultimately, this process is hypothesised to constrain the evolution of behavioural isolation between populations inhabiting downstream sites (Magurran 2001; see also Parker & Partridge 1998).

Regrettably, various properties of upstream sites make it impossible to convincingly test Magurran's (2001) prediction using comparative analyses (Magurran 2001). As a result, the potential importance of sexual conflict has to be inferred indirectly, and is perhaps most profitably investigated by testing for the presence of post-insemination selection mechanisms occurring in the female reproductive tract. One possible mechanism entails the preferential loss of sperm from sneaky matings from the tract, over those from accepted copulations. This possibility could be tested using histological analyses to contrast patterns of sperm fate in the female tract following single bouts of either forced or accepted copulations. Alternatively, competitive insemination trials could be conducted to examine whether sperm from sneaky matings are disadvantaged when females are already in receipt of sperm from accepted matings. These latter trials would require sperm from different males to be differentially labelled, a requirement that is perhaps best met using radioisotope labelling protocols described in Luyten & Liley (1991). Specifically, radioactive tracers such as thymidine-$_{14}$C can be injected into the male abdominal cavity, where they will eventually become incorporated into sperm. Should sperm from a sneaking male, say, be labelled in this way, they can then be distinguished from non-labelled sperm from males that have participated in accepted copulations.

5.3 Duration of speciation processes in guppies

Given the stochastic nature of the evolution of speciation, it is impossible to predict the duration of the speciation process in relation to specific taxa. (This duration is termed the *transition time for biological speciation*, and refers to the "time required to evolve strong reproductive isolation once the evolution of that isolation has begun" (Coyne & Orr 2004)) Moreover, only vague assistance is available from those theoretical works that have considered transition times. For instance, the genetic theory of Orr & Turelli (2001), which describes the accumulation of Dobzhansky-Muller incompatibilities between allopatric populations, is heavily dependent upon unknown parameters and only treats a single reproductive barrier such that transition times were probably overestimated. So, non-

genetic models seem the only theoretical recourse. These models have indicated that divergent sexual or natural selection can yield short transition times for allopatric populations, from several hundred to several thousand generations, depending on assumptions regarding the costs of female preferences and the particular mechanism of sexual selection involved. In contrast, allopatric divergence can be extremely slow when genetic drift is solely present, at least for large populations (Nei et al. 1983). Because selection appears to be involved in the accumulation of intrinsic incompatibilities (see Table 1.2) and in the divergence of traits causing other forms of isolation (Turelli et al. 2001), it is unlikely that drift alone determines transition time. So, the only reasonable inference possible is that the transition time for Caroni and Oropuche guppies is probably shorter than that predicted by drift alone.

Rather than use transition times, relevant empirical work has concentrated on alternative metrics, including the *Net Diversification Interval* (NDI). This measures the change in the number of surviving lineages per unit time, and is estimated from molecular phylogenies that have been calibrated with a molecular clock. The NDI therefore incorporates the transition time, in addition to any time elapsing between episodes of lineage splitting. In fish, there is a paucity of NDI estimates, which range from 5.6 to 2.2 myr^{-1} for N. American cyprinids (Stanley 1998) and *Rivulus* (McCune & Lovejoy 1998) respectively. Unfortunately, the NDI is difficult to interpret because of several serious caveats regarding its efficacy (Coyne & Orr 2004; p. 422). In any case, comparable estimates are unavailable from *Poecilia* species because of the absence of a calibrated molecular clock for that genus.

Another metric is the *Biological Speciation Interval* (BSI). The BSI measures the "average period elapsing between the origin of a new lineage and the next branching event in that lineage (Coyne & Orr 2004)". The BSI can be estimated from comparative studies that have investigated the relation between the strength of reproductive isolation and the amount of sequence divergence between sister taxa. It ranges from 1.1 to 5.5 myr in birds, frogs, *Drosophila* and lepidoptera (Coyne & Orr 1989, 1997; Sasa et al. 1998; Price & Bouvier 2002; Presgraves 2002), although these values were probably overestimated because reproductive barriers other than intrinsic isolation were ignored and because the taxa considered had already speciated. Unfortunately, equivalent data are absent for fish since Russell (2003) did not consider pairs of sister taxa.

In summary, all that can be said is that speciation generally takes millions of years, and that there is presently no indication that Trinidadian guppy populations are deviating grossly from this pattern. This inference, however, has depended on the assumption that Caroni and Oropuche guppies separated a long time ago, perhaps 2 mya, as judged by the application of an uncalibrated molecular clock to mitochondrial DNA sequences (see Appendix I). Fossil or biogeographical data are badly needed to help determine the true divergence time of these populations.

5.4 The genetic architecture of reproductive barriers

The genetic architecture of reproductive isolation has been intensively studied, leading to the identification of several phenomena. But this work has largely used *Drosophila* species, such that the generality of characterised phenomena remains uncertain. For instance, the magnitude of sex chromosome differentiation necessary to cause the large-X effect is unknown, since studies have previously only considered groups with highly differentiated sex chromosomes (e.g., *Drosophila*, rats, anophiline mosquitos). Another uncertainty concerns the number of loci responsible for initially causing speciation. This latter uncertainty exists because empirical work has concentrated on inter-specific crosses, even though incompatibilities are expected to accumulate rapidly following speciation (Orr 1995; Orr & Turelli 1995). Thus, although inter-specific crosses have occasionally implicated over fifty loci in causing intrinsic isolation (e.g., Presgraves 2003; Tao & Hartl 2003), work using less diverged lineages, namely different subspecies of *D. pseudoobscura*, suggests the involvement of much fewer genetic factors (Orr & Irving 2001).

Guppies may be useful in helping to resolve these uncertainties. The worth of this work is already indicated by the conformation of guppies to Haldane's Rule, an observation suggesting that guppies would provide a conservative test of the role of the Dominance theory, versus faster male evolution, in causing reproductive isolation (see Chapters 1.3.3 and 2.5.2). With regard to evaluating the number of loci contributing to intrinsic isolation, the large chromosome number of guppies ($2n = 48$) may present some technical challenges. This is because a large number of markers would be required to maintain an adequate mapping density. A practical solution might be to identify those chromosomes

harbouring QTL, as a prelude to targeted mapping. Such work has at least the potential to determine if many genetic factors (up to 48) are involved.

Field studies

Several methods are available for exploiting hybrid zones to make inferences on the genetic architecture of reproductive isolation. Each is complementary to the clinal analyses discussed in section 5.1. One is to test for differential patterns of introgression at multiple loci (e.g., Jiggins et al. 1997) or for patterns of segregation distortion in segregating hybrid populations. In both instances, those loci (and linked markers) contributing to RI should introgress less readily or be less frequent than expected by chance, thus providing conservative estimates of the number of genetic factors involved in causing RI. Should the markers be genetically mapped, then inferences regarding the location of individual QTL are also possible (e.g., Machado et al. 2002; Kauer et al. 2003; Panithanarak et al. 2004).

An alternative method involves the use of association mapping to obtain correlations between mapped markers and traits conferring RI. This method allows estimates of the numbers, locations and effect sizes of QTL, along with the genetic actions involved. It is therefore the most appropriate choice for *in situ* evaluations of the genetic architecture of RI. Moreover, it allows the efficacy of individual barriers to gene flow to be tested in the wild (Rieseberg et al. 1999; see Rieseberg & Buerkle 2002 for review). The approach is also advantageous as hybrid zones already contain representatives from multiple recombinant classes, circumventing the laborious procedures associated with establishing laboratory populations. Preferably, the genomic map used to identify QTL should be constructed independently from genetic analysis of the hybrid zone (i.e., in the laboratory; Rieseberg & Buerkle 2002).

Nonetheless, there may problems with such QTL work, including difficulties in distinguishing drift from selection given that only one hybrid zone is available (though the approach does appear to be reliable and repeatable; Rieseberg et al. 1999). Also, considerable improvements are required in the methodology, particularly with regard to the statistical association methods (Rieseberg et al. 1999; Rieseberg et al. 2000; Rieseberg & Buerkle 2002). Should these problems prove insurmountable, laboratory investigations of genetic architectures would be preferable.

Behavioural isolation

Besides intrinsic isolation, the genetic architecture of behavioural isolation may also be productively examined using QTL mapping. Of particular interest is the possibility that genes underlying female mating preferences are physically linked to those determining sexually selected male traits. Theoretical work has predicted that genetic correlations between these gene sets can facilitate the evolution of behavioural isolation, and that these correlations are most easily maintained when they are based on physical linkage (Fisher 1958). It has also been predicted that genes creating sexually antagonistic phenotypes should preferentially accumulate on the X chromosome (Rice 1984). To date, quantitative genetic and pedigree analyses have established that attractive traits in male guppies are predominantly either Y linked, or are capable of recombining between the sex chromosomes. A minority are X linked (Lindholm & Breden 2002). Because guppy sex chromosomes are only slightly differentiated, the species therefore provides a conservative test of the ability of (physical) linkage to engender behavioural isolation. More generally, such work would help to settle doubt over whether female preference traits are often disproportionately X-linked due to the effects of sexual antagonism (e.g., Moehring et al. 2004)

Perhaps the most appropriate cross for investigating behavioural isolation involves the Venezuelan Cumana guppy (Alexander & Breden 2004) and the more common, 'typical' guppy morph, of which Trinidadian populations were considered in this thesis. Preliminary experiments suggested that behavioural isolation between these taxa could be considerable (Alexander & Breden 2004), although there is currently uncertainty as to the reliability of this observation (see Chapter 2.5.3). By contrast, behavioural isolation between Trinidadian populations seems too marginal for their sole use to be productive (e.g., Magurran et al. 1996).

5.5 Additional considerations of reproductive isolation

Developmental basis of intrinsic isolation

The developmental basis of intrinsic isolation has only rarely been characterised (see Coyne & Orr 2004 for review). But it is feasibly explored in guppies. For instance, the recent development of protocols allowing the *in vitro* culturing of guppy embryos (C. Dreyer, personal communication) could allow the identification of developmental defects

leading to hybrid embryo inviability. In addition, *Drosophila* experiments have established that hybrid sterility generally results from post-meiotic defects, which are often manifested as problems in sperm motility and bundling (Coyne & Orr 2004). Whether the same factors are responsible for guppy hybrid sterility could also be explored. For example, hybrid sperm motility could be assayed using protocols developed for the poeciliid genus *Gambusia* (Adriaenssens et al. 2004).

Extrinsic isolation

Potential also exists for exploring extrinsic, environmentally mediated, reproductive isolation. Although the behavioural sterility shown by F_1 guppy males is probably intrinsic, it is likely to contribute to extrinsic isolation, since females may be less liable to mate with these males (see Chapter 2.5.1 for discussion). Similarly, disease resistance in hybrids may be reduced due to intrinsic genetic incompatibilities, with clear repercussions for extrinsic ecological fitness (e.g., Bert et al. 1993). These are possibilities that are readily amenable to laboratory investigation. Extrinsic isolation is usually conceived of as entailing the reduced ability of a hybrid to survive or reproduce in the divergent habitats of their parental forms, simply as the hybrid possesses an intermediate phenotype (Coyne & Orr 2004). Because guppy populations from the Caroni and Oropuche Drainages do not occur in overtly divergent habitats (Magurran 2001), it is unlikely that this particular form of extrinsic isolation exists in guppies, at least when the parental populations share the same predation regime.

Gametic isolation

Artificial insemination experiments have revealed the presence of post-copulatory selection mechanisms at the intra-specific (Evans et al. 2003a) and inter-specific (A.M. Ludlow, personal communication) levels. Both studies analysed patterns of sperm precedence using competitive assays involving the simultaneous insemination of females with sperm from two males. Evans et al. (2003a) showed that sperm from the more attractive male somehow fertilised a greater proportion of offspring within single broods. (Anna Ludlow's work is summarised in Chapter 5.1.) The mechanism underlying such differential selection is not known. Some possible answers are suggested by recent work in *Drosophila*, where it has been shown that gametic isolation can result from the differential incorporation of sperm into sperm storage sites (Price et al. 2001) or the differential loss of

sperm from these storage sites (Price et al. 1999, 2000, 2001). Whether the same factors operate in guppies could be explored using sperm labelling experiments.

A further advantage of such experiments would be an extension of Evans et al.'s (2003a) work, which only considered two competing males. This is despite the fact that female guppies are sexually receptive for several days following parturition, allowing multiple (>> 2) mating opportunities. Hence, the intensity of directional and non-directional (i.e., genetic complementarity) selection simulated by Evans et al. (2003a) may have differed from that occurring naturally. So, the effective magnitude of postcopulatory selection in nature remains uncertain. That the intensity of directional selection was underestimated is suggested by several observations: (1) in laboratory populations the median number of sires per brood is two, even though females typically mate with more than two males (Becher & Magurran 2004), and (2) newly delivered sperm must also compete against stored sperm from previous brood cycles. Similarly, because a greater diversity of sperm genotypes are likely to be tested by females in natural populations, non-directional selection may also be more important than suggested by Evans et al. (2003a). These may be problems that are commonly encountered during competitive fertilisation experiments, yet their true importance is wholly unknown.

What labels can be used for such work? Lipophilic carbocyanine dyes that stain the cell membrane seem particularly suited since they are non-toxic, persist in vivo for up to several days, and are especially effective in staining dissociated cells that are to be introduced into foreign organisms or tissues (Dzuik 1996; Vercelli et al. 2000). Moreover, their efficacy in competitive fertilisation experiments has already been established for ungulates (Miller et al. 1998), murines (Youakin et al. 1994) and birds (fowl and turkey; King et al. 2002).

5.6 Miscellaneous

Effective mate number

This thesis has described the development of a novel protocol that allows sperm within the female reproductive tract to be genotyped at microsatellite loci. This protocol could readily be extended to include the sperm storage sites that occur adjacent to developing oocytes in guppies (Constantz 1984; Kobayashi and Iwamatsu 2002). It may then be possible to

estimate the number of males that have been successful in transferring sperm to the female, whether through sneaky matings or accepted copulations. This number constitutes the effective mate number (M_E). The statistical inference of M_E would be based on genotypic data from the female and sperm, and must account for shared alleles amongst different sperm donors. Currently, procedures are available that allow the frequency of multiple matings in a sample population of females to be calculated from such data (MateSoft v.1.0; Moilanen et al. 2004). None is yet available for the estimation of frequencies of particular values of M_E, although they could be developed from algorithms detailed in Pedersen & Boomsma (1999). One possible solution is the use of these algorithms in conjunction with a truncated Poisson distribution, should the latter accurately describe the underlying distribution of mate number. This particular solution is soon to be incorporated into the MateSoft package (J.S. Pedersen, personal communication).

M_E has important evolutionary consequences. For example, polyandry can enhance progeny fitness and number, perhaps through the replenishment of dwindling sperm reserves, or through the testing and preferential use of competitively superior sperm (Arnquist & Nilson 2000; Singh et al. 2002). In guppies, multiple mating results in offspring with more effective anti-predator behaviour, and in shorter gestation periods and larger broods (Evans & Magurran 2000; A.F. Ojanguren, personal communication). Experimental data indicate that guppy broods are sired by a median of two males (Becher & Magurran 2004), although observations show that mate number is considerably larger (A.E. Magurran, personal communication), suggesting that post-copulatory sexual selection is present. The parallel quantification of M_E using sperm genotypic data from natural populations, and of the number of males that sire individual broods (see Neff & Pitcher 2002), should provide a clearer picture of the strength of such selection (e.g., see Fernándes-Escudero et al. 2002).

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APPENDIX I

Phylogenetic analysis

Methods

The only gene for which sequence data are freely available in sufficient quantity to allow reasonably detailed phylogenetic analyses of guppy populations is the mitochondrial control gene (*CR*). The *CR* is the major non-coding region of the animal mitochondrial genome, and has a role in the replication and transcription of mtDNA. The vertebrate *CR* is commonly subdivided into three domains that differ in base composition, and in the rate and mode of evolution. While the central domain of the *CR* contains the heavy (H)-strand's origin of replication and is relatively conserved, the flanking domains (I and II) are typically hypervariable (Lee et al. 1995). Consequently, the *CR* is often useful in resolving relatively shallow relationships.

Largely complete sequences (~890 bp) of the *CR*, from individuals distributed across the guppy's natural range in S. America and Trinidad, were obtained from GenBank (Table I.1; Figs. 1.4 and I.1). Sequences were also obtained from both putative sister species to guppies (*Poecilia picta* and *P. parae*) and from three outgroups (*P. formosa*, *P. mexicana* and *Gambusia affinis*).

Fig. I.1. Provenance of S. American samples.

Species	Sampling locality			Genbank Accession no.
	Country	Drainage	River	
Poecilia reticulata	Trinidad	Oropuche	Turure	AY135469.1
			Rio Grande	AF170270.2
			Quare	AF529246.1
			Oropuche	AF193899.3
		Caroni	Arima	AY135452.1
			Aripo	AY135470.1
			Guanapo	AY135472.1
		Northern	Yarra	AY135461.1
		flowing	Paria	AY135453.1
		("Northern")	Marianne	AY135456.1
		drainages	Madamas	AF170262.3
	Venezuela	?	near Carapito*	AF538280.1
		?	Guanare	AY135457.1
		?	Isla Margarita	AF228610.2
		?	Poza de Azufre (near Carapito)*	AY135468.1
	Guyana	?	Bartica*	AF170257.3
		?	New-Amsterdam*	AF228609.2
		?	Springlands*	AF228608.2
	Suriname	?	Lelydorp*	AF228605.2
P. parae	Guyana	?	?	AF033050.1
P. picta	Trinidad	Caroni	?	AH005666.1
P. formosa		?	?	AY305353.1
P. mexicana		?	?	AY305343.1
Gambusia affinis		?	?	NC_004388.1

Table I.1. Details of sequences used in the phylogenetic analysis. *, sampling locations are towns.; ?, information missing from GenBank accessions.

Sequences were first aligned with Clustal X v.1.81 (Thompson et al. 1997). General characteristics of the data-set, including base content, transition and transversion ratios, and pairwise corrected and uncorrected (p) distances, were estimated using MEGA v.2 (Kumar et al. 2001) and PAUP* v.4b10 (Swofford 2001).

Considerable debate surrounds the relative efficacy of different methods for tree topology reconstruction. So, consensus topologies were estimated using maximum likelihood (ML), maximum parsimony (MP) and distance-based (neighbour joining; NJ) methods, and then compared to identify shared characteristics that may constitute the most reliable inferences of the true topology.

First, the best-fitting model of sequence evolution for the complete dataset was determined using the combined AIC-hierarchical likelihood-ratio test (NucModelCompare) in Hy-Phy v.0.99b (Pond et al. 2002). This test evaluated all 203 reversible nucleotide models, and determined that the best-fitting model was HKY85 + G (Hasegawa et al. 1985). This model was then used to reconstruct NJ and ML phylogenies using PAUP* v.4b10. In the NJ analysis, 1000 boostraps were used to infer confidence values. In the ML analysis, an NJ tree was initially constructed to optimise the transition/transversion rate ratio and the shape parameter of the gamma distribution in the nucleotide model, in relation to empirical base frequencies (T = 31.9%, C = 20.7%, A = 32.8%, G = 14.6%). The resulting model was used to obtain tree topologies with ten random addition replicates and TBR branch swapping. Confidence values were obtained using 100 branch-and-bound bootstraps. A representative non-bootstrapped ML tree was constructed to illustrate branch lengths.

The MP tree was constructed using MEGA v.2.1. Here, close-neighbour interchange searching (search level = 3) was based on trees obtained with the Min-Mini heuristic algorithm. 1000 boostraps were performed. All trees were edited using TreeView v.1.6.6 (Page 1996) or MEGA v.2.

To evaluate rate constancy of the molecular clock, the program MolClockAllRoots in Hy-Phy v.0.99b (Pond et al. 2002) was used. This program performs a likelihood ratio test of the null hypothesis of a global molecular clock, on every possible rooted version of a predefined tree (an NJ tree in this case). The HKY85 + G nucleotide model was again used. The analysis was conducted with all sequences present, but also with guppy

sequences only to see if a molecular clock could appropriately be applied to the two principal guppy clades that were identified in the phylogenetic analysis.

Results and Discussion

Pairwise uncorrected and corrected distances are very strongly linearly correlated, and there is no suggestion that transitions or transversions may asymptote with time (Fig. I.2; uncorrected distances are given in Table I.2). Thus, sequence saturation is negligible. Out of 891 sites, 156 were parsimoniously informative and 334 were variable for the complete dataset. The respective values for guppy populations only were 43 and 71.

The exact interpretation of bootstrap values is ambiguous. However, simulation studies suggest that they may provide conservative measures of node support, in which case a threshold of 70% may often be appropriate for defining significance (Zharkikh & Li 1992; Hillis & Bull 1993). This threshold is adopted here.

The MP, NJ and ML consensus trees (Figs. 1.7, I.4 and I.5 respectively) show some common attributes. For example, two reciprocally monophyletic guppy clades occur in all analyses: an 'Eastern' clade generally containing populations from the Oropuche Drainage only, and a 'Western' clade containing populations from the Caroni and Northern Drainages of Trinidad, in addition to those from mainland S. America. Node support for these clades is usually significant, although that for the Western clade in the ML analysis was only 61%. There were several incongruously placed sequences: the Turure sample (Oropuche Drainage) consistently grouped with western samples (specifically, as the sister lineage to Guanapo), probably reflecting an artificial introduction of Guanapo fish into the Turure (see Chapter 3 for further details). This is the case with another upper Turure sample too (AF327266; data not shown). Similarly, a Northern drainage sample, Madamas, was consistently placed in the Eastern clade. This may also reflect population admixture, since the two populations are geographically close, facilitating introductions by natural agents (e.g., birds or storms; Houde 1997) or humans.

Otherwise, though, relationships remained poorly resolved, and this is particularly the case for S. American samples. For instance, although the two Guyanese (New Amsterdam and Springlands) and the one Suriname sample clustered strongly together (with bootstrap

support > 90%), their relationship to other western samples remained unresolved. Moreover, the Venezuelan and Guyanese Bartica samples typically formed polytomies. Hence, our phylogeographic understanding of guppy populations is presently very crude. Broader geographical sampling and different (nuclear) genes are needed to obtain stronger inferences.

Likewise, relationships amongst the outgroups could not be determined. *P. picta* and *P. parae* were included in this study because they had grouped together with *P. reticulata* in a *NADH2*-based phylogeny of *Poecilia* species (Breden et al. 1999), suggesting that either was the sister species to guppies. However, the present analysis also failed to determine which is the more likely sister species.

Finally, the null hypothesis of a global molecular clock was consistently rejected, regardless of how the NJ tree was rooted, and of whether outgroup sequences were considered. To gain some appreciation of the divergence time (and numbers of generations) separating Caroni and Oropuche Drainage guppies, I examined the only available *NADH2* sequences from Genbank. Accession numbers are: AF178033 ("Caroni Drainage"), AF031394 (Aripo R.) and AF178032, AF178031 and AF031393 (Oropuche R.). The average Caroni-Oropuche pairwise uncorrected (*p*) distance was 5.2%. Although considerable rate variation in mitochondrial evolution exists between lineages, the mitochondrial "clock" is generally assumed to be ~ 2% myr^{-1} for non cold-tolerant fishes (Avise 1994). This clock translates into a divergence time of ~ 2.58 mya, or ~4-6 million generations. With this sample set, clock rate heterogeneities were tested using multiple relative rate tests (Tajima 1993) in MEGA v.2, and the null hypothesis of clock-like evolution could not be rejected. However, relative rate tests lack power, particularly when in-group sequences show only moderate levels of rate variation and modest numbers of variable sites (i.e., < 400 out of 1000; Bromham et al. 2000). These caveats make it likely that relative rate tests are unreliable for guppy sequences. Either way, robust estimates of divergence times require calibration of the clock with independent fossil or biogeographical evidence, both of which are currently lacking for Poeciliids.

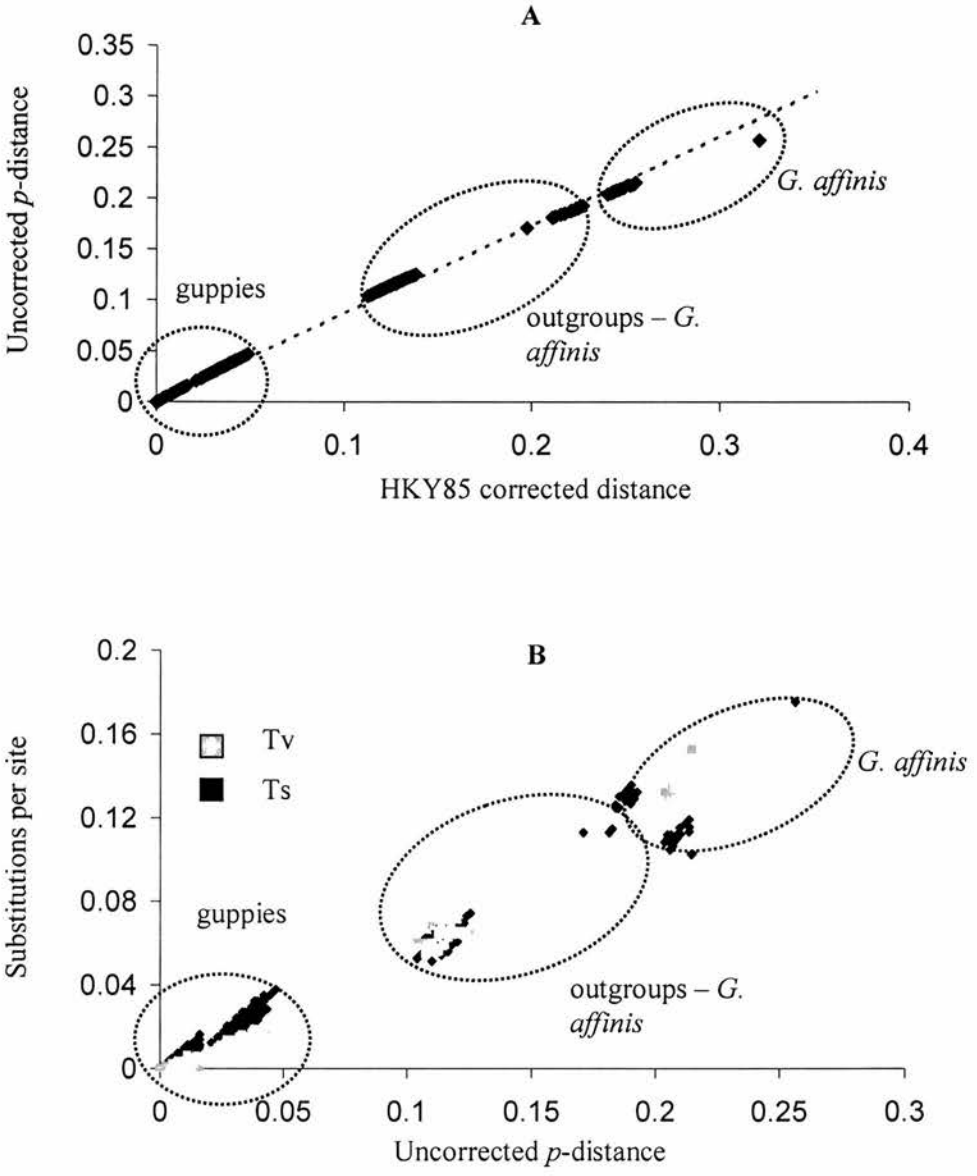

Fig. I.2. Graphs evaluating possible sequence saturation: **A,** uncorrected and corrected pairwise sequence distances, where deviation from the X = Y dashed line indicates saturation; **B,** mean transition and tranversions per site estimated with the HKY85 model in pairwise sequence comparisons, and plotted against uncorrected pairwise distances. Three groups have been marked corresponding to guppy - guppy, guppy - outgroup (minus *G. affinis*) and guppy - *G. affinis* comparisons.

Population / Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Madamas [1]																							
Rio Grande [2]	0.01																						
Quare [3]	0.01	0.01																					
Oropuche [4]	0	0.01	0																				
New Amsterdam [5]	0.03	0.04	0.03	0.03																			
Springlands [6]	0.03	0.04	0.04	0.03	0																		
Suriname [7]	0.04	0.04	0.04	0.04	0.01	0.01																	
Bartica [8]	0.04	0.04	0.04	0.04	0.01	0.01	0																
Guanapo [9]	0.04	0.05	0.04	0.04	0.01	0.01	0	0															
Turure [10]	0.04	0.04	0.04	0.04	0.01	0.01	0.01	0.01	0.01														
Aripo [11]	0.03	0.04	0.04	0.03	0.01	0.01	0.01	0.01	0.01	0.02													
Arima [12]	0.03	0.04	0.03	0.03	0	0.01	0.01	0.01	0.01	0.01	0.01												
Yarra [13]	0.03	0.04	0.03	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0											
Paria [14]	0.03	0.04	0.03	0.03	0	0.01	0.01	0.01	0.01	0.01	0.01	0	0										
Isla Margarita [15]	0.03	0.03	0.03	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01									
Poza de Azufre [16]	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.02	0.03								
Guanare 17]	0.03	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.03	0.02							
Carapito [18]	0.03	0.04	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0						
Marianne [19]	0.04	0.05	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.01	0.01					
P. picta [20]	0.1	0.1	0.1	0.1	0.1	0.11	0.11	0.11	0.11	0.11	0.11	0.1	0.1	0.11	0.11	0.1	0.11	0.11	0.11				
P. formosa [21]	0.1	0.1	0.1	0.1	0.1	0.11	0.11	0.11	0.11	0.11	0.11	0.1	0.1	0.1	0.1	0.1	0.11	0.1	0.11	0			
P. mexicana [22]	0.17	0.17	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.17	0.17		
P. parae [23]	0.09	0.1	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.1	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.1	0.13	0.13	0.16	
G. affinis [24]	0.18	0.19	0.19	0.19	0.19	0.19	0.2	0.2	0.2	0.19	0.2	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.21	0.2	0.25	0.2

Table I.2. Pairwise uncorrected *p*-distances between mitochondrial control region sequences. *p*-distances represent the proportion of nucleotides that differ between sequences. See Figs. 1.4 and I.1 for geographical provenance of samples.

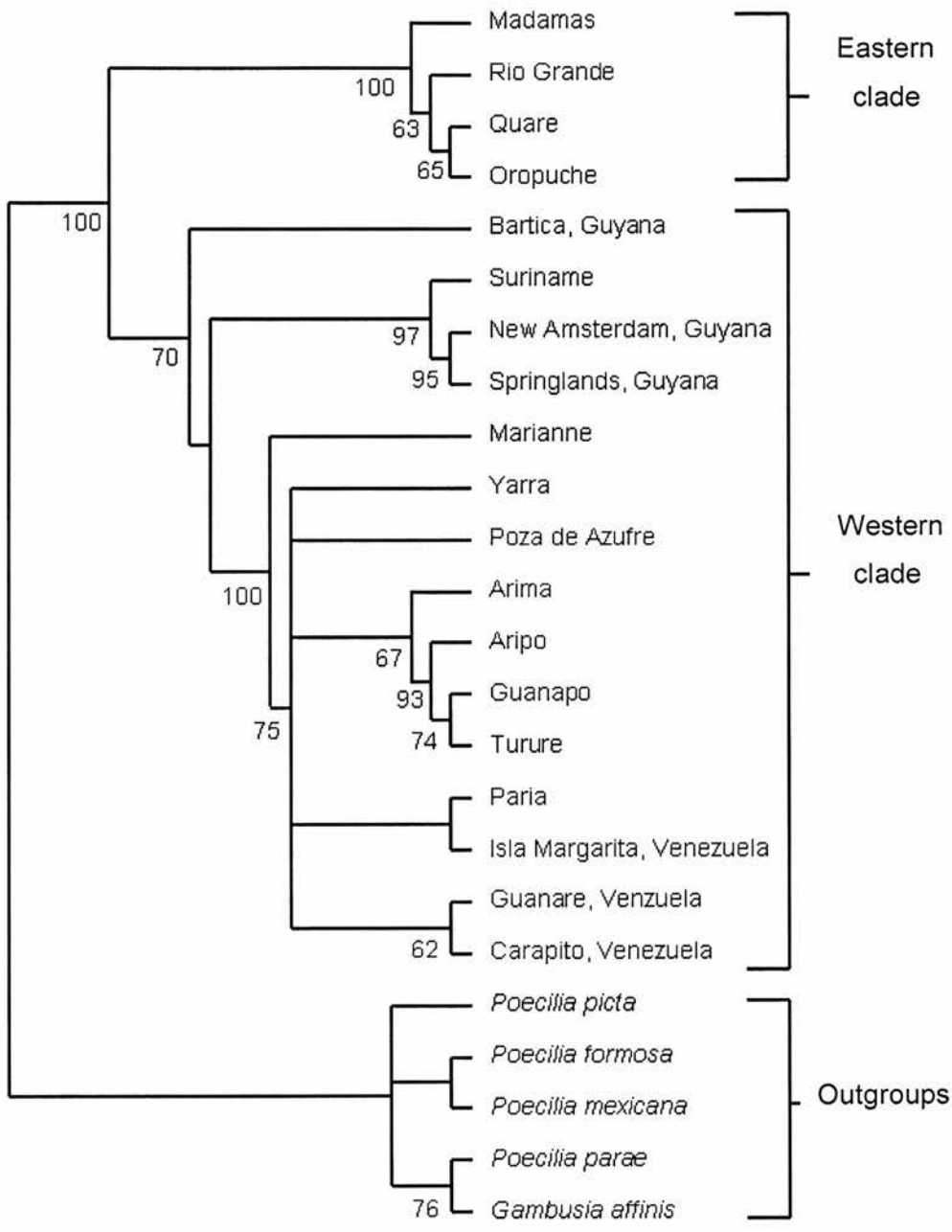

Fig. I.4. 50% majority-rules NJ consensus tree. Phylogeny estimated with HKY85 + G nucleotide model, and node support with 1000 bootstraps.

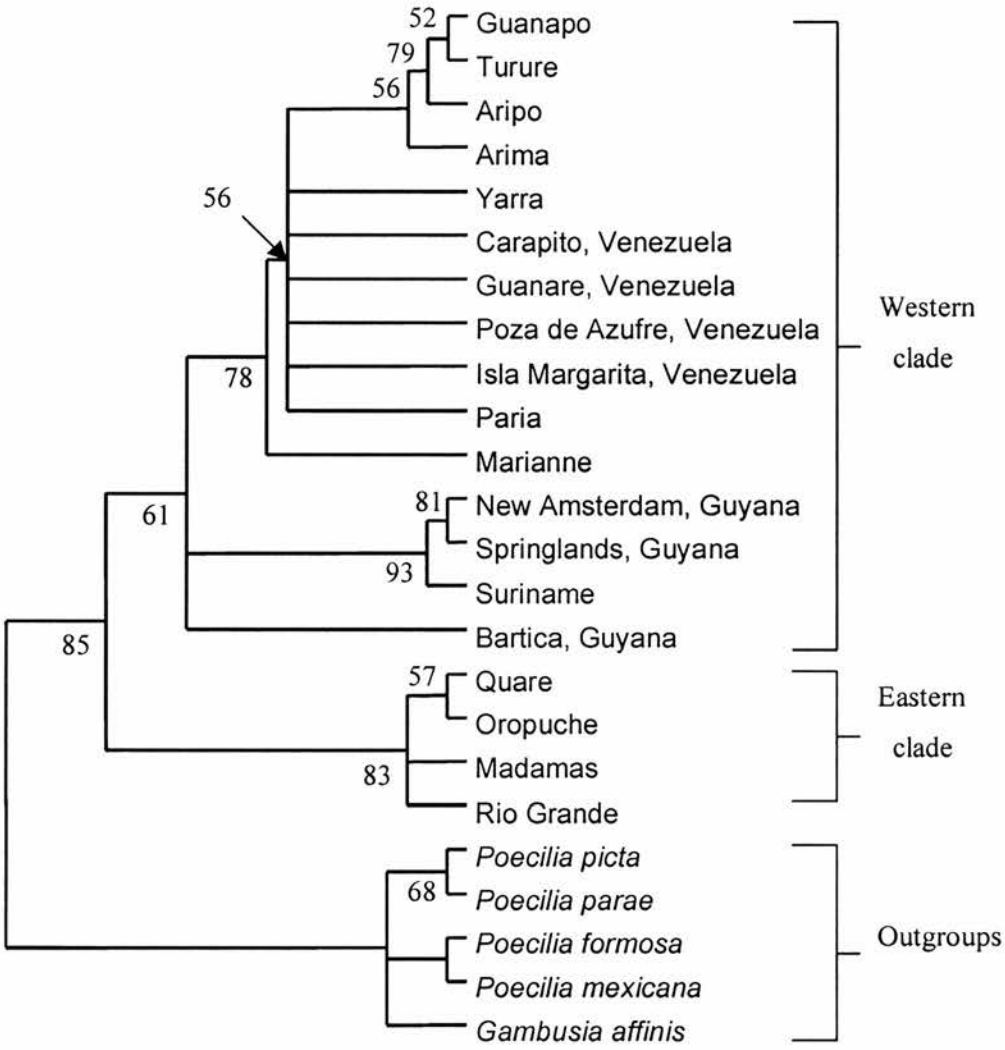

Fig. I.5. 50% majority-rules ML consensus tree. Phylogeny estimated with HKY85 + G nucleotide model, and node support with 100 bootstraps.

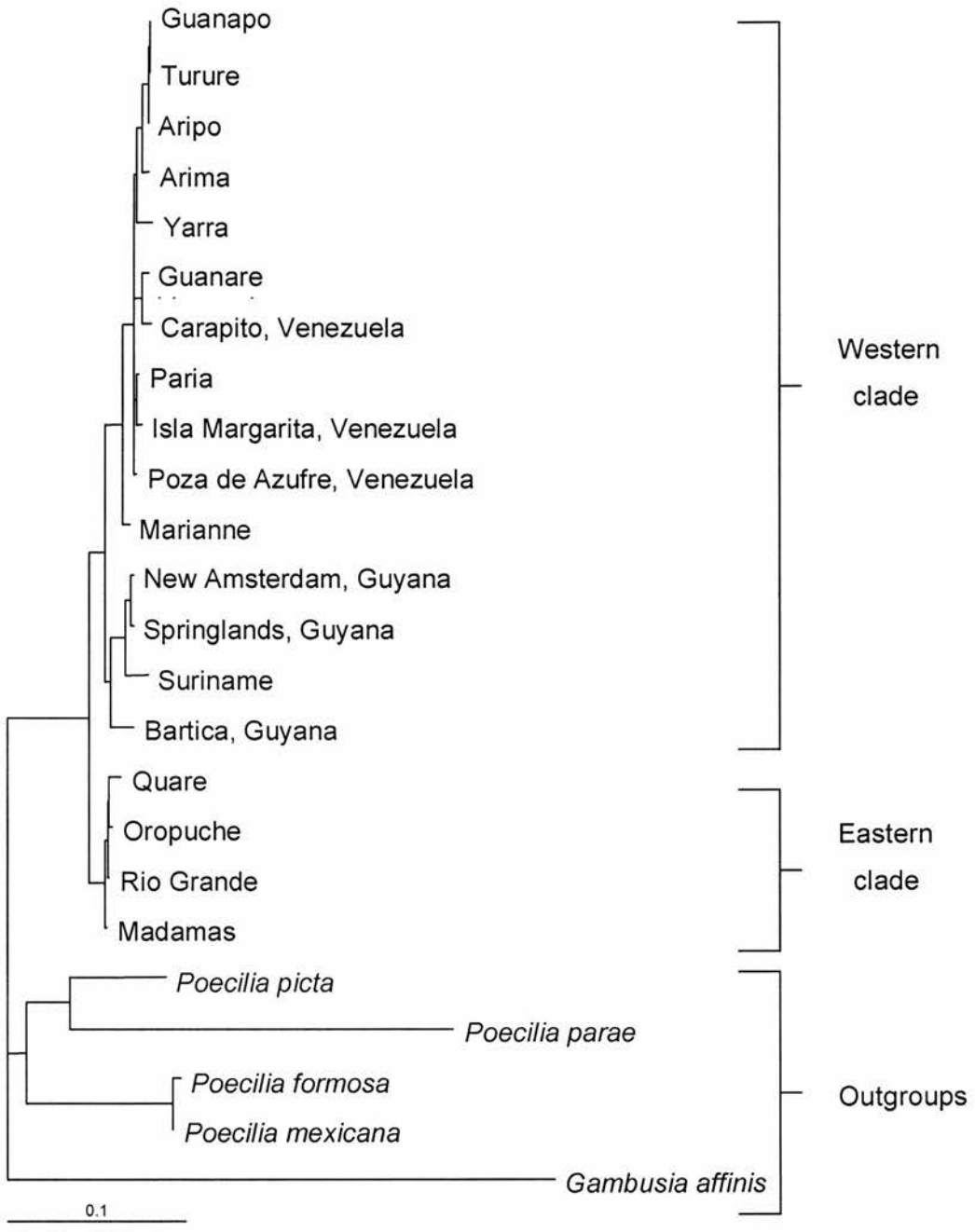

Fig. I.6. Representative ML tree illustrating branch lengths. Estimated using a HKY85 + G nucleotide model.

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