Fine-scale genetic pattern and evidence for sex-biased dispersal in the túngara frog, *Physalaemus pustulosus*

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Abstract

Túngara frogs (*Physalaemus pustulosus*) are a model system for sexual selection and communication. Population dynamics and gene flow are of major interest in this species because they influence speciation processes and microevolution, and could consequently provide a deeper understanding of the evolutionary processes involved in mate recognition. Although earlier studies have documented genetic variation across the species' range, attempts to investigate dispersal on a local level have been limited to mark–recapture studies. These behavioural studies indicated high mobility at a scale of several hundred metres. In this study we used seven highly polymorphic microsatellite loci to investigate fine-scaled genetic variation in the túngara frog. We analysed the influence of geographical distance on observed genetic patterns, examined the influence of a river on gene flow, and tested for sex-biased dispersal. Data for 668 individuals from 17 populations ranging in distance from 0.26 to 11.8 km revealed significant levels of genetic differentiation among populations. Genetic differentiation was significantly correlated with geographic distance. A river acted as an efficient barrier to gene flow. Several tests of sex-biased dispersal were conducted. Most of them showed no difference between the sexes, but variance of Assignment Indices exhibited a statistically significant male bias in dispersal.

Keywords: gene-flow barrier, male-biased dispersal, metapopulation, microsatellite marker, population genetics

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Introduction

Species are often patchy and unevenly distributed and may be divided into numerous populations that vary in the rate of exchange of individuals with other populations (Avise 2000; Newman & Squire 2001). Genetic exchange within such subdivided populations is a critical variable in evolutionary divergence. Since molecular markers allow precise estimates of gene flow, metapopulation systems are once again a focus of interest (review in Pannell & Charlesworth 2000). Metapopulations are defined as local populations that interact via individuals moving among populations (Hanski & Gilpin 1991). Understanding metapopulation dynamics is important for understanding speciation and microevolution and also has a more applied value for conservation.

Estimating gene flow using molecular methods has become a tempting alternative to gene-flow estimates based on field data, e.g. mark–recapture studies. While field experiments can be very time consuming and difficult to perform, molecular techniques using bi-parentally inherited neutral genetic markers, such as microsatellites, have recently become more practicable. In addition, genetic data provide information on the genetic influence and reproductive success of dispersing individuals, rather than just a measurement of their mobility, and recently developed statistical approaches (review in Prugnolle & de Meeus 2002) allow the inference of sex-biased dispersal from population genetic markers.

Several hypotheses can account for differences in migration between the sexes. They relate sex-bias in dispersal to sex-specific differences in the advantages that philopatry (= nondispersal) conveys to males and females, or to sex-specific impact of fecundity costs. The theories can be classified into three major categories: competition for resources (Greenwood 1980), competition for mates (Dobson 1982; Perrin & Mazalov 2000), and avoidance of inbreeding (Pusey 1987; Perrin & Mazalov 2000). All theories, however, conclude that the best prediction of

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sex-biased dispersal derives from the species' mating system. For example, in mammals, which are mostly polygynous, males tend to be the dispersing sex, while in birds, which are usually monogamous, females disperse farther from their birth place. Little is known, however, about other animal groups (Goudet *et al*. 2002).

Amphibian population studies have contributed to our understanding of ecology and evolution. The recent decline in many species and concern about their conservation has focused attention on their population dynamics (Breden 1987; Alford & Richards 1999); they are now widely used as models for metapopulation investigations (e.g. Rowe *et al*. 2000). Amphibians have traditionally been considered to have low dispersal ability (Driscoll 1998a), but recent molecular studies have found high levels of genetic exchange between geographically distant populations (Seppä & Laurila 1999; Newman & Squire 2001). Túngara frogs (*Physalaemus pustulosus*) are abundant throughout the dry lowland forests in Middle America and inhabit a wide range of habitat types (Ryan 1985). Their preference for disturbed areas and their abundance make them tractable for studies of ecology, evolution and behaviour. They exhibit a lek-mating system and deposit their eggs in foam nests in small temporary ponds (Ryan 1985). Túngara frogs have been an especially productive system for studies of sexual selection and communication (review in Ryan 1998). Little is known, however, about their population ecology. Although allozyme markers have been used to examine genetic variation across the species-range (Ryan *et al*. 1996), less is known about the population structure and dynamics on a local scale. Mark–recapture studies by Marsh *et al*. (1999, 2000) showed that within the same habitat type adult frogs are quite mobile, regularly changing breeding ponds over distances up to 200 m. Males seem more likely to disperse, but a sex-bias in dispersal was not detected, perhaps because of low recapture rates, especially in females. The studies by Marsh *et al*. (1999, 2000) suggested overall high dispersal rates in túngara frogs regardless of sex. Despite the frog's lek mating system, which might imply a female bias in dispersal, males are expected to be more mobile because the frogs are definitely polygynous (Ryan 1985) which in other taxa has been shown to predict male-biased dispersal.

In this study, molecular data were used to test the hypotheses of overall high dispersal rates and male-biased migration by quantifying genetic patterns among populations of túngara frogs and documenting the geographical scale at which populations are genetically distinguishable. Special emphasis was given to sex-biased dispersal and the possible role of a river as a genetic barrier. Seven highly polymorphic microsatellites developed for túngara frogs (Pröhl *et al*. 2002) allowed high-resolution analysis of population structure and dispersal in 17 breeding populations located within a radius of 10 km around Gamboa, Panama.
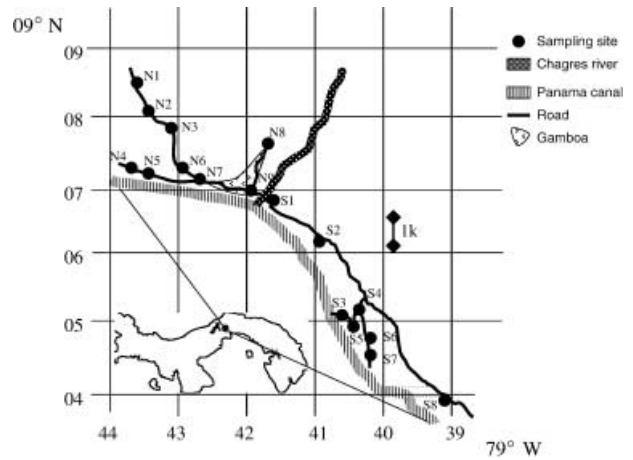

Fig. 1 Map of túngara frog sampling sites around Gamboa. Grey dots indicate the sampled populations. The Panama Canal (vertical stripes) and the Chagres River (dark pattern) are shown as well as the main roads (black line) and the township of Gamboa (light pattern).

Materials and methods

Sampling

Adult túngara frogs were collected between 8 June and 26 August 2002 from breeding ponds within a radius of about 10 km around Gamboa, Panama. All sampling sites were situated at the east side, very close to the Panama Canal (Fig. 1). Nine breeding sites north of the Chagres river (Gamboa side) (N1–N9) and eight sites south of the Chagres River (Summit side) (S1–S8) were sampled (Fig. 1). A bridge at the entrance of Gamboa spans the Chagres River. Toe clips were used to mark frogs individually and were also used as tissue samples for genetic analysis. Seventeen different sites were sampled ranging in distance between 260 m and 11.8 km. At least 40 individuals, including a minimum of 10 females, were collected from each site except for sites S5 (30 males, five females) and S1 (15 males, nine females). Frogs were released after toe clipping, otherwise unharmed, at the point of capture. Tissues from a total number of 668 individuals were stored in 20% ethylenediaminetetraacetic acid/Sarcosyl buffer until processing in the laboratory at the University of Texas at Austin.

Sample processing

DNA was extracted using the DNeasy Tissue kit (Qiagen). Polymerase chain reactions (PCR) were performed with seven (CA 120, CA 298, A #3.11, A #19.11, C #30.11, ATG 159, ATG 263) of the eight microsatellite primers pairs identified by Pröhl *et al*. (2002). The eighth primer (GA 240) could not be analysed because of complex stutter bands of variable heights. A slightly modified PCR profile was used

(30 s denaturing; 30 s annealing; 30 s elongation) but in all other aspects (e.g. annealing temperature) the technique followed the protocol of Pröhl *et al.* (2002). One primer of each primer pair was labelled with fluorescent dye to allow fragment size analysis on an ABI Prism®3100 capillary sequencer. The ABI Sequencer output was automatically analysed with an adapted GENESCAN ANALYSIS program (Applied Biosystems) and fragment size was determined using the GENOTYPER program (PE Biosystems).

Statistical analysis

Variation within populations. Statistical analyses were conducted using ARLEQUIN 2.0 (Schneider *et al.* 2000). Allele frequencies were estimated, as were the observed and expected heterozygosities. A one-tailed probability of departure from the Hardy–Weinberg equilibrium was calculated to test for deficiency of heterozygotes. Deviation from Hardy–Weinberg was tested using a Markov Chain approximation with 100 000 steps and 1000 dememorization steps.

Interpopulation variation. Estimates of pairwise F_{ST} values were calculated to analyse population differentiation in allele frequencies. ARLEQUIN 2.0 (Schneider *et al.* 2000) was used for this analysis and it also estimated the probability of nondifferentiation over 3000 randomizations. To estimate the number of migrants (*Nm*) between the sites, Wright's (1943) equation, $Nm = (1 - F_{ST})/4 \times F_{ST}$, was used. Values of *Nm* were calculated to illustrate the dispersal patterns between sites, although these calculation methods have serious limitations (Avise 2000).

Isolation by distance. To analyse the relationship of geographical distance (km) and genetic distance (F_{ST}) between populations, Mantel tests were performed using MANTEL version 2.0 (Liedloff 1999). Ten thousand iterations were used to determine the statistical significance of the results. The genetic data were analysed on two different geographical scales: first across all populations and second separately for the populations on each side of the Chagres River. In addition, the relationships of genetic and geographical distance for all frogs, as well as for male and female frogs separately were analysed. To estimate the potential influence of the Chagres River as a barrier to gene flow, a categorical matrix was generated describing whether the populations investigated were on the same (= 0) or different (= 1) sides of the Chagres River and this matrix was then used in Mantel tests (Legendre & Legendre 2000) to determine if this aspect of biogeography co-varied with genetic distance. A partial Mantel test was also performed using the program FSTAT 293 (Goudet 2001), as river side and geographical distance were highly correlated.

As one-dimensional F_{ST} statistics might be insufficient to describe multivariate multilocus population genetic data adequately (Stenton *et al.* 2002), a principal component analysis (PCA) based on individual genotypic data from all populations was conducted using PCA-GEN version 1.2 (Goudet 1999). The statistical significance associated with each axis was calculated over 10 000 randomizations.

Sex-biased dispersal. FSTAT 293 was used (Goudet 2001) to calculate five different tests for sex-biased dispersal: F_{IS}, F_{ST}, relatedness, mean Assignment Index and variance of Assignment Indices. Positive F_{IS} values were predicted for the dispersing sex because F_{IS} is a measure of how well the genotype frequencies within the population match Hardy–Weinberg expectations (Hartl & Clark 1997). The dispersing sex at one site will be a mixture of immigrants and residents and thus consist of two 'separate' populations at any one site. This hidden subdivision will lead to a heterozygote deficiency and a positive F_{IS} (Wahlund effect). A lower relatedness was also predicted for the dispersing sex (Knight *et al.* 1999; Surridge *et al.* 1999). Lower F_{ST} values for the dispersing sex follow because F_{ST} expresses the proportion of the total genetic variance attributable to among-population differentiation (Hartl & Clark 1997). The dispersing sex should be less differentiated in its allelic frequencies. Finally, we chose Assignment Indices to test for differences in the mean values and in variance of Assignment Indices between the sexes. The Assignment Index statistic calculates the probability for each genotype to be represented in the sampled population. Allele frequencies at each locus, after a correction for multilocus probabilities, provide a corrected Alc value for each individual (l being the number of loci) (Paetkau *et al.* 1995; Favre *et al.* 1997; Goudet *et al.* 2002). Alc values are centred around 0. Resident individuals probably have genotypes that are more likely than average to occur in the sample and should therefore have positive Alc values, while immigrant genotypes are less likely to occur in the sample and therefore have negative Alc values (Goudet *et al.* 2002). The mean Assignment Index should be higher in the philopatric sex, while the variance of Alc (vAlc) should be higher in the dispersing sex, because sampled members of this sex should include dispersed and resident genotypes with positive and negative vAlc values. FSTAT version 2.9.3 (Goudet 2001) was used to calculate individual Alc values and to test for significant differences in vAlc between the sexes.

Results

Variation within populations

A total number of 668 frogs, 430 males and 238 females, were captured at the 17 breeding sites. No differences in

Table 1 Numbers of alleles (*Na*) and observed and expected heterozygosities (H_O, H_E) for each locus in each population. In addition, results over all populations and average for all populations are shown. Significant heterozygosity deficiencies are given in bold type

Pop.	Ind.	Ca 120		Ca 298		A 3.11		A 19.11		C 30.11		Atg 159		Atg 263	
		Na	H_O/H_E	*Na*	H_O/H_E	*Na*	H_O/H_E	*Na*	H_O/H_E	*Na*	H_O/H_E	*Na*	H_O/H_E	*Na*	H_O/H_E
N1	40	13	0.7436/0.7925	14	**0.5897/0.8565**	18	0.7750/0.8712	14	**0.4500/0.8082**	20	0.8205/0.9231	14	0.7750/0.8114	7	0.8500/0.7820
N2	41	14	0.7317/0.7630	10	**0.3590/0.7925**	14	0.6342/0.7733	15	**0.3784/0.7038**	20	0.7805/0.9073	14	**0.7561/0.8817**	8	0.7073/0.6745
N3	40	16	0.8205/0.8125	10	**0.4211/0.7126**	14	**0.5897/0.8062**	17	**0.3947/0.8411**	17	0.7750/0.9111	13	0.8000/0.8687	7	0.7250/0.7010
N4	41	14	**0.5250/0.8073**	11	**0.4390/0.8016**	15	0.7000/0.8611	13	**0.4474/0.8133**	16	**0.6750/0.9139**	13	0.8537/0.8308	7	0.6250/0.6937
N5	40	14	0.8000/0.8171	12	**0.5429/0.8485**	15	**0.6500/0.8544**	16	**0.4250/0.7905**	17	0.8000/0.9146	12	0.9000/0.8585	10	0.6500/0.6731
N6	40	14	**0.6500/0.8329**	12	**0.3611/0.8173**	15	0.7750/0.8291	16	**0.5000/0.8600**	15	0.7949/0.9151	11	0.7750/0.8082	7	0.8250/0.7405
N7	41	14	0.7805/0.8425	13	0.6750/0.8174	15	0.8781/0.8242	14	**0.3846/0.7832**	17	0.7750/0.8842	13	0.8293/0.7946	8	0.7805/0.7564
N8	40	17	0.8250/0.8491	17	**0.4000/0.8677**	16	0.7692/0.8788	12	**0.3684/0.6856**	21	0.7750/0.9294	12	0.8250/0.8348	6	0.8718/0.7975
N9	42	10	0.6829/0.8157	12	**0.4762/0.7983**	18	0.8781/0.8443	12	**0.3750/0.7317**	13	0.9500/0.8750	15	0.7619/0.8511	7	0.8537/0.7561
S1	24	14	0.8750/0.8750	15	**0.6667/0.8936**	12	0.7500/0.8307	13	0.7273/0.8763	16	0.7083/0.8963	9	0.7917/0.8351	6	0.7826/0.6821
S2	40	12	0.7180/0.8542	16	**0.7000/0.7788**	18	0.8000/0.8696	15	**0.4595/0.8671**	22	0.9460/0.9237	12	0.7500/0.7788	6	**0.4500/0.6573**
S3	40	12	0.6579/0.7481	11	**0.6154/0.8512**	17	0.8250/0.8658	14	**0.5455/0.8546**	20	0.9231/0.8971	13	0.9250/0.8184	7	0.5750/0.6965
S4	40	14	0.7027/0.8197	15	**0.3750/0.7832**	19	0.6500/0.7696	15	**0.7105/0.8344**	18	0.9487/0.9237	13	0.8000/0.8108	5	0.6500/0.6728
S5	35	12	0.6857/0.8348	13	**0.7143/0.8605**	12	**0.6000/0.7917**	16	**0.6857/0.8551**	20	0.8485/0.9268	13	0.8000/0.8327	6	0.8286/0.6311
S6	41	14	0.7180/0.8888	15	**0.6829/0.8732**	19	0.7500/0.8279	14	**0.5641/0.8691**	18	0.8438/0.9241	14	**0.7317/0.8257**	6	0.5854/0.6555
S7	42	15	0.6829/0.8708	17	**0.6667/0.8434**	15	0.6410/0.7799	16	**0.6667/0.8637**	16	0.8333/0.9117	13	0.8095/0.8276	7	0.7857/0.7235
S8	41	15	0.7805/0.8684	15	**0.6500/0.8275**	18	0.7805/0.7808	15	**0.5250/0.8430**	18	0.7750/0.9253	15	0.7805/0.8458	5	0.8293/0.6167
mean	39	14	0.72823/0.82896	13	0.54911/0.82493	16	0.73210/0.82697	15	0.50634/0.81651	18	0.82191/0.91190	13	0.80378/0.83027	7	0.72793/0.70060
all	668	28		42		32		29		36		22		12	

Table 2 Estimates of population pairwise genetic distance (F_{ST}) and number of migrants (Nm). Below diagonal: pairwise F_{ST} values for all populations (bold values were significant after Bonferroni correction). Above diagonal: estimate of Nm

	N1	N2	N3	N4	N5	N6	N7	N8	N9	S1	S2	S3	S4	S5	S6	S7	S8
N1	*	36	27	28	163	166	25	29	15	8	10	31	8	11	11	16	7
N2	0.007	*	324	658	80	48	22	18	11	6	7	25	8	19	10	13	8
N3	0.009	0.001	*	568	53	?	3571	51	32	7	10	77	12	36	20	23	12
N4	0.009	0.000	0.000	*	93	56	38	24	14	8	10	49	12	59	15	20	13
N5	0.002	0.003	0.005	0.003	*	58	27	29	16	12	12	34	9	14	10	17	10
N6	0.002	0.005	0.000	0.004	0.004	*	69	51	22	7	10	31	10	21	26	24	11
N7	0.010	0.011	0.000	0.007	0.009	0.004	*	64	32	7	11	44	11	21	16	15	11
N8	0.008	**0.014**	0.005	0.010	0.008	0.005	0.004	*	37	8	9	18	9	14	16	16	9
N9	**0.016**	**0.022**	0.008	**0.018**	**0.015**	0.011	0.008	0.007	*	8	9	20	8	8	11	10	8
S1	**0.029**	**0.039**	**0.034**	**0.031**	**0.021**	**0.033**	**0.034**	**0.031**	**0.031**	*	11	9	10	9	9	13	9
S2	**0.025**	**0.035**	**0.023**	**0.024**	**0.021**	**0.024**	**0.022**	**0.027**	**0.028**	**0.021**	*	13	18	9	15	88	23
S3	0.008	0.010	0.003	0.005	0.007	0.008	0.006	**0.014**	0.012	**0.028**	**0.020**	*	15	61	21	24	12
S4	**0.029**	**0.032**	**0.021**	**0.020**	**0.027**	**0.024**	**0.023**	**0.027**	**0.029**	**0.023**	0.014	0.017	*	60	129	96	?
S5	**0.021**	0.013	0.007	0.004	**0.018**	0.012	0.012	**0.018**	**0.029**	0.028	**0.027**	0.004	0.004	*	?	34	29
S6	**0.022**	**0.024**	0.012	**0.016**	**0.024**	0.010	0.015	**0.015**	**0.023**	**0.027**	**0.016**	0.012	0.002	0.000	*	?	101
S7	**0.016**	**0.018**	0.011	0.012	**0.015**	0.010	**0.016**	**0.016**	**0.023**	0.019	0.003	0.010	0.003	0.007	0.000	*	271
S8	**0.033**	**0.031**	**0.020**	0.019	**0.026**	**0.022**	**0.023**	**0.027**	**0.030**	**0.028**	0.011	0.020	0.000	0.008	0.002	0.001	*

DNA extraction or primer performance were detected between the populations. All seven loci were highly polymorphic, with up to 22 alleles in one locus in just one population. Overall allele numbers varied between 12 (locus ATG 263) and 42 (locus CA 298) (Table 1). Two loci showed significant heterozygote deficiencies for more than 50% of all populations (CA 298, A #19.11; Table 1). As the observed deviations from Hardy–Weinberg equilibrium might be the result of null alleles, these loci were excluded from all following analyses. Heterozygote deficiencies in other loci were rare and restricted to single populations (Table 1).

Interpopulation variation

All single microsatellite loci provided similar F_{ST} values (mean: 0.025, 0.013, 0.019, 0.007 and 0.018 for CA 120, A #3.11, C #30.11, ATG 159 and ATG 263, respectively). Overall F_{ST} values varied between 0.0 and 0.03922 (Table 2; lower part). There was significant genetic differentiation in 48% of all possible 136 site combinations. Females seemed to have slightly higher interpopulation F_{ST} values (0.0– 0.06341) than males (0.0–0.04271), but these differences were not statistically significant (see sex-biased dispersal). The number of migrants between populations was calculated directly from the F_{ST} values (Wright 1943) and is given in Table 2 (upper part). They ranged between six individuals (N2 to S1) and 3571 individuals per generation (N3 to N7) with a median of 16 individuals per generation changing between sites. Predictions of number of migrants were not calculated for field sites (N3–N6, S4–S8, S5–S6, S6–S7) that were not genetically differentiated (i.e. had an estimated $F_{ST} = 0$).

Isolation by distance

A graph plotting genetic distance vs. geographical distance showed an overall positive correlation between these two factors (Fig. 2). These graphs only illustrate a potential relationship between geographical and genetic distance, a regression was not calculated because of pseudo-replication (each population occurs 16 times in the graph). Mantel tests, however, revealed a strong influence of geographical distance on the genetic distance in all frogs, as well as in males and females separately (Table 3). Testing populations from both sides of the Chagres River independently and therefore creating areas without an obvious gene-flow barrier revealed a substantially stronger correlation between geographical distance and genetic distance (Fig. 2b, Table 3). Correlations between geographical distance and genetic distance on both sides of the river were, however, not significant when separated for sex (Table 3).

A PCA (Fig. 3) of allelic frequencies in the populations revealed one significant horizontal axis ($P = 0.0032$), while the vertical axis was not significant ($P = 0.6369$). The populations were separated in two major groups: one group included all populations on the north side of the Chagres River, the other almost all populations on the south side of the river (Fig. 3). Population S3, situated very close to the Panama Canal, seemed genetically more similar to the northern populations than to the rest of the southern populations.

The Chagres River was a major influence on the genetic diversity, as expected. The genetic distance between populations separated by the Chagres River was significantly higher than the genetic distance between populations on
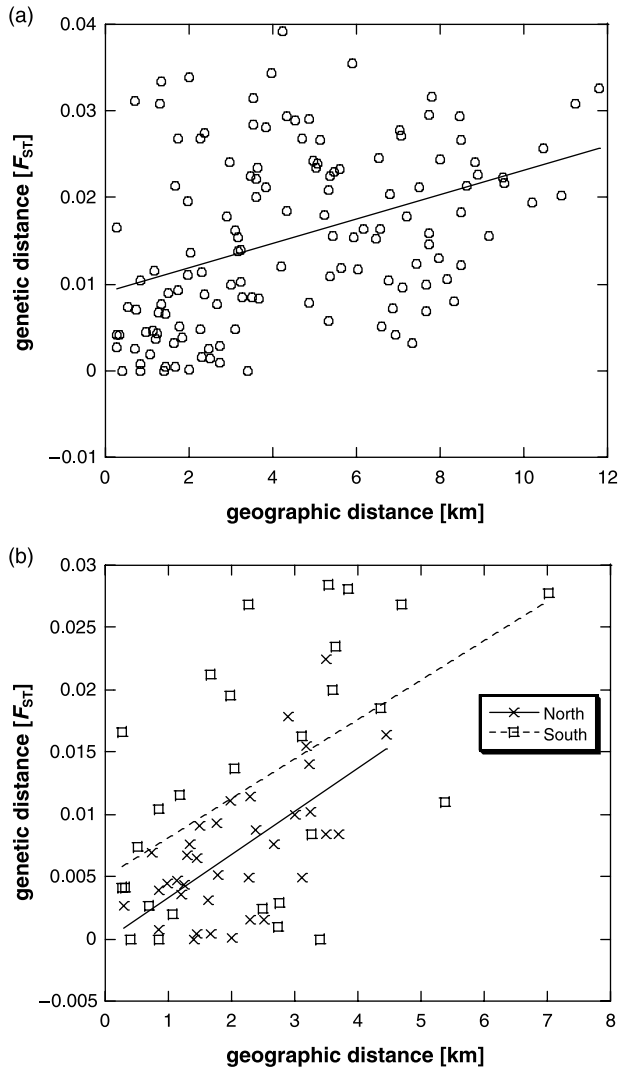
(a)

(b)

Fig. 2 (a) Overall correlation of geographical distance in km (*x*-axis) vs. genetic distance given as F_{ST} values (*y*-axis). The line represents the linear regression, although pseudo-replication in the data precludes proper statistical analysis. (b) Correlation of geographical distance (km) and genetic distance (F_{ST}) for túngara frogs from both the north and south sides of the Chagres River. Lines are regressions for the two different groups.

the same side of the river (Table 3). The estimated *r*- and *P*-values were even higher than those for geographical distance vs. genetic distance. The correlation between river side and genetic distance seemed stronger in males than in females (Table 3), but a test of homogeneity among two or more correlation coefficients (Sokal & Rohlf 1995; p. 582) revealed a nonsignificant difference between the *r*-values of the sexes ($t = 3.46$; $P = 0.09$).

Chagres River side and geographical distance were not independent. Populations that were separated by the Chagres River were usually also farther apart from each other. There were, however, a few cases of sites being equi-

Table 3 Mantel test results. (a) Isolation by distance: correlation between geographical distance (km) and genetic distance (F_{ST}), (b) influence of the Chagres River on genetic distance

Group	*g*-value	Z-value	*r*-value	*P*-value
(a) Isolation by distance*				
All frogs, all sites (668)	3.986	21.5032	0.4676	0.0004
Females, all sites (238)	4.238	20.7786	0.2438	0.0119
Males, all sites (430)	1.17	22.2653	0.3379	0.0028
All frogs, north (365)	3.14	1.5071	0.6191	0.001
All frogs, south (303)	1.83	2.2418	0.5467	0.028
Females, north (145)	1.3832	1.7366	0.2705	0.091
Males, north (220)	1.5754	1.2533	0.2971	0.067
Females, south (93)	1.4081	3.1236	0.4273	0.1420
Males, south (210)	1.3769	1.5555	0.3424	0.1050
(b) Chagres River†				
All frogs (668)	6.7957	3.0236	0.4848	0.0001
Females (238)	2.8072	3.0746	0.1957	0.0006
Males (430)	6.7957	3.4279	0.5463	0.0001

*Data were subdivided by sex and tested separately for the two distinct areas (north and south of the Chagres river) found with the PCA. *P*-values were derived from 10 000 iterations. Significant results ($P < 0.05$) are given in bold type.
†Genetic distance (F_{ST}) was tested against a categorical matrix coding populations located on the same Chagres River side as 0 and populations on different Chagres sides as 1. *P*-values were derived from 10 000 iterations. Significant results ($P < 0.05$) are given in bold type. The number of frogs included in each test is given in parentheses in the leftmost column.
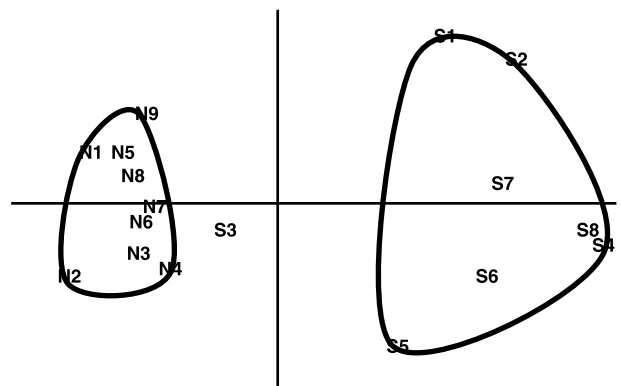

Fig. 3 Principal component analysis of allele frequencies in the transect populations. The horizontal axis is statistically significant ($P = 0.003$), while the vertical axis is not significant ($P = 0.637$). Statistical significances of the axes were calculated over 10 000 randomizations.

distant from a focal site but located on different sides of the river. Although the geographical distance from the focal population to the two other populations was similar, the population on the different river side always had a higher F_{ST} value than the population on the same river side (Table 4). A nonparametric Friedman test revealed a

Table 4 Chagres River as a barrier to gene flow

Focus	Distance	Chagres side	
		Same	Different
S1	4.2 km	0.027 (S6)	0.039 (N2)
S1	3.9 km	0.028 (S5)	0.034 (N3)
S1	1.8 km	0.021 (S2)	0.027 (N8)
N5	3.3 km	0.008 (N8)	0.021 (S1)
N4	3.5 km	0.010 (N8)	0.031 (S1)
N9	4.4 km	0.016 (N1)	0.029 (S4)

The columns list the focus population, the geographical distance to both other sites and the F_{ST} values for the population on the same and on the other side of the Chagres River (population names in parentheses).

significant difference between the two groups ($P = 0.014$; d.f. = 1). It was therefore concluded that the river presented a stronger dispersal barrier than predicted by distance alone.

As river side and geographical distance were highly correlated, a partial Mantel test was performed that revealed a significant correlation for the geographical distance and genetic distance ($r = 0.40$; $P = 0.0005$) and only a slightly weaker correlation for Chagres River side and genetic distance ($r = 0.30$, $P = 0.002$). The overall variation in genetic distance explained by the model was 25.2%.

Sex-biased dispersal

Although females had a slightly lower mean assignment index, pointing towards a female bias in dispersal, the four other measurements for genetic differentiation indicated a male bias in dispersal (lower F_{IS} values in males, higher F_{ST} values in females, higher relatedness values among females, higher variance of assignment values in males). Only one test, however, turned out to be significant: the variance of Assignment Index test revealed a significant difference between male and female frogs ($P = 0.004$; Fig. 4), identifying males as the predominantly dispersing sex in túngara frogs (Table 5, Fig. 4) even after Bonferroni correction for multiple testing of the data set (adjusted critical P-value = 0.01).

Discussion

Population genetic analysis of microsatellite variation at five loci refutes the hypothesis of high dispersal rates in the túngara frog, *Physalaemus pustulosus*. The data from this study provide clear evidence for a high level of genetic population structure. Even at a fine scale of only a few kilometres, significant differences in allelic frequencies could be detected. Furthermore, the Chagres River constitutes a major gene-flow barrier. The results did offer some
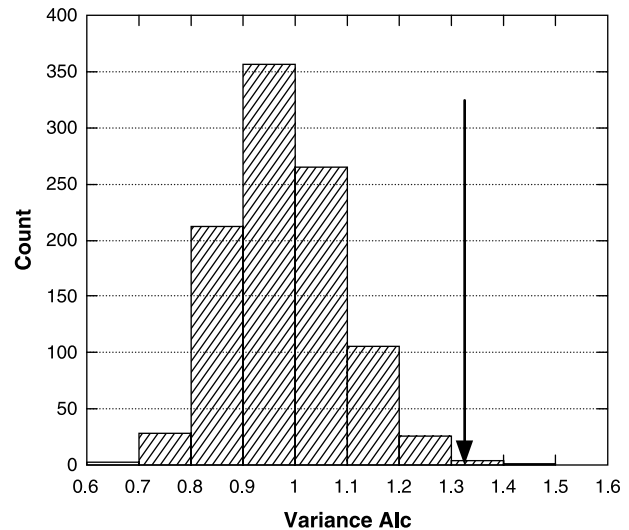
Fig. 4 Distribution of the randomized statistics of the variance of assignment index test (vAlc) for male and female túngara frogs (results of 1000 random permutations). The arrow corresponds to the observed value of the statistic (vAlc = 1.3226) indicating a significant sex-bias in dispersal. The direction of the bias (males are the dispersing sex) becomes clear from Table 5.

Table 5 Test results for sex-biased dispersal in the túngara frogs

	n	F_{IS}	F_{ST}	Rel.*	Assignment indices	
					Mean	Variance
Females	238	0.0690	0.0144	0.0267	−0.08440	**6.32694**
Males	430	0.0569	0.0136	0.0254	0.04671	**8.36789**
P-value		0.7170	0.3850	0.3960	0.7170	**0.0040**

*Rel., relatedness.
All values were calculated using FSTAT 293 (Goudet 2001).
Probabilities were calculated using 1000 permutations. Significant results ($P < 0.05$) are given in bold type.

tentative support for the hypothesis that males are the more mobile sex in túngara frogs.

Most studies of intraspecific genetic variation in amphibians have been conducted across large scales, of several hundred kilometres, and have found substantial variation among populations (Phillips 1994; Rowe *et al*. 1998; Barber 1999). For túngara frogs, an allozyme study along a 5000-km transect throughout the species' range (Mexico to Venezuela) revealed high levels of genetic variation (Ryan *et al*. 1996). Mitochondrial DNA sequence data from the same sample sites are consistent with these results (Weigt, Rand & Ryan, in preparation). More recently, a number of studies of amphibians concentrated on a smaller scale of several kilometres and also found high levels of genetic differentiation. Rowe *et al*. (2000), using microsatellites, found an average F_{ST} value of 0.11 for *Bufo*

calamita sampled at distances between 0.5 and 9.0 km. Other studies report even higher F_{ST} values using single-strand conformation polymorphism and allozymes (Shaffer *et al.* 2000: *Bufo canorus* F_{ST} = 0.2; Driscoll 1998b: *Geocrinia rosea* F_{ST} = 0.6). These values are substantially higher than the values found in this study for *P. pustulosus*, which showed a median F_{ST} value of 0.015 for populations that were on average 3.75 km apart from each other. Our F_{ST} values resemble the F_{ST} values for wood frog (*Rana sylvatica*) populations found by Newman & Squire 2001 (F_{ST} = 0.014, scale: 0.5–21 km) although that study did not reveal significant genetic differentiation between populations. It was possible to resolve small genetic differences as the microsatellite loci used in this study were highly variable (Pröhl *et al.* 2002). Allele numbers varied between 12 and 36 per locus with a maximum of 22 alleles in one locus at one site. In contrast, Newman & Squire (2001), using five different microsatellites with two, three, nine, 10 and 18 alleles, failed to find small-scale genetic population structures in *R. sylvatica*, possibly because of a lack of sufficient variation in their microsatellite loci.

Despite our finding of overall population differentiation, the migrants between populations [as derived directly from the F_{ST} values (Wright 1943)] indicate some level of gene flow between the populations. The median of 16 migrants per generation seems somewhat high compared to other studies (~1.5 in Favre *et al.* 1997) but this is probably the result of some sites being separated by only several hundred metres. High levels of gene flow seem unlikely for longer distances because all breeding sites were distinguishable in PCA and differences in allelic frequencies were found to increase with geographical distance. Túngara frogs therefore seem to be substructured in a metapopulation system (Hanski & Gilpin 1991) with genetically differentiated populations that are connected by some gene flow.

It appears that the main factor determining the genetic structuring of these populations is isolation by distance. There was a significant correlation between geographical distance and genetic distance that was confirmed by a number of Mantel tests. A PCA of individual allelic frequencies revealed one major source of variation that subdivided the data into two major groups one on each side of the Chagres River (Fig. 3). It was suspected that the Chagres River would be a major influence on gene flow because allozyme data already suggested a separation into northern and southern population groups. Ryan *et al.* (1996) found allozyme variation on different river sides within several hundred metres, but not on the same river side within in a few kilometres. Moreover, since the river was dammed in 1912, as part of the construction of the Panama Canal, it is at least 100 m wide and contains water all year round. This massive water way should therefore prevent the small túngara frogs (body length ~30 mm) from crossing it easily. However, the estimated number of migrating

individuals (*Nm*) indicated some gene flow across the Chagres River. As there is a bridge across the Chagres River at the entrance of Gamboa, the frogs could use the bridge for crossing. They may also swim through the river, or, perhaps more likely, be unintentionally transported by humans (e.g. in building material). The frogs' preference for anthropogenically disturbed areas makes human transport likely and could be a reason for the unexpectedly close genetic relation of population S3 to the northern populations rather than to the rest of the southern populations. The influence of the Chagres River as a gene-flow barrier is supported by the Mantel tests and by the partial Mantel test, where the absence or presence of the river barrier between sites revealed significant correlations for the genetic distance between sites (r = 0.30). In addition, a comparison of sites that were equally distant from a focus site but located on different river sides revealed the strong influence of the river as a gene-flow barrier. Equidistant populations across the river always had a higher F_{ST} value than equidistant populations on the same river side. In contrast to other studies, the Chagres River did not reduce the geographical distance necessary for genetic differentiation. Significant genetic differentiation occurred on the same river side (no genetic barrier) between sites that were separated by 3.8 km. Genetically significantly different sites from different river sides (genetic barrier present) were separated by 6 km, Hitchings & Beebee (1997), in a study of the common frog *Rana temporaria*, reported genetic differentiation at a 2.3-km scale in isolated urban ponds, while in barrier-free rural areas genetic differentiation occurred at a level of 41 km. This implies that although the Chagres River is a major influence on the genetic differentiation found between populations of túngara frogs around Gamboa, geographical distance without an obvious gene-flow barrier is sufficient to create genetic substructuring at a very fine scale.

There was some evidence for male-biased dispersal, as predicted from the frogs' polygynous mating system. The variance of Assignment Indices (vAIc) test revealed a significant difference between males and females (P = 0.004). This difference was still significant after correction for multiple tests of the same data set (corrected critical P = 0.01).

Four other sex-biased dispersal tests did not show significant differences between the sexes. Other authors were able to find evidence for sex-biased dispersal using F_{ST} values (Favre *et al.* 1997, female-biased dispersal in *Crocidura russula*, a monogamous mammal) or relatedness tests (Piertney *et al.* 1998, female-biased dispersal in red grouse; Knight *et al.* 1999, male-biased dispersal in cichlid fish; Surridge *et al.* 1999, male-biased dispersal in European wild rabbits). Goudet *et al.* (2002), however, tested several methods for inferring sex-biased dispersal from molecular data and concluded that the variance of assignment index test (the only test that revealed a significant dispersal difference

between the sexes in this study) is the most powerful of all tests when dispersal rates are low and when the sex-bias is not very strong (see also Mossman & Waser 1999). As the dispersal rates in túngara frogs seem high on a small scale, it is likely that females are not entirely philopatric, and therefore weaken the sex-bias in dispersal. Female túngara frogs probably disperse less frequently and over smaller distances than males (Marsh *et al*. 1999). This behaviour would explain the known ability of túngara frogs to exploit new breeding ponds rapidly, documented in various studies (Marsh *et al*. 1999, 2000). Also, assignment tests only detect very recent migration events, where the migrants are still alive. They do not reflect migration events that happened in the past, as the bi-parentally inherited microsatellites are passed on to both sexes in the next generation, compensating a possible differentiation in allele frequencies in the parents (Prugnolle & de Meeus 2002).

In summary, although túngara frogs seem to be quite mobile on a small scale of several hundred metres, there was a high level of genetic population differentiation at a geographical scale of several kilometres. The Chagres River provided a major gene-flow barrier but migration still occurred, perhaps because of unintentional human transport. It was possible to estimate precisely the proportion of genetic variation because of the gene-flow barrier, but geographical distance without obvious gene-flow barriers was still sufficient to create genetic population substructures at a scale of a few kilometres. In general, dispersal seemed to be slightly male-biased in túngara frogs, but female philopatry was not exclusive. Future studies should clarify the causal mechanisms for the genetic patterns found in this study, for example by detailed observation of an individually marked population. In addition, more sampling and genetic analysis, especially of females, will help resolve the gene-flow patterns in túngara frogs in even more detail.

Acknowledgements

References

Alford RA, Richards SJ (1999) Global amphibian declines: a problem in applied ecology. *Annual Review of Ecology and Systematics*, **30**, 133–165.

Avise JC (2000) *Phylogeography: the History and Formation of Species*. Harvard University Press, Cambridge, USA.

Barber PH (1999) Patterns of gene flow and population genetic structure in the canyon treefrog, *Hyla arenicolor*. Molecular Ecology, 8, 563–576.

Breden F (1987) The effect of post-metamorphic dispersal on the population genetic structure of Fowler's toad, *Bufo woodhousei fowleri*. Copeia, **1987**, 386–395.

Dobson F (1982) Competition for mates and predominant juvenile male dispersal in mammals. *Animal Behaviour*, **30**, 1183–1192.

Driscoll DA (1998a) Genetic structure, metapopulation processes and evolution influence the conservation strategies for two endangered frog species. *Biological Conservation*, **83**, 43–54.

Driscoll DA (1998b) Genetic structure of the frog *Geocrinia lutea* and *Geocrinia rosea* reflects extreme population divergence and range changes, not dispersal barriers. *Evolution*, **52**, 1147–1157.

Favre L, Balloux F, Goudet J, Perrin N (1997) Female-biased dispersal in the monogamous mammal *Crocidura russula*: evidence from field data and microsatellite patterns. *Proceedings of the Royal Society Series B*, **264**, 127–132.

Goudet J (1999) *PCA-GEN*, Version 1.2. Institute of Ecology, Biology Building, UNIL, Lausanne, Switzerland.

Goudet J (2001) Fstat, a program to estimate and test gene diversities and fixation indices (Version 2.9.3). Available from http://www.unil.ch/izea/softwares/fstat.html. Updated from Goudet (1995).

Goudet J, Perrin N, Waser P (2002) Tests for sex-biased dispersal using bi-parentally inherited genetic markers. *Molecular Ecology*, **11**, 1103–1114.

Greenwood P (1980) Mating systems, philopatry and dispersal in birds and mammals. *Animal Behaviour*, **28**, 1140–1162.

Hanski I, Gilpin M (1991) Metapopulation dynamics: a brief history and conceptual domain. *Biological Journal of the Linnean Society*, **42**, 3–16.

Hartl DL, Clark GC (1997) *Principles of Population Genetics*. Sinauer Associates, Sunderland, MA.

Hitchings SP, Beebee TJC (1997) Genetic substructuring as a result of barriers to gene flow in urban *Rana temporaria* (common frog) populations: implications for biodiversity conservation. *Heredity*, **79**, 117–127.

Knight ME, Van Oppen MJH, Smith HL *et al*. (1999) Evidence for male-biased dispersal in lake Malawi cichlids from microsatellites. *Molecular Ecology*, **8**, 1521–1527.

Legendre P, Legendre L (2000) *Numerical Ecology*. Elsevier, Amsterdam.

Liedloff A (1999) *Mantel, Version 2, Mantel nonparametric test calculator*. http://www.sci.qut.edu.au/nrs/mantel.htm.

Marsh DM, Fegraus EH, Harrison S (1999) Effects of breeding pond isolation on the spatial and temporal dynamics of pond use by the túngara frog, *Physalaemus pustulosus*. *Journal of Animal Ecology*, **68**, 804–814.

Marsh DM, Rand AS, Ryan MJ (2000) Effects of inter-pond distance on the breeding ecology of túngara frogs. *Oecologia*, **122**, 505–513.

Mossman CA, Waser PM (1999) Genetic detection of sex-biased dispersal. *Molecular Ecology*, **8**, 1063–1067.

Newman RA, Squire T (2001) Microsatellite variation and fine-scale population structure in the wood frog (*Rana sylvatica*). *Molecular Ecology*, **10**, 1087–1100.

Paetkau D, Calvert W, Stirling I, Strobect C (1995) Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology*, **4**, 347–354.

Pannell JR, Charlesworth B (2000) Effects of metapopulation processes on measures of genetic diversity. *Philosophical Transaction of the Royal Society of London Series B*, **355**, 1851–1864.

Perrin N, Mazalov V (2000) Local competition, inbreeding, and the evolution of sex-biased dispersal. *American Naturalist*, **155**, 116–127.

Phillips CA (1994) Geographic variation of mitochondrial DNA variants and the historical biogeography of the spotted salamander, *Ambystoma maculatum*. *Evolution*, **48**, 567–607.

Piertney SB, MacColl ADC, Bacon PJ, Dallas JF (1998) Local genetic structure in red grouse (*Lagopus lagopus scoticus*): evidence from microsatellite DNA markers. *Molecular Ecology*, **7**, 1645–1654.

Pröhl H, Adams RMM, Mueller U, Rand AS, Ryan MJ (2002) Polymerase chain reaction primers for polymorphic microsatellite loci from the túngara frog *Physalaemus pustulosus*. *Molecular Ecology Notes*, **2**, 341–343.

Prugnolle F, de Meeus T (2002) Inferring sex-biased dispersal from population genetic tools: a review. *Heredity*, **88**, 161–165.

Pusey AE (1987) Sex-biased dispersal and inbreeding avoidance in birds and mammals. *Trends in Ecology and Evolution*, **2**, 295–299.

Rowe G, Beebee TJC, Burke T (1998) Phylogeography of the natterjack toad *Bufo calamita* in Britain: genetic differentiation of native and translocated populations. *Molecular Ecology*, **7**, 751–760.

Rowe G, Beebee TJC, Burke T (2000) A microsatellite analysis of natterjack toad, *Bufo calamita*, metapopulations. *Oikos*, **88**, 641–651.

Ryan MJ (1985) *The Túngara Frog, a Study in Sexual Selection and Communication* University of Chicago Press, Chicago.

Ryan MJ (1998) Sexual selection, receiver biases, and the evolution of sex differences. *Science*, **181**, 1999–2003.

Ryan MJ, Rand AS, Weigt LA (1996) Allozyme and advertisement call variation in the túngara frog, *Physalaemus pustulosus*. *Evolution*, **50**, 2435–2453.

Schneider S, Roessli D, Excoffier L (2000) *Arlequin, Version 2.000: Software for Population Genetic Data Analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

Seppä P, Laurila A (1999) Genetic structure of island populations of the anurans *Rana temporaria* and *Bufo bufo*. *Heredity*, **82**, 309–317.

Shaffer HB, Fellers GM, Magee A, Voss SR (2000) The genetics of amphibian declines: population substructure and molecular differentiation in the Yosemite toad, *Bufo canorus* (Anura, Bufonidae) based on single-strand conformation polymorphism analyis (SSCP) and mitochondrial DNA sequence data. *Molecular Ecology*, **9**, 245–257.

Sokal RR, Rohlf FJ (1995) *Biometry: the Principles and Practice of Statistics in Biological Research*, 3rd edn. W. H. Freeman, New York.

Stenton AG, Malhorta A, Thorpe RS (2002) Population differentiation and nuclear gene flow in the Dominican anole (*Anolis oculatus*). *Molecular Ecology*, **11**, 1679–1688.

Surridge AK, Ibrahim KM, Bell DJ *et al.* (1999) Fine-scale genetic structuring in a natural population of European wild rabbits (*Oryctolagus cuniculus*). *Molecular Ecology*, **8**, 299–307.

Wright S (1943) Isolation by distance. *Genetics*, **28**, 139–156.