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Population genetics of *Glossina morsitans submorsitans* (Diptera: Glossinidae)

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Abstract

Breeding structure of *Glossina morsitans submorsitans* Newstead was evaluated by using genetic markers in mitochondrial DNA where diversity was scored at two loci in five natural populations from The Gambia and two populations in Ethiopia (form *ugandensis* Vanderplank), countries separated by *c*. 5450 km. Twenty six haplotype combinations were found, of which 17 were shared among two or more populations. Nine haplotypes were found in The Gambia and 23 haplotypes in Ethiopia. There were 12 unique haplotypes. Only six haplotypes were shared between the two countries. Populations in The Gambia ($h_e = 0.26 \pm 0.04$) showed less than a third of the diversity of populations in Ethiopia ($h_e = 0.84 \pm 0.03$). This suggests recovery from an earlier reduction in population. In a nested analysis of molecular variance of haplotype frequencies, 65% of the variance was due to differences within populations, 34% to differences between populations grouped by country, and only 1% was due to differences among populations within countries. In terms of gene flow, the fixation index $F_{ST} = 0.35$, which leads to an estimate by Wright's island model of less than one reproducing migrant per generation exchanged between the eastern and western *submorsitans* populations. Nei's genetic similarity measure showed a deep division between Gambian and Ethiopian populations.

Introduction

Tsetse flies (Diptera: Glossinidae) are most unusual insects. They feed- exclusively on blood and have among the least reproductive rate of any insect species (Rogers & Randolph, 1985). They are presently found in sub-Saharan Africa, although Oligocene fossils identified as *Glossina* have been found in Colorado, USA (Ford, 1971).

The most widespread species is *Glossina morsitans* Westwood. Three allopatric subspecies occur (Jordan, 1993), the northernmost of which is *G. m. submorsitans* (Newstead). *Glossina m. submorsitans* is found in all west African countries except Liberia; it occurs also in Cameroun, Democratic Republic of Congo, the Central African Republic, and in Sudan, Ethiopia, Uganda and Kenya (Jordan, 1993). A race of this subspecies, *Glossina m. submorsitans ugandensis* Vanderplank (1949), is recognized based on characteristics of the male genitalia (Machado, 1970). *Glossina m. submorsitans ugandensis* inhabits the easternmost part of the subspecies' range, in Uganda and it is this form that may be present in Ethiopia. Indeed, Hadis *et al*. (1995) refer to *G. m. ugandensis* but Ford's (1971) map indicates the Ethiopian population is isolated from the Ugandan.

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The genetics of hybridization of *Glossina morsitans* sensu lato have been investigated; hybrid males are sterile and hybrid females sterile or semisterile (Curtis, 1972; Gooding, 1985, 1990, 1993).

Tsetse populations are distributed discontinuously and this fact, together with their low reproductive rates, predicts that there would be pronounced genetic differentiation of populations. Indeed, 11 natural populations of *Glossina pallidipes* Austen from Kenya, Zambia, Zimbabwe and Mozambique showed remarkably high levels of genetic differentiation at allozyme loci (Krafsur *et al.*, 1997) and even greater levels of differentiation at mitochondrial loci (Krafsur & Wohlford, 1999). Patterns of mitochondrial variation were examined also in *G. m. morsitans* (Wohlford *et al.*, 1999) and high levels of differentiation were observed. Genetic differentiation of *G. m. centralis* Machado populations, however, was the most pronounced of all (Krafsur *et al.*, unpublished data).

Glossina m. submorsitans occurs in the Sudanese, sub-Sudanese, Northern Guinea and Southern Guinea vegetation zones (Keay *et al.*, 1958). It may occur where annual rainfall is between 800 and 1350 mm. The bioclimatic optimum temperature and saturation deficit for this subspecies in West Africa is 24-26°C and 6-10 mm Hg, respectively (Rogers & Randolph, 1986). Seasonal drought makes large areas uninhabitable for tsetse hosts and seasonally high saturation deficits cause a periodically hostile environment (Rogers & Randolph, 1985). Hence, the distribution of *G. m. submorsitans* contains many significant gaps, although the subspecies occupies a wide band across the continent. The eastern *G. m. submorsitans* is particularly isolated from populations in west Africa according to maps (Ford, 1971).

We obtained samples of *G. m. submorsitans* from the eastern and western extremes of its range, The Gambia and Ethiopia. The populations were separated by about 5450 km. *Glossina m. submorsitans* has been recorded in The Gambia since the first survey in 1911; five foci were recorded in a recent survey by Rawlings *et al*. (1993), who observed little change in its distribution over the past 45 years. Canopied savanna woodland favours *G. m. submorsitans* and its preferred host in The Gambia is warthog (Rawlings *et al*., 1993). Solid information on the distribution of *G. m. submorsitans* in Ethiopia is lacking, with only a few recent surveys in two areas (Leak & Mulatu, 1993;Hadis *et al.*, 1995). However, the species is widely distributed in the southwest quarter according to maps in Hutchinson (1971).

Here we ask, are the populations represented by these samples freely interbreeding? Are they descended from small, isolated populations or are they part of one continentally distributed population? When populations become small and discontinuous, drift will cause gene frequencies to diverge in proportion to numbers and time since divergence. The forces of drift and selection, however, are compensated by migration and so it is appropriate to ask, what levels of exchange occur among these sampled populations thousands of kilometres apart?

We measured and partitioned mitochondrial DNA (mtDNA) variation within and among populations to estimate gene flow. Mitochondrial DNA is useful in population analysis because it is single copy, non-recombining, maternally inherited, and is subject to a high mutation rate that leads to ample diversity. Typically, among-population mtDNA variation is greater than within-population variation (Avise, 1994).

We used the polymerase chain reaction (PCR) to amplify conserved mitochondrial loci, and the single stranded conformational polymorphism (SSCP) technique was used to reveal polymorphisms (Black & DuTeau, 1996). The universal DNA primers we used are reviewed in Simon *et al*. (1994).

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Materials and methods

Sampling—Tsetse flies were sampled at five sites in The Gambia by using Epsilon F3 cloth traps baited with acetone, phenol, and octenol. The flies were killed, frozen in liquid nitrogen, and shipped to Ames, USA. The westernmost sampling site was Kenaba (*c*. 10° 22′ N, 10° 30′ W), about 70 km from the Atlantic coast. Proceeding eastwards, distances between sampling sites were 81 km from Kenaba to Dankunku, a further 36 km to Kudang, and another 32 km to Kunting and Bansang, which were within 15 km of each other, some 195 km from the Atlantic coast.

Flies were collected in Ethiopia near the small town of Chanka in the West Wollega Zone of the Oromia Region. *Glossina m. submorsitans* is presently widespread in *Acacia-Combretum-Terminalia* woodland and savannah. *Glossina pallidipes* and *Glossina fuscipes* Newstead are also present in riverine forest. *Glossina m. submorsitans* appears to have colonized the area only recently, roughly between 1970 and 1990 (unpublished surveys of the National Tsetse and Trypanosomiasis Information and Control Centre, Bedelle). Although agricultural activities are now altering the landscape (e.g. coffee is present in the understory in riverine areas, savannah near villages is burned during the dry season), much of the area remains in its natural state. There have been no tsetse control activities and there have been only haphazard efforts to treat livestock for trypanosomiasis (CONCERN, 1994). However, *G. m. submorsitans* were abundant in the 1960s and 1970s further to the west in the region encompassing the Rivers Baro, Akobo and Gilo (Balis & Bergeon, 1968; Hutchinson, 1971).

Tsetse were collected in late 1997 from two areas about 10 km apart. The River Ketto site was in relatively undisturbed habitat near Village 6, a few km off the highway to Dembidolo, at the Ketto river (8° 46.3′ N, 35° 3.1′ E, 1300 m asl). The Chanka site was near the town of Chanka along a secondary road between Villages 8 and 12 (8° 43.1′ N, 35° 7.7′ E, 1200 m asl). Flies were collected in odour-baited cloth traps and were killed with chloroform before identification. After sorting, they were placed in 95% ethanol, which was then changed at least twice over the next few hours. Flies were stored in a fresh change of ethanol until shipment to Ames about a month after collection.

Processing of samples—DNA extraction, primers and polymerase chain reactions (PCR), polyacrylamide gel electrophoresis of the amplified DNA, and silver staining of single strand conformational polymorphisms were as described previously (Krafsur & Wohlford, 1999; Wohlford *et al*., 1999).

Sequencing—To see if putative alleles inferred from patterns on acrylamide gels correspond to unique nucleotide sequences, DNA from two or three flies showing the same allele were amplified in 30 μl PCR reactions. Each PCR fragment was sequenced in both directions and chromatograms were assembled and conflicting base calls were corrected by using Auto Assembler 1.4.0. Seven presumptive alleles at *12S* and 12 at *16S2* were sequenced by using methods set forth elsewhere (Krafsur & Wohlford, 1999; Wohlford *et al.*, 1999). Two presumptive alleles at each locus were found to be duplicates and the data were adjusted accordingly.

Data analysis—Homogeneity of allele frequencies among populations was tested by chi-square contingency. The unbiased estimate of genetic diversity h_e over x_k alleles is $h_e = n(1-\Sigma x_k^2)/(n - 1)$, where n is the number of individuals (Nei, 1987). It is the probability that two randomly chosen individuals in the sampled population have different haplotypes. Chakraborty's (1990) neutrality test and the Ewens-Watterson homozygosity test were used to test the hypothesis that haplotypes were selectively neutral. The calculations were made with Arlequin version 1.1 software (Schneider *et al.*, 1997).

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Genetic similarities were estimated according to the prescription of Nei (1987) and a dendrogram made by the unweighted pair group methods. The genetic similarity index varies from zero, when no alleles are shared, to one, where populations have identical gene frequencies. BIOSYS-1 was used to do the calculations (Swofford & Selander, 1981).

Wright's (1978) fixation index F_{ST} was calculated. It is the standardized variance in allele frequencies among populations. An analogous measure of F_{ST} is given by θ, the 'co-ancestry coefficient' of Weir & Cockerham (1984). θ, like F_{ST}, can be viewed as the correlation of two random haplotypes in subpopulations relative to the total population. θ was estimated at three hierarchical levels: populations within regions, regions within the total, and populations relative to the total. Analysis of variance (Excoffier *et al.*, 1992) was used on the combined dataset by using Arlequin (Schneider *et al.*, 1997). Pairwise θ also were calculated by using Arlequin. Henceforth, θ will be identified as F_{ST} because they measure the same thing.

F_{ST} allows estimates of the average amount of gene flow among populations. The theoretical mean number of reproducing migrant females exchanged among populations per generation *Nm* can be obtained from the relationship, $Nm \simeq (1–F_{ST})/2F_{ST}$ (Avise, 1994). The principal assumptions are selective neutrality of alleles, mutation rates much less than migration rates, spatially homogeneous densities, and equilibrium populations (Whitlock & McCauley, 1999). Because some of these and other assumptions may not hold, *Nm* can be viewed safely as the equivalent number of reproducing migrant females consistent with the F_{ST} estimates.

The allelic data are presented in combined format as haplotypes because mitochondrial loci are linked tightly so that alleles at a locus are not independent of alleles at the other loci, but are fixed and inherited matrilineally.

Results

Sequencing presumptive alleles over *c.* 395 base pairs at *12S* and 315 bp at *16S2* showed 43 variable positions at *12S* and 22 variable positions at *16S2* - equal to *c.* 9% of the nucleotides. The commonest alleles differed from each other at one to 26 positions. Two to five copies were sequenced for each of seven common presumptive haplotypes (a total of 23 copies). Two gel phenotypes at *12S* each were found to consist of two variants. Thus, phenotypes on gels underestimate diversity.

In the final analysis, a total of 11 phenotypes (alleles) were found at *12S* with a mean locus diversity $h_e = 0.49$. Six phenotypes were found at *16S2* with a mean locus diversity $h_e = 0.22$. The commonest allele at *12S* accounted for 70% of the total and was recorded in all samples. The most frequent allele at *16S2* accounted for 88% of the total and occurred in each population.

Allelic combinations were scored as haplotypes. Twenty-six haplotypes were recorded in a total of 282 flies (table 1), of which 14 (54%) were confined to single populations. Nine haplotypes were represented by single individuals. Nine haplotypes were found in The Gambia and 23 haplotypes in Ethiopia. Only six of the 26 (23%) haplotypes were shared between the two countries. Although only 27% of the flies came from Ethiopia, 65% of the haplotypes were found only there, and 11% occurred only in The Gambia. The commonest haplotype occurred in 69% of the 282 flies examined. This haplotype was unequally distributed between eastern and western populations where it accounted for 27 and 85% of the flies examined, respectively ($\chi^2_1 = 88.5$, $P << 0.001$).

The diversity index h_e (table 1) is the chance that any two randomly chosen flies have different haplotypes. Diversities varied from 0.12 at Dankunku to 0.91 at the River Ketto. Flies from The Gambia possessed much less mitochondrial variation ($h_e = 0.27 \pm 0.04$) than flies from Ethiopia ($h_e = 0.84 \pm 0.03$).

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Nei's unbiased genetic identity measures are presented as a dendrogram in fig. 1. It shows that within-country identities were close to unity while between country identities averaged 0.65 ± 0.02.

Chi-square analysis of haplotype frequencies showed that most variance (51%) occurred between the eastern and western populations (table 2). The two samples from southwestern Ethiopia did not differ significantly in their haplotype frequencies but samples from The Gambia did differ. The insignificant heterogeneity term (table 2) confirms that the samples grouped by country were more alike within than between countries.

Chakraborty's (1990) amalgamation test for selective neutrality in each population showed three significant deviations of observed frequencies from the expected frequencies ($P < 0.03$ or less). The populations were Chanka, Kudang and Kunting. These deviations were caused by high frequencies of singular haplotypes. Selective neutrality tests by the Ewens-Watterson homozygosity procedure showed no departures from haplotype distributions expected on hypotheses of neutrality and equilibrium.

Wright's (1978) fixation index F_{ST} measures the correlation of haplotypes within populations relative to that overall. The estimate over the seven populations was $F_{ST} = 0.184$. When the haplotypes were pooled by country, F_{ST} became 0.167, a value that suggests an equivalent migration rate between The Gambia and Ethiopia of 2.5 reproductive tsetse every generation. By using Weir & Cockerham's (1984) method, $F_{ST} = 0.35$ (table 3). It predicts an equivalent migration rate of one tsetse every 0.94 generations. The related fixation indices include F_{SC} which estimates the probability that two randomly chosen flies within population in The Gambia or in Ethiopia have the same haplotypes; the realized value was only 0.016 suggesting unlimited gene flow (table 3). F_{CT} estimates the chances that two randomly chosen flies in a country have the same haplotypes relative to the total. Most of the genetic variance, 65%, was within populations and a third, 34%, was between countries. The within country, among-populations variance component was small, 1%, but statistically significant.

F_{ST} was calculated for all pairwise comparisons and used as a measure of genetic distance; the corresponding hypothetical migration rates were also estimated (table 4). The migration rates are the number of reproducing flies per generation that would account for observed levels of genetic differentiation. Substantial F_{ST}s (i.e. >> 0) were recorded between The Gambia and Ethiopia. Between population F_{ST}s in The Gambia differed from zero in the Kenaba-Kunting and Dankunku-Bansang, Kunting and Kudang comparisons. The pairwise equivalent migration rates between The Gambia and Ethiopia varied from 0.8 to 1.69. Within country migration rates were very much greater.

Discussion

Diversities compared

The average haplotype diversity over all *G. m. submorsitans* populations was 0.44 ± 0.12 compared with 0.80 ± 0.04 averaged over five *G. m. morsitans* populations (Wohlford *et al.*, 1999), 0.22 ± 0.08 averaged over six *G. m. centralis* populations (Krafsur *et al*., unpublished data), and 0.38 ± 0.06 averaged over 21 *G. pallidipes* populations (Krafsur & Wohlford, 1999). These mean diversities obviously differ greatly. *Glossina m. submorsitans* showed the largest variance, caused by the grossly unequal diversities between eastern and western populations.

Indeed, diversities were *c*. three-fold greater in Ethiopian than in Gambian *G. m. submorsitans*. Nine of 26 haplotypes were unique, only one of which occurred in The Gambia. The high diversities in Ethiopian flies are indicative of permanent, continuously large

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populations. The lesser diversities in The Gambia, on the other hand, probably reflect earlier, substantial reductions in population sizes relative to the Ethiopian populations. Forest and woodland cover in The Gambia fell from 60% in 1946 to *c*. 3% by the early 1980s, caused by clearance for farming and firewood by a human population that grew more than two-fold in that interval. Moreover, there has been a declining trend in rainfall in The Gambia throughout the 20th century. The Gambia suffers annual, prolonged dry seasons in which tsetse populations greatly contract in range and density (Rawlings *et al.,* 1993). The foregoing changes could, in principle, account for much of the relative depauperization of mitochondrial diversity in Gambian *G. m. submorsitans* populations. Unfortunately, biological, agricultural, and meteorological records for Ethiopia are virtually nil, but the high diversities recorded in its *G. m. submorsitans* testify to historically robust populations.

Gene flow

Estimates of gene flow are based on the F_{ST} statistic, the correlation of two randomly chosen haplotypes in subpopulations relative to the total population. We obtained two estimates and they differed nearly two-fold, 0.18 by Wright's (1978) method, and 0.35 by Weir & Cockerham's (1984) method as modified by Excoffier *et al*. (1992) and programmed in Arlequin. These two estimates lead to equivalent migration rates of 2.2 and 0.94, respectively. The differences are substantial. Both methods are supposed to measure the same thing and were applied to the same data. They differ because Weir & Cockerham (1984) index haplotypes as zero or one and this makes for less efficient estimation when there is much unshared variation among populations. It seems unlikely, however, that variance statistics were overestimated by using Weir and Cockerham's prescription in Arlequin. We therefore expect that the fixation indices F_{ST} and F_{CT} in table 3 may be the more accurate. The inference is supported by the large differences between the eastern and western populations brought out in the chi-square statistics and ANOVA, where 51% and 34% of the variance respectively was attributed to differences between the regional groups.

Dispersal minimizes genetic differences between populations. In principle, numerically little exchange between populations prevents differentiation at selectively neutral loci, and about one reproducing migrant per generation is the 'critical' level (Wright, 1978). Our inferences from the mitochondrial genome suggest that gene flow between the easternmost and westernmost *G. m. submorsitans* populations are less than the critical level and predict that haplotypes in the two regions will continue to drift further apart. We do not imagine that flies have been directly exchanged between The Gambia and Ethiopia; rather, over time, gene flow between adjacent demes allows the spread of genes among all breeding units.

Contrasts in gene flow with other morsitans group tsetse

Estimates of F_{ST} based on mitochondrial variation varied from 0.87 in *G. m. centralis*, 0.48 in *G. pallidipes* (Krafsur & Wohlford, 1999), to 0.09 in *G. m. morsitans* (Wohlford *et al.,* 1999). These F_{ST}s are substantial in magnitude. They indicate that tsetse fly populations are highly structured into demes (local breeding units) among which there is rather less gene flow than may be inferred from ecological research on dispersal (Rogers, 1977). But, why such large differences in F_{ST}s among tsetse fly species?

The geographic scale of sampling accounts for some of the difference. *Glossina pallidipes* were sampled over an area that included Ethiopia, Kenya, Zambia, Zimbabwe and Mozambique. The seven *G. m. centralis* populations samples showed an extraordinary degree of subdivision which may be explained by the earlier rinderpest epizootic (Ford, 1971) and intensive control measures practised subsequently in the Okavango of Botswana. The lowest F_{ST} was recorded in *Glossina m. morsitans*, which was sampled from a relatively small area of one fly belt that extended from Zimbabwe north to the Kakumbi park in northeastern Zambia.

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Glossina m. submorsitans were taken from the easternmost and westernmost parts of the range separated by *c*. 5450 km. Maps show *G. m. submorsitans* as discrete, discontinuously distributed populations (Ford, 1971). Only 23% of the haplotypes were shared between The Gambia and Ethiopia. Samples from additional populations are required to confirm the picture of gene flow presented here. Examination of genomic variation in these same samples will provide an interesting contrast because genetic recombination and bi-parental transmission may show a higher degree of gene flow than the exclusively maternal patterns shown by mitochondrial loci. We are developing microsatellite loci for just such an investigation.

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Fig. 1.
Unbiased genetic identity measure (Nei, 1987) by the unweighted pair group method. The standard deviation was 2.5% and the cophenetic correlation was 0.995.

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Table 1

Haplotype diversities in *Glossina m. submorsitans.*

Haplotype	The Gambia					Ethiopia		Total
	Kenaba	Bansang	Kunting	Kudang	Dankunku	River Ketto	Chanka	
1	0	0	0	0	0	2	0	2
2	0	0	0	0	0	1	2	3
3	0	0	0	0	0	0	2	2
4	0	0	0	0	0	1	0	1
5	0	0	1	0	0	0	0	1
6	0	0	0	0	0	2	0	2
7	0	0	0	0	0	1	1	2
8	0	0	0	0	0	0	1	1
9	0	0	0	0	0	0	2	2
19	0	0	0	0	0	1	1	2
11	0	0	0	0	0	1	0	1
12	0	0	0	0	0	1	0	1
13	0	0	0	0	0	0	1	1
14	0	2	1	0	0	1	1	5
15	0	0	0	0	0	1	0	1
16	0	0	0	0	0	1	0	1
17	0	0	2	1	0	1	0	4
18	0	0	0	1	0	1	0	2
19	3	0	0	4	0	0	0	7
20	38	26	33	34	45	6	14	196
21	0	0	0	0	0	6	17	23
22	0	0	1	1	3	0	1	6
23	0	0	0	3	0	0	0	3
24	0	0	0	0	0	0	1	1
25	0	0	0	0	0	0	3	3
26	0	4	4	0	0	0	1	9
Total	41	32	42	44	48	27	48	282
Diversity	0.137	0.325	0.374	0.393	0.118	0.892	0.785	0.509

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Table 2

Chi-square tests of homogeneity in haplotype frequencies in *Glossina m. submorsitans.*

Location or contrast	No. samples	No. haplotypes	Chi-square	d.f.	*P*
The Gambia	5	9	62.16	32	0.001
Ethiopia	2	23	28.12	22	0.171
Between countries	2	26	169.58	26	<< 0.001
Heterogeneity	-	-	74.04	70	0.348
Total	7	26	333.90	150	<< 0.001

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Table 3

Analysis of variance in *Glossina m. submorsitans* haplotype frequencies by the method of Excoffier et al. (1992). All haplotypes are considered equidistant.

Source	d.f.	Variance	% of total	F-statistics	Equivalent no. migrants	Significance[1]
Among groups[2]	1	0.10833	33.8	F_{CT} = 0.338	0.98	< 0.050
Among populations within groups	5	0.00333	1.0	F_{SC} = 0.016	31.40	< 0.017
Within populations	275	0.20904	65.2	F_{ST} = 0.348	0.94	< 0.001
Total	281	0.32070				

[1] Probability of obtaining a greater variance and *F* by chance.

[2] Groups are The Gambia and Ethiopia.

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Table 4

Pairwise genetic distance measures in terms of F_{ST} in the upper diagonal and equivalent migration rates[1] in the lower diagonal.

	Kenaba	Bansang	Kunting	Kudang	Dankunku	River Ketto	Chanka
Kenaba	-	0.054	0.047	0.031	0.014	0.383	0.347
Bansang	8.71	-	0	0.021	0.065	0.251	0.242
Kunting	10.11	Infinite	-	0.008	0.052	0.238	0.232
Kudang	15.68	23.63	61.1	-	0.059	0.231	0.228
Dankunku	36.12	7.15	9.15	7.97	-	0.415	0.371
River Ketto	0.8	1.49	1.6	1.66	0.71	-	0.003
Chanka	0.94	1.57	1.66	1.69	0.85	171.34	-

[1] Average no. reproducing migrants = $(1 - F_{ST})/2F_{ST}$.