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Population genetics of the schistosome intermediate host *Biomphalaria pfeifferi* in the Zimbabwean highveld: implications for co-evolutionary theory

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Co-evolutionary theory proposes that polymorphisms in co-evolved traits may be maintained through differential selection in spatially heterogeneous environments. Spatial heterogeneity of the schistosome intermediate host, *Biomphalaria pfeifferi*, was investigated here, using RAPD markers. Overall, 256 individuals, collected at 32 sites on 13 rivers in the Chiweshe region of Zimbabwe, were analysed. Significant genetic differentiation was demonstrated, both between populations from the different rivers and between populations collected at different sites on each of several of the rivers investigated. However, the presence of spatial differentiation between populations from individual rivers varied with river type. It was not apparent in permanent, deep, fast-flowing rivers, where high levels of migration, through passive dispersal along the rivers, are likely. The snails collected from shallow, semi-permanent rivers not only showed relatively high levels of population subdivision but also high levels of within-site genetic diversity, consistent with the existence of 'co-evolutionary hot-spots' where schistosome infection may be prevalent. These results are discussed with reference to the population biology of *B. pfeifferi* and to host–parasite co-evolution.

The concept of the metapopulation, with its focus on the existence of multiple interacting populations, has revolutionized our understanding of the ways in which hosts and parasites interact. Different populations of many host–parasite systems show varying geographical patterns of genetic variation in resistance and infectivity (Burdon and Thrall, 1999). Parasites may significantly affect the fitness of their hosts, reducing reproductive success and/or longevity. At a population level, such

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changes to individual hosts may alter the size, density and genetic compositions of host populations and thereby affect the selection pressures acting on the parasites themselves. However, it is only when such changes within individual populations are placed in an ecological framework—of ongoing processes of population establishment and extinction, of varying gene flow between different areas in the interconnected network of populations, and of variable environmental suitability—that the complexities of host and parasite co-evolution can begin to be understood (Burdon *et al.*, 1999).

Freshwater snails of the genus *Biomphalaria* are intermediate hosts for *Schistosoma mansoni*, the causative agent of human intestinal schistosomiasis. *Biomphalaria* populations are considered to be part of a 'metapopulation' (Woolhouse, 1995). They are susceptible to extreme fluctuations in population size and genetic 'bottlenecks' caused by drought or molluscicide treatments (Woolhouse and Chandiwana, 1989; Woolhouse, 1992; Davies *et al.*, 2001). Moreover, flooding following heavy rains can promote passive dispersal of snails along rivers and result in the recolonization of depleted sites (Woolhouse, 1988; Davies *et al.*, 2001).

A feature of schistosome epidemiology is the patchiness of transmission to humans within endemic areas (Wilkins, 1987). This is possibly the result of the ecology and genetics of interactions between the schistosomes and their intermediate hosts (Chandiwana, 1988). Geographical variation in snail–schistosome compatibility, for example, has been widely reported (Manning *et al.*, 1995). A compatible snail infection is defined as one which results in the release of the human-infective cercariae (Wright, 1971). Reciprocal cross-infection studies have demonstrated that schistosomes may be adapted to local populations of their snail hosts, such that sympatric host–parasite combinations are generally more compatible than allopatric combinations (Morand *et al.*, 1996; Webster and Woolhouse, 1998; Woolhouse and Webster, 2000; Webster and Davies, 2001).

Since co-evolutionary interactions commonly occur in heterogeneous environments, it is important to understand both the processes influencing the development of such variability and the consequences such heterogeneity might have for co-evolutionary trajectories. Patterns of migration and gene flow in hosts and parasites are very important in the development of co-evolving associations and for determining the geographical scale over which any such interactions will occur (Burdon and Thrall, 1999). The geographical-mosaic theory of co-evolution was developed from exploration of how the raw materials of co-evolution are organized (Thompson, 1994). This suggests that there is a selection mosaic among populations, favouring different evolutionary directions to interactions in different populations. Furthermore, it assumes that there are 'co-evolutionary hot-spots', such that reciprocal selection need not occur in all populations. Finally, if this hypothesis is correct, there is probably a continuous remixing of the range of co-evolving traits, resulting from the selection mosaic, co-evolutionary hot-spots, gene flow, random genetic drift and the local extinction of populations (Thompson, 1994).

Genetic studies of populations can be used to investigate the spatial distribution of genetic structure and estimate the relative power of the forces, such as mutation, genetic drift and local adaptation, which promote genetic differentiation of local populations (Slatkin, 1987), and of the opposing effect of gene flow (Slatkin and Barton, 1989). In the present study, RAPD markers were used to investigate the genetic structure of *B. pfeifferi* populations sampled at more than 30 field locations along several rivers and their tributaries in the Zimbabwean highveld. This expands upon an earlier survey (Hoffman *et al.*, 1998) of one Zimbabwean river, and apparently represents the most comprehensive cross-sectional survey performed on a snail metapopulation. The main aim of the present study was to see whether an environmental variable (river type) could be associated with differences in population structure, thereby supporting the mosaic theory of selection in host–parasite co-evolution.

ANIMALS AND METHODS

Study Site and Sampling Methodology

The study was set in the Chiweshe highveld in north–eastern Zimbabwe, an area endemic for human *S. mansoni* infection. Sampling sites were located along three major river systems, within which snails were collected from 32 sites (Fig. 1). At each site, snails were collected by two to four experienced samplers

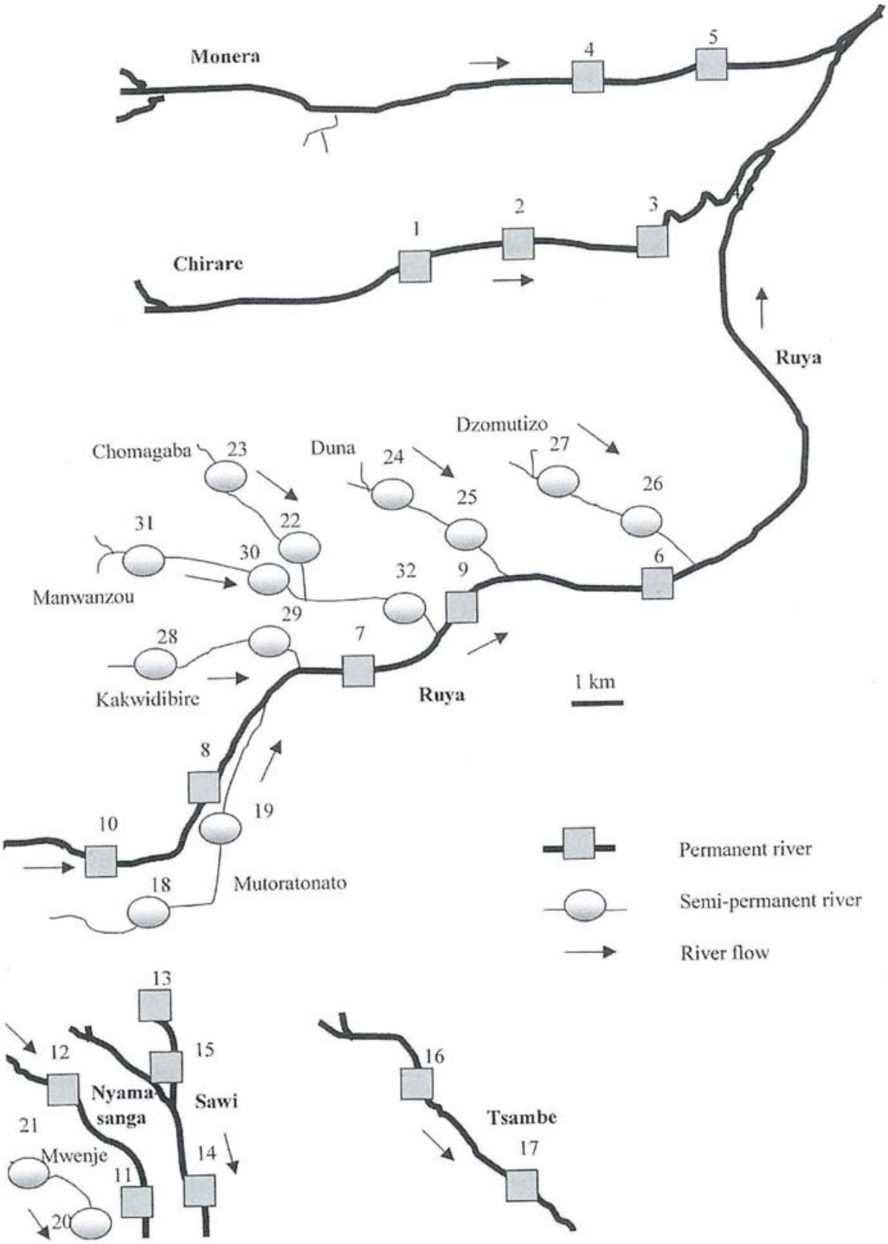

Fig. 1. Map showing the 32 collection sites along 13 rivers.

using 2-mm-mesh scoops, within an area of 5 m^2. Sampling intensity was approximately 10 scoops/m of riverbank.

Each river was 1–8 m wide and normally between 0.5 and 1.5 m deep, with a mud or rock substrate (Woolhouse, 1995). Geographical distances between collection sites ranged from 1 to 42 km. The climate of the study region is subtropical. Annual rainfall is 500–1400 mm and air temperatures vary from 3–32°C, resulting in mean water temperatures of 15–21°C. All the snail samples were collected in August 1995, at the end of the cold, dry season.

People in the study area make substantial use of the natural water bodies for domestic activities such as the washing of clothes and recreational bathing, although water contact tends to be confined to a number of discrete sites (Chandiwana, 1988; Woolhouse, 1995). The prevalence of human infection with schistosomes is strongly related to the spatial patterns of water contact rather than simply the duration of such contact (Chandiwana, 1988). Likewise, the prevalence of snail infection with schistosomes is known to be strongly related to spatial patterns of human water-contact (Woolhouse and Chandiwana, 1989). In the present study, the prevalence of snails infected with *S. mansoni* was estimated, from a subset of rivers, following each day's sampling. Each snail checked was placed in a glass vial containing approximately 25 ml fresh water. All the vials were then exposed to natural sunlight for 2 h, within an open-topped box that had been lined with aluminium foil to enhance reflection, and the number of snails releasing cercariae was then recorded. Whilst this method will not reveal pre-patent infections, the aim was to compare relative measures of snail infection rather than absolute measures between the 'permanent' and 'semi-permanent' rivers (Woolhouse, 1995) studied. 'Permanent' rivers are deep, fast-flowing water bodies that typically have steep banks, making access to the river difficult and thereby limiting human contact sites. 'Semi-permanent' rivers are shallower, narrower and much slower flowing, with lower turbidity, than the permanent rivers (Woolhouse and Chandiwana, 1989; Woolhouse, 1995). The banks of the 'semi-permanent' rivers are of gentle incline and permit frequent human–water contact (Woolhouse and Chandiwana, 1989; Woolhouse, 1995; Hoffman *et al.*, 1998). Moreover, the present snail collections were made during the dry season, when many of these rivers become partially dry, creating small isolated pools.

RAPD Analysis

At the end of each day's sampling, the field-collected snails were relaxed in menthol, to prevent bursting of the hepatopancreas, and then preserved in molecular-grade ethanol prior to genetic analysis.

Eight snails of each population were processed (giving a total of 256 snails); it was considered desirable to match sample sizes between populations in order to conduct a comparative study, and sample size was limited by snail availability at certain sites. [Although use of such small sample sizes may lead to overestimates of the absolute measures of population subdivision (Apostol *et al.*, 1996), it was the comparative population structure of rivers of different types, and hence relative rather than absolute measures, that were of interest in this study. Furthermore, the results of previous pilot studies at a single site (Ruya river) within the present study area indicated that estimates of intrapopulation similarity were not strongly influenced by varying population size from eight to 64 snails, with mean (S.E.) values for Jaccard's similarity coefficients of 0.89 (0.019) and 0.89 (0.013), respectively (Davies *et al.*, 2001; see below).]

Genomic DNA was extracted from the tip of the head-foot region of each snail (hence avoiding potential contamination by any schistosome stages present), using a standard phenol–chloroform procedure modified to overcome the problems associated with DNA degradation from gastropod nucleases (Vernon *et al.*, 1995). PCR amplification conditions and reaction mixtures were as described by Okamura *et al.* (1993). Nine randomly chosen oligonucleotide primers (each of 10 bp), which always produced clear and reproducible bands in the RAPD–PCR analysis, were selected. There was no bias towards selecting primers producing polymorphic loci or not, which would otherwise affect estimates of diversity. Seven of the selected primers [R&D8 (CGCACGCTTC), R&D9 (AGCAGCGTGG), R&D10 (TAGCAGCGGG), R&D12 (ATGGATCCGC), R&D15 (CTGGCGGCTG), R&D17 (GTGATCGCAG) and R&D18 (AATCGGGCTG)] were from R&D Technologies (Abingdon, U.K.) and the other two [OPR1 (TGCGGGTCCT) and OPR4 (CCCGTAGCAC)] were from Operon Technologies (Alemada, CA).

Controls were run without primer, without snail DNA or without Taq polymerase, and snail DNA samples were re-run in order to verify repeatability.

The products of amplification were resolved by electrophoresis in 1.4% agarose gels stained with 0.001% ethidium bromide (Sigma) and photographed under ultraviolet light using a Polaroid MP-4 land camera loaded with Polaroid 667 film (Polaroid Corporation, Cambridge, MA).

Data Analysis

Overall, 129 clearly reproducible bands were scored. [Intra-observer reliability was investigated by re-scoring five population/primer combinations selected at random. Two of the selected 376 bands were scored differently, which suggests an acceptable scoring reliability of approximately 99.5% (Martin and Bateson, 1986)]. As measures of phenotypic relatedness (Jaccard, 1908), pair-wise Jaccard's similarity coefficients (JS) were calculated as:

$$\mathrm{JS} = a/(a + b + c)$$

where a is the number of shared bands between individuals 1 and 2, b the number of bands present in individual 1 but absent from individual 2, and c the number of bands present in individual 2 but absent from individual 1.

Similarities were converted to distances, for use in the analysis of molecular variance (AMOVA; see below), by subtraction from unity. RAPD markers are thought to be predominantly inherited in a Mendelian dominant fashion (Williams *et al.*, 1990) and thus allele frequencies, classically used in population genetic analysis, cannot be calculated without assuming Hardy–Weinberg equilibrium (Lynch and Milligan, 1994). Though many *Biomphalaria* populations are thought to outcross preferentially (Vianey-Liaud *et al.*, 1987, 1989; Vernon *et al.*, 1995), they are also capable of self-fertilization such that populations can be re-established from a single individual (Woolhouse, 1995). Since the relative rates of selfing *versus* outcrossing in the different river types were not known, analysis methods were chosen that make no assumptions of Hardy–Weinberg analysis.

AMOVA (Excoffier *et al.*, 1992) was used to quantify the amount of variation between individuals from the same and differing collection sites, and between the rivers. AMOVA is analogous to the classical analysis of variance and is suitable for RAPD phenotypes (Huff *et al.*, 1993; Gabrielsen *et al.*, 1997). The analysis was conducted using version 1.55 of the WINAMOVA software (Excoffier *et al.*, 1992) and the significance levels of the variance components were based on 1000 permutations. Analysis was performed on a matrix of Jaccard distances between haplotypes, where a haplotype was defined as a single multi-locus banding pattern. Bartlett's heteroscedasticity index (B; Bartlett, 1937) was used to investigate whether there were differences in the amount of variation within rivers and collection sites. Fixation indices between pairs of populations (Φst) were calculated and tested for a significant difference from zero, using 1000 random permutations (where a significant result represents the presence of population differentiation).

The data were further investigated using principal co-ordinate analysis, a procedure for projecting multi-dimensional data into, in this case, a two-dimensional space, in order to reveal intrinsic patterns (Gower, 1966). Individual snails are plotted as points in such a way as to make the distance between every pair of points as equal as possible to their similarity. Intrapopulation genetic similarities were visualized as the mean Jaccard similarities among snails comprising each population. These analyses were conducted using the R Package (Legendre and Vaudoir, 1991).

RESULTS

Within the sample sub-set investigated, snail densities, as measured by the approximate number of snails/10 scoops [$\chi^2 = 263$; degrees of freedom (df) = 2; $P < 0.001$], and the proportions of those snails found to be shedding *S. mansoni* cercariae ($\chi^2 = 4.63$; df = 1; $P = 0.03$), whilst low, were significantly higher within the 'semi-permanent' rivers than within the 'permanent' (Table 1).

Of those snails from each of the 32 sites examined by RAPD analysis (Fig. 1), 210 different multi-locus banding patterns (haplotypes) were identified from the 256 individuals. The results of the AMOVA are shown in Table 2. There was an overall significant genetic difference between populations collected in the different rivers ($P = 0.009$) and also between populations collected at different sites within rivers ($P < 0.001$). Approximately 60% of the variation was, however, between individuals within sites. Bartlett's heterocedasticity index (which measures variance heterogeneity) showed that rivers demonstrated significant differences in the amount of variance contained ($B = 19.6$; df = 12; $P < 0.009$), but there was no further heterogeneity of variance between sites within rivers ($B = 6.11$; df = 31; $P = 0.99$). Intrapopulation similarity was high in the 'permanent' rivers and lower (i.e. diversity was higher) in the 'semi-permanent' rivers ($U = 333$; $P = 0.02$; Figs 2 and 3).

Table 3 shows the range of fixation indices between populations within rivers and the river habitat types. There was no significant population differentiation in any of the six 'permanent' rivers. There was, however, significant differentiation in four of the eight rivers classified as 'semi-permanent'. Moreover, it appears that the lack of significant differentiation, as measured by the *F*-statistic, in the remaining 'semi-permanent' rivers was simply the result of the high intra-population diversity recorded within these rivers. That all of the 'semi-permanent' rivers did show spatial substructure was indicated by the results of the principal coordinate analysis, which was further used to investigate data patterns in six rivers where fixation indices (Φst) indicated that there was no spatial differentiation between the different populations: the 'permanent' Chirare, Monera and Ruya and the 'semi-permanent' Manwanzou, Mutoratoanoto and Mwenje (Fig. 4). Individual snails from sites within the 'permanent' rivers showed high levels of similarity to individuals from other sites within the same river. Indeed individuals with banding patterns identical at all 129 marker alleles were identified at mul-

TABLE 1

The numbers of Biomphalaria pfeifferi *scooped, and found to be shedding* Schistosoma mansoni, *from sites on three 'permanent' rivers and three 'semi-permanent' rivers in the Chiweshe region of Zimbabwe*

*River**	*Sites**	*River type*	*No. of snails collected*	*No. and (%) of snails infected†*
Ruya	6 and 9	Permanent	66	0 (0)
Nyamasanga	11 and 12	Permanent	61	2 (3)
Sawi	13 and 14	Permanent	163	3 (1)
Manwanzou	30 and 31	Semi-permanent	420	16 (4)
Kakwidibire	28 and 29	Semi-permanent	309	9 (3)
Duna	24 and 25	Semi-permanent	82	12 (15)

* See Figure 1.

† Snail densities, as measured by number of snails/10 scoops [$\chi^2 = 263$; degrees of freedom (df) = 2; $P < 0.001$], and the proportion of snails found infected ($\chi^2 = 4.63$; df = 1; $P = 0.03$) were both significantly higher on the 'semi-permanent' rivers than on the 'permanent'.

TABLE 2

Analysis of molecular variance for Biomphalaria pfeifferi *collected at 32 sites in the Chiweshe region of Zimbabwe (eight snails were analysed/site)*

Source of variation	*Sum of squares*	*Degrees of freedom*	*Variance component*	*% of total variance*	P
Between rivers	5.69	12	0.0142	17.24	0.009
Among sites within rivers	3.76	19	0.0185	22.52	<0.001
Among individuals within sites	11.10	224	0.0496	60.24	

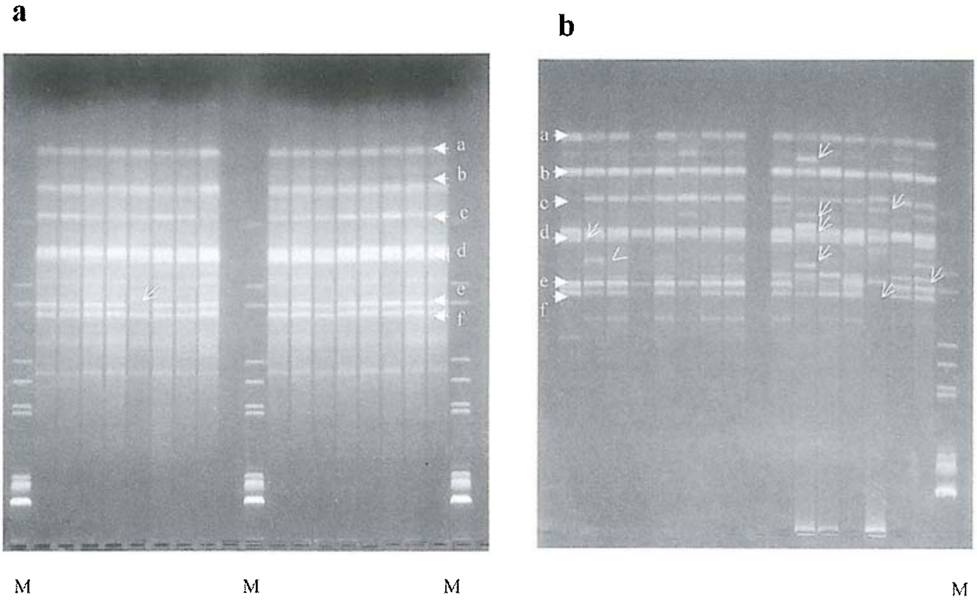

Fig. 2. RAPD gels showing low levels of intra- and inter-population genetic diversity amongst snails from each of two 'permanent' river sites (a), and high levels of intra- and inter-population genetic diversity amongst snails from each of two 'semi-permanent' river sites (b). Example bands are labelled a–f, and examples of additional polymorphic bands are indicated with arrows. All the RAPD–PCR shown were run simultaneously using the same R&D17 primer (GTGATCGCAG). A lambda DNA EcoRI HindIII digest (Sigma) was used as a source of markers (M).

tiple sites in the Monera, Chirare, Ruya, Sawi and Tsambe rivers. In contrast, no individuals with identical phenotypes were present in neighbouring sites in any of the shallow 'semi-permanent' rivers. Principal co-ordinate plots also revealed that there was no overlap between populations collected at the different sites in the Manwanzou, Mutoratanoto or Mwenje rivers.

DISCUSSION

In this study, consistent variations in snail population structure were identified between 'permanent' and 'semi-permanent' rivers within a small geographical area. Snail populations from the 'permanent', deep and fast-flowing rivers showed little or no evidence of population subdivision and there were gener-

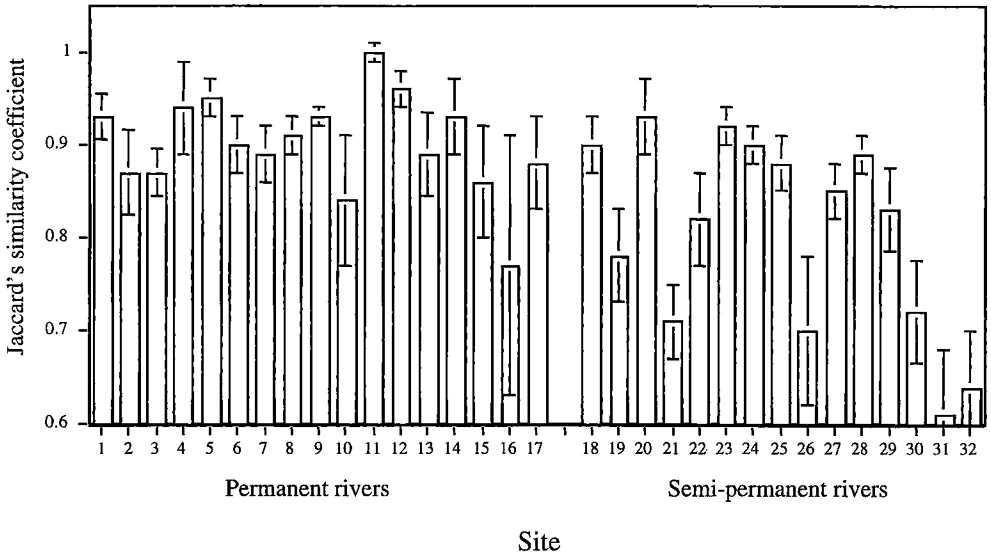

Fig. 3. Mean values for the pair-wise Jaccard's similarity coefficients between individuals within collection sites on 'permanent' and 'semi-permanent' rivers. The vertical lines indicate 95% confidence limits.

TABLE 3

River habitats and range of fixation indices (Φst) between populations within each river system

*River**	*Sites**	*River type*	*Φst*	P†
Chirare	1–3	Permanent	− 0.09– − 0.01	0.54–0.99
Monera	4–5	Permanent	− 0.01	0.39
Ruya	6–10	Permanent	− 0.14– − 0.04	0.57–0.99
Nyamasanga	11–12	Permanent	− 0.04	0.74
Sawi	13–15	Permanent	− 0.14– − 0.12	0.96–0.99
Tsambe	16–17	Permanent	− 0.14	0.99
Chomagaba	22–23	Semi-permanent	0.59	< 0.001
Duna	24–25	Semi-permanent	0.75	< 0.001
Dzomutizo	26–27	Semi-permanent	0.48	< 0.001
Kakwidibire	28–29	Semi-permanent	0.77	< 0.001
Manwanzou	30–32	Semi-permanent	− 0.14	0.96–0.99
Mutoratanoto	18–19	Semi-permanent	− 0.14	0.99
Mwenje	20–21	Semi-permanent	− 0.01	0.47

* See Figure 1.

† Significance levels were tested using 1000 random permutations.

ally high levels of similarity between individuals both within and between populations. In contrast, populations from the 'semi-permanent', shallow rivers were genetically distinct, and genetic diversity within sites was relatively high (see Figures 2 and 3 and Table 3). These patterns may reflect, in part, relatively high levels of migration in the deeper 'permanent' rivers and relatively limited migration between the populations of the shallower, 'semi-permanent' rivers that become partially dry during each dry season. More

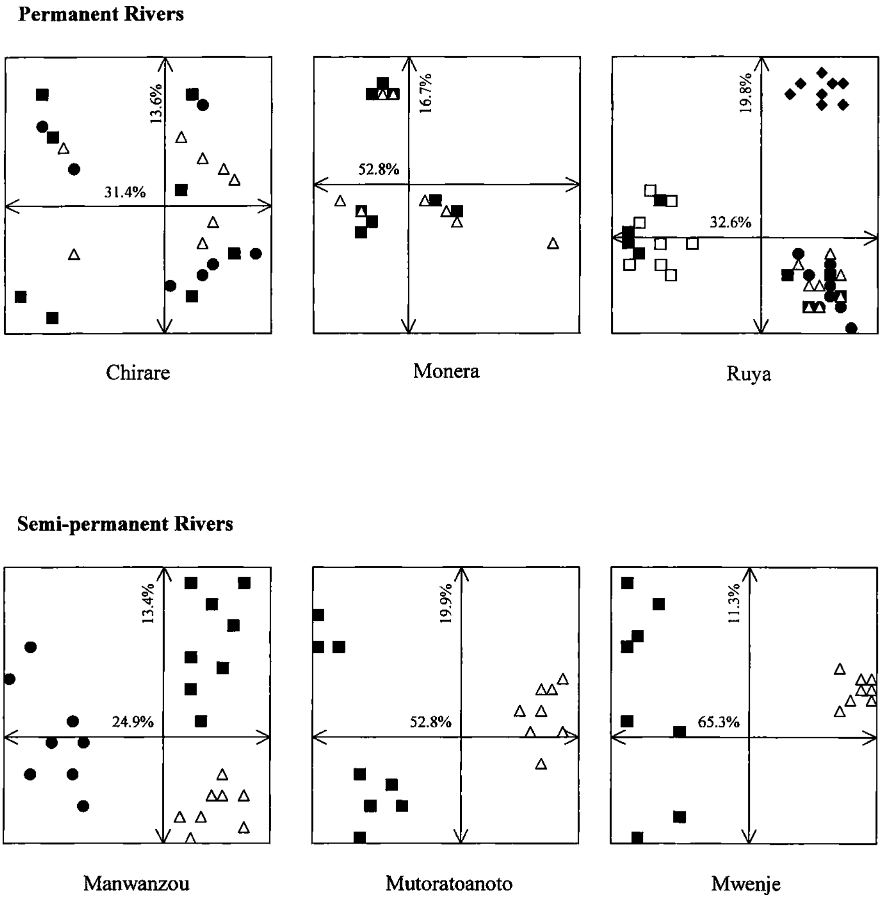

Fig. 4. Results of the principal components analysis, showing patterns of similarity between snails within each of the three 'permanent' rivers (Chirare, Ruya and Monera) compared with those between snails within each of the three 'semi-permanent' rivers (Manwanzou, Mutoratanoto and Mwenje). Lack of population differentiation was indicated in each of these six rivers by fixation indices (Φst). Various symbols are used to represent individual snails collected at sites 1 (■), 2 (△) and 3 (•) on the Chirare, 4 (■) and 5 (△) on the Ruya, 6 (■), 7 (△), 8 (•), 9 (□) and 10 (◆) on the Monera, 30 (■), 31 (△) and 32 (•) on the Manwanzou, 18 (■) and 19 (△) on the Mutoratanoto, and 20 (■) and 21 (△) on the Mwenje.

over, repeated population extinctions over time when rivers run dry may also promote population differentiation in the 'semi-permanent' rivers, and are indeed a feature of *B. pfeifferi* (Woolhouse, 1995); Davies *et al.* (2001) give the results of a temporal study of a sub-set of the populations studied here, and describe such localized extinctions. Finally, 'semi-permanent' rivers when full offer more favourable habitats for the snails than the fast-flowing 'permanent' rivers (Utzinger *et al.*, 1997) and hence would then be expected to support high snail densities. Even within the cold-dry season of the current study, significantly higher snail densities were found within the 'semi-permanent' rivers than the 'permanent' (Table 1). Such high population densities promote more opportunities for out-crossing and reducing genetic drift, and could thereby further enhance the high levels of intrapopulation diversity observed here within the 'semi-permanent' rivers (Table 3).

The theory that co-evolution occurs within a geographical mosaic assumes that there is a structured population of interacting populations, where gene flow and the force of selection can vary among populations, thereby leading to a mosaic of traits in space (Thompson, 1994, 1999). The present results indicate that the *B. pfeifferi–S. mansoni* system may be well described by such characteristics. For example, co-evolution of host resistance and parasite infectivity is thought to generate local adaptation in compatibility in this system (Morand *et al.*, 1996). A prerequisite for local adaptation is spatial differentiation in host populations (Gandon and von Zandt, 1998). Thus the scale over which local adaptation is likely to occur will vary where patterns of spatial differentiation differ, as demonstrated, in the present study, between rivers of different general characteristics. Moreover, theoretical models have predicted that increased host migration may promote local maladaptation (Gandon *et al.*, 1996). This is analogous to a prediction of the geographical mosaic theory, that gene flow between co-evolving populations will result in transient mismatches of traits or maladaptation (Lively, 1999; Woolhouse and Webster, 2000). Accordingly, in snail–schistosome interactions, there has been a significant minority of cases where allopatric host–parasite combinations have been found to be more compatible than sympatric ones (Morand *et al.*, 1996). One explanation for this is that, in local, frequency-dependent co-evolutionary dynamics, there is a time lag before parasites adapt to evolving host populations, such that some populations may be out of phase (Morand *et al.*, 1996; Woolhouse and Webster, 2000). An alternative, albeit not incompatible explanation is that differential selection pressure in different areas, and regional patterns of gene flow and migration, will also maintain local maladaptation in co-evolving interactions.

An associated premise of co-evolutionary models, such as that of the geographical mosaic theory, is that there exist co-evolutionary 'hot-spots' and 'cold-spots' within the distribution of a given species, such that reciprocal adaptation may only be occurring in particular populations. The observation of variation in the spatial patterns of human–water contact (Woolhouse and Chandiwana, 1989), in the contamination of rivers with excreta, and in environmental factors favouring successful infection of host snails with schistosome miracidia (Prah and James, 1977) indicates that such heterogeneities in co-evolution are likely in snail–schistosome interactions. Shallow, 'semi-permanent' rivers may represent the 'hot-spots', as they experience greater human–water contact, and hence parasite pressure, than do the 'permanent' rivers (Woolhouse, 1995). Indeed, although the prevalences of (patent) snail infection with *S. mansoni* observed during the present study were low, as is characteristic of *B. pfeifferi* (Woolhouse and Chandiwana,1989), the proportion of the tested snails from the 'semi-permanent' rivers which were found infected was significantly higher than that of their counterparts from the 'permanent' rivers (Table 1). Such a difference may thus further account for the relatively higher intrapopulation genetic diversity observed amongst the snails collected from 'semi-permanent' rivers during the present study (Table 3) and, previously, amongst the *B. pfeifferi* populations collected at, or immediately downstream from, human–water contact sites in the same region (Hoffman *et al.*, 1998).

In summary, *B. pfeifferi* populations were differentiated between rivers within a relatively small area of the Chiweshe region of north-eastern Zimbabwe. The extent of population differentiation within rivers, however, varied with river type, being high in the 'semi-permanent' rivers but more limited in the deeper, 'permanent' rivers. The spatial heterogeneity in *B. pfeifferi* associated with environmental variability will probably promote geographical variation in snail–schistosome compatibility.

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