Genetic variation in a Pacific Island land snail: population history versus current drift and selection

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Previous studies of *Partula* land snails from the Society Islands, French Polynesia, have shown that there can be striking differences in shell shape, colour and banding pattern between nearby populations, even in the absence of any obvious geographical barriers to the movement of snails, or environmental gradients. Elsewhere, there may be relative uniformity over large distances. Analysis of a mitochondrial gene from *Partula taeniata* (Mörch) shows a similar pattern. The relative frequencies of two mitochondrial haplotypes change abruptly over small distances, seemingly independent of the environment. Although the transition roughly coincides with clines in the frequencies of some morphological characteristics, it appears to be unrelated to others. It is likely that many of the differences accumulated while populations were isolated from one another, through the effects of random genetic drift and selection. Isolation of populations may have occurred as a result of demographic changes, or during the process of colonization if occasional long-distance migrants establish populations ahead of the main invading front. Current genetic drift, even without restrictions to gene flow, may contribute to genetic patchiness on a small scale, although it is likely that conspicuous characteristics such as shell colours and banding patterns are also influenced by selection.

Keywords: land snail; population structure; *Partula*; 16S ribosomal DNA

1. INTRODUCTION

Land snails of the genus *Partula* in the Pacific Ocean have given excellent opportunities to study adaptation and speciation (Crampton 1916, 1925). Some of the best-studied *Partula* come from the island of Moorea, in the Society Islands of French Polynesia (Murray & Clarke 1980). Moorean *Partula* are particularly interesting because they have apparently radiated into a suite of species within a single island only 15 km across (Johnson *et al.* 1986). One striking feature is that they show a large amount of intraspecific variation, which, at least in terms of morphology, colour and banding patterns, is distributed in patches. High frequencies of particular types occur in discrete areas, in a spatially recurring pattern (Clarke & Murray 1969).

The species *Partula taeniata* (Mörch) is distributed more or less continuously throughout the island of Moorea, with few large barriers to the movement of snails. Despite this distribution, regions in the north-west of the island, where individuals are squat and thick-shelled (*P. t. nucleola* Bailey), are very close to regions with more elongated, thinner-shelled forms (*P. t. simulans* Crampton) (see Clarke & Murray 1969). At the borders between such patches there may be sharp spatial changes in the proportions of different shell colours and patterns (Crampton 1932; Clarke 1968; B. Clarke and J. Murray, unpublished data). There is a cline in the frequencies of orange- and pink-shelled individuals, running from north-east to south-west within the north-western region, and an area with high frequencies of dark purple-brown morphs in Faatoai valley, to the north-west of the orange-pink cline (Clarke 1968). The

observed transitions between types do not occur along any obvious environmental gradients, and are not associated with any notable change in the frequencies of allozyme genotypes (J. Stephens, unpublished data).

One proposal to explain the patterns, both on a large scale (changes from *P. t. nucleola* to *P. t. simulans* and other forms) and on a smaller one (changing proportions of coloured or banded types within the *nucleola* or *simulans* regions), is that co-adapted gene complexes have evolved in physical isolation (Clarke 1968). This theory is attractive, given that snails have a low mobility. Differences between populations might persist even after evidence about how they came to be isolated (for example, through arid zones) has disappeared. However, a large number of isolates are necessary to explain all the differences observed in *P. taeniata*. The present paper investigates variation at a mitochondrial locus in populations of *P. taeniata* from the north-west of Moorea, and compares the results with earlier data on morphology, shell colour, shell banding and allozymes, in order to illuminate the origins of these striking patterns.

2. MATERIAL AND METHODS

Moorean *Partula* are now thought to be extinct in the wild, due to a mismanaged biological control programme that aimed to eliminate the giant African land snail, *Achatina fulica* (Bowdich), using a carnivorous snail, *Euglandina rosea* (Férussac) (Clarke *et al.* 1984). It is no longer possible to collect natural populations of Moorean *Partula*. The samples reported here were taken in 1982 from 21 sampling sites in five valleys from the north-west of Moorea (see figures 1 and 2). For convenience, all references to Moorean *Partula* will be made in the present tense. Samples were stored at -20°C or pickled in ethanol. Samples were collected by B. C. Clarke and J. J. Murray from areas of ca. 10 m $\times$ 10 m, which is smaller than the estimated neighbourhood size of *P. taeniata* (Murray & Clarke 1984). The number of

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Figure 1. Location of valleys on Moorea, Society Island, French Polynesia, from which *P. taeniata* samples were taken (boxed region, which is shown in more detail in figure 2). Internal lines mark the positions of mountain ridges, which divide the island into a series of steep-sided valleys.

individuals studied from each site ranged between five and 14 (see table 1).

DNA was extracted from individual snails using a modified version of the hexadecyltrimethylammonium bromide (CTAB) protocol (Stine 1989; Terrett *et al.* 1994). CTAB is regarded as an effective remover of mucopolysaccharides commonly associated with snail DNA, which can inhibit reactions such as PCR. For each sample a small piece of foot tissue (*ca.* 25 mm³) was sliced finely and placed in 300 µl CTAB solution (100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 0.2% β-mercaptoethanol) with 0.01 mg proteinase K. The sample was vortexed, incubated at 60 °C for 2–3 h and vigorously shaken every hour. Proteins were removed by extraction first with 300 µl chloroform, then twice with an equal volume of phenol and chloroform in the ratio 1:1, and finally again using chloroform alone. The extracts were centrifuged at 10 000 *g* for 10 min and the aqueous layer removed for further extraction at each stage. DNA was precipitated using ethanol, pelleted by centrifuging at 10 000 *g* for 15 min, washed in 70% ethanol, dried and resuspended in 50 µl distilled water.

Primers 16S1 and 16S2, known to work well with land-snail DNA (Thomaz *et al.* 1996) were used to amplify 400 base pairs of the mitochondrial 16S ribosomal RNA gene (16S1: 5'-TGAC TGTGCAAAGGTAGC-3'; 16S2: 5'-CTGGCTTACGCCGTC TG-3'). PCR reactions were carried out in a total volume of 25 µl containing 1 unit of Taq, 2.5 mM MgCl₂, 0.5 mM of each dNTP, 400 nM of each primer and 1 µl DNA solution, in a buffer of 10 mM Tris-HCl, 500 mM KCl pH 8.3 (20 °C). An initial denaturation at 94 °C for 1 min was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 20 s and extension at 72 °C for 30 s.

PCR products were then subjected to single-strand conformation polymorphism (SSCP) analysis. This is a technique that separates different PCR products according to both sequence and size, and is a relatively quick and cost-effective method for identifying different sequence variants that reduces the need for sequencing. For SSCP, the DNA is denatured and then plunged swiftly onto ice, with the result that each of the denatured single strands adopts a sequence-specific secondary structure through re-annealing only with itself. This structure determines the rate of migration on an acrylamide gel.

For SSCP analysis, 10 µl of products from the PCR reaction were precipitated using 100% isopropanol. DNA was resuspended in 12 µl of a formamide, bromophenol blue 1%, 0.5 M EDTA solution in the ratio 250:12.5:10, heated to 94 °C for 5 min then immediately plunged into ice before loading onto a non-denaturing, glycerol-containing polyacrylamide gel with 0.054 M Tris-borate, 0.0012 M EDTA buffer and run at 300 V for *ca.* 17 h. Gels were fixed (10 min in 50% methanol, 10% glacial acetic acid, 40% distilled water), washed twice (2–3 min in 10% ethanol, 0.5% glacial acetic acid) stained (10 min in 0.1% silver nitrate), developed (up to 10 min in 1.5% sodium hydroxide containing 400 µl formaldehyde and 200 µl sodium borohydride at 100 mg ml⁻¹) and neutralized (20 min in 0.75% sodium carbonate), according to a standard protocol using *ca.* 100 ml solution at each stage (Ainsworth *et al.* 1991).

PCR products to be sequenced were cleaned using Qiagen PCR product purification columns. Approximately 50 ng DNA in a volume of 10 µl were used for direct sequencing using a Sequenase v.2 PCR product manual sequencing kit (Amersham Laboratories, Buckinghamshire, UK), which uses ³⁵S as a radioactive label, or for automated sequencing using the Perkin-Elmer ABI (Warrington, UK) dRhodamine terminator cycle sequencing kit.

Haldane's test (Haldane 1939) was used to test for heterogeneity in the data as a whole. This test is suitable when the number of categories is large, but the expected values are small. Pairwise comparisons of the frequencies of haplotypes in different populations were made using Fisher's exact tests from the program GENEPOP (Raymond & Rousset 1995). This program uses a Markov chain method for estimating the chances of observing a data set more extreme than the input. A dememorization number (1000) was used in order to avoid bias. Fifty thousand iterations (divided into 50 batches in order to compute standard errors) were performed. Genetic distances between haplotypes were calculated according to the method of Tajima & Nei (1984). The software package ARLEQUIN (v.1.1 (Schneider *et al.* 1999)) was used to investigate genetic structure by partitioning variation within populations, between populations and between groups of populations.

(a) *Nucleotide sequence accession numbers*

Nucleotide sequences reported in this study have been assigned the GenBank accession numbers AF311866–AF311874.

3. RESULTS

One hundred and seventy-nine individuals of *P. taeniata* from six sites in Faatoai valley, ten in Urufara valley, three in Aareo valley, one in Vaitapi valley and one in Moruu valley were haplotyped. An attempt was made to extract DNA from many more specimens, but without success. Age and storage probably contributed to the low success rate at obtaining good-quality DNA (< 10%).

SSCP analysis identified two common variants (T6 and T10), and eight others at low frequencies (table 1). Haplotype T7 was the next most frequent after T6 and T10. It was found in populations 7 and 8 in Faatoai and populations 11 and 12 in northern Urufara. A diagrammatic representation of the proportions of each haplotype at each sampling site is given in figure 3. Haplotypes are classed as T6, T10 or other, the last category including haplotypes T5, T7, T8, T9, T11, T12, T13 and X. The approximate transition between the subspecies *P. t. nucleola* to *P. t. simulans* is shown

Figure 2. Location of *P. taeniata* sample sites in the north-west of Moorea. Each sample site is ca. 10 m × 10 m.

Table 1. The number of different *P. taeniata* haplotypes found in 179 individuals from a total of 21 sample sites (*n* denotes sample sizes for each site. Haplotypes are numbered T5–T13, with an additional haplotype ‘X’.)

sample site	T5	T6	T7	T8	T9	T10	T11	T12	T13	X	<i>n</i>
1	0	0	0	0	1	5	0	0	0	0	6
2	0	2	0	0	1	10	0	1	0	0	14
3	1	0	0	0	0	8	0	0	0	0	9
4	0	8	0	0	0	1	1	0	0	0	10
5	0	9	0	0	0	0	1	1	0	0	11
6	0	8	0	0	0	0	0	0	0	0	8
7	0	4	1	0	0	0	0	0	0	0	5
8	0	9	1	0	0	0	0	0	0	0	10
9	0	9	0	0	0	0	1	0	0	0	10
10	0	4	0	0	0	0	0	0	2	1	7
11	0	5	1	0	0	0	0	0	0	0	6
12	0	4	2	0	0	0	0	0	0	0	6
13	0	7	0	0	0	1	0	0	0	0	8
14	0	4	0	0	0	5	0	0	0	0	9
15	0	2	0	0	0	5	0	0	0	0	7
16	0	0	0	0	0	11	0	0	0	0	11
17	0	2	0	0	0	2	1	0	0	0	5
18	0	3	0	0	0	3	0	0	0	0	6
19	0	4	0	0	1	5	0	0	0	0	10
20	0	2	0	0	0	10	0	0	0	0	12
21	0	4	0	1	0	4	0	0	0	0	9
total	1	90	5	1	3	70	4	2	2	1	179

in figure 2. All haplotypes apart from type ‘X’, found only in one individual in Moruu valley, were sequenced.

SSCP analysis may not detect all sequence variants if some base changes do not sufficiently alter DNA

secondary structure. However, four individuals typed as having the same haplotype were sequenced and found to be identical for each of haplotypes T6, T7, T10 and T11. Two haplotypes differing only by a single C–T

Figure 3. Distribution of *P. taeniata* 16S haplotypes in the north-west of Moorea. Pie charts represent the location of the different sample sites. Haplotypes identified in each population are shown in different colours. Populations are numbered 1–21 for reference (see also table 1).

transversion were detected in this experiment. These observations suggest that the amount of undetected variation is small.

Considering all north-western samples together, Haldane's test for heterogeneity in the data was highly significant ($p < 0.001$, normal deviate 6.4, 180 d.f). Pair-wise comparisons using the exact test in GENETOP showed that populations 1, 2, 3, 15, 16 and 20 (group A) are significantly different from populations 4–13 (group B) at $p < 0.05$. Populations within each group are not significantly different from each other, nor are they significantly different from the remaining populations (14, 17, 18, 19 and 21), group C. The results confirm that the distribution of haplotypes is not random. Differences between populations in groups A, B and C, calculated using AMOVA in ARLEQUIN, accounted for *ca.* 55% of the total variation, with no significant differences between populations within each group.

The genetic distance between haplotypes T6 and T10 is 0.065. Distances between T6, T7, T8 and T9 range from 0.010 to 0.020. Distances between T10, T11, T12 and T13 range from 0.003 to 0.027. None of the haplotypes detected in these populations from the north-west of Moorea were found in 89 specimens taken from other parts of the island (Goodacre 2001). The overall level of variation found within *P. taeniata* haplotypes T5–T13 and a further 15 types from other valleys on the island is 0.084 (Goodacre 2001). Phylogenetic analysis using a distance-based neighbour-joining method of all 24 haplotypes shows that, with the exception of haplotype T5, *P. taeniata* haplotypes fall into two main groups. (These are supported by high bootstrap values and by other methods of tree construction such as

maximum parsimony.) One group includes both north-western haplotypes (T6, T7, T8 and T9) and other haplotypes found predominantly in western valleys on Moorea. The other group includes both north-western haplotypes (T10, T11, T12 and T13) and also haplotypes found predominantly in central, southern and eastern valleys on Moorea.

(a) ***The relationship between clines in shell shape, colour and banding, and that in molecular data***

The abrupt change in the frequencies of mitochondrial haplotypes is approximately concordant with a change in the frequencies of orange- and pink-shelled morphs from north-east to south-west in Urufara valley (B. Clarke and J. Murray, unpublished data). However, it is not coincident with many other clines in the frequencies of other banded shell morphs, such as *frenata* and *lyra*, that occur in Urufara valley (Crampton 1932; Clarke 1968). Nor is it coincident with changing frequencies of shell colour around an area with a high frequency of purple-shelled morphs (Clarke 1968). It does occur, however, close to the region where there is a change in morphology: a transition from the subspecies *P. t. nucleola* to *P. t. simulans* (Crampton 1932; Clarke & Murray 1969). Interestingly, no similar change in mitochondrial types is observed at transitions between other *P. taeniata* subspecies, such as between *P. t. simulans* and *P. t. elongata* in the south of Moorea (Goodacre 2001). Data from six polymorphic allozyme loci (in samples from all populations but the one in Vaitapi) show no clear changes in the frequency of alleles, or in heterozygosity along the cline in mitochondrial haplotypes (J. Stephens, unpublished data).

4. DISCUSSION

The geographical pattern of variation and the existence of very different mitochondrial haplotype frequencies can be explained in several ways, not all of which are mutually exclusive. The first is that the patterns reflect historical events (such as drift and selection in populations that became isolated from one another), rather than current evolutionary processes. The second is that the different mitochondrial haplotypes are influenced by selection, and that this is sufficient to maintain the clines. The third is that random genetic drift in an uninterrupted series of populations has been sufficient to prevent homogenization of gene frequencies.

Isolating events are potentially important means by which populations can become 'structured', since genetic drift or local selection can then allow differences to accumulate between them. In cases where the original range of an organism lies over an environmental cline, peripheral populations that have been prevented by gene flow from reaching their ecological optima can be released to evolve to an increased fitness by the imposition of a barrier (Garcia-Ramos & Kirkpatrick 1997). The barrier might be physical or ecological, or the result of habitat expansion and contraction. The latter is thought to be responsible for the geographical distribution of mitochondrial DNA haplotypes in salamanders (Templeton *et al.* 1995), green turtles (Bowen *et al.* 1992) and the land snails *Cepaea nemoralis* (Davison & Clarke 2000) and *Albinaria* spp. (Douris *et al.* 1998).

Isolation can also occur during the colonization process. The dispersal of snails is believed to be leptokurtic. Most individuals do not migrate very far, but there are occasional long-distance migrants, potentially assisted by birds or climatic events. Leptokurtic dispersal has been shown to increase genetic patchiness, since initial rates of inbreeding in demes formed by long-distance migrants are high (Ibrahim *et al.* 1996). Thereafter, when an area becomes densely populated, long-distance migrants no longer contribute substantially to genetic variation because they are small in number relative to the populations they invade. Variation between expanding populations, founded by a few long-distance migrants can be explained by drift alone, and need not necessarily involve selection (though founder events themselves may be selective). If there are repeated cycles of colonization and expansion followed by extinction, then the effects of drift may be magnified and complicated.

After secondary contact between hitherto isolated populations, the time taken for neutral alleles to disperse to form a cline of a known width (w) can be calculated using the equation $t = 0.35(w/\theta)^2$ (Endler 1977), where w is $1/\text{maximum gradient of the cline}$ and θ is the dispersal distance per generation. θ has previously been calculated for *P. taeniata* as 4 m per generation (Murray & Clarke 1984). An upper limit for the width of the cline in mitochondrial haplotypes is *ca.* 1.1 km (using the distance between populations 13 and 16 and a change from 100% to 14% T10 type to calculate w). This gives an upper estimate of t as 26 500 generations. Since the generation time of *P. taeniata* is thought to be about two years, this corresponds to *ca.* 53 000 years. This calculation shows

that clines, like that observed in mitochondrial haplotypes, can persist long after secondary contact. By this time the reasons for previous separation may no longer be apparent. If populations develop different adjustments between nuclear and mitochondrial genes while they are separated, selection can radically slow the process of homogenization. Thus the secondary contact could have occurred longer ago than the calculated upper limit.

The genetic difference between mitochondrial types T6 and T10 in *P. taeniata* (0.065) is large relative to the overall level of variation found within the species (0.084) (Goodacre 2001). This observation and the fact that another Moorean snail, *P. suturalis*, changes in morphology in the same region as the mitochondrial cline in *P. taeniata*, suggest a historical separation of populations either side of the cline (Clarke & Murray 1969). A large number of *P. taeniata* colour morphs appear in the transition zone (B. Clarke and J. Murray, unpublished data). Novel genotypes arising through hybridization between individuals from previously isolated populations is a possible explanation for this.

It is unlikely that random genetic drift in an uninterrupted series of populations could be responsible for the observed mitochondrial data, since particular morphs predominate over areas far greater than the panmictic unit (calculated to be between 400 and 200 m²). Previous experiments on *P. taeniata* have suggested that gene flow between populations is insufficient to prevent localized responses to environmental selection (Murray & Clarke 1984). However, selection without restricted gene flow between populations also appears to be an unlikely explanation for the mitochondrial data, since there is no evidence of any ecological gradient coincident with the cline in mitochondrial haplotypes in Urufara valley. Indeed, the T10 haplotype is found both on the Urufara ridge, where it is particularly wet, and in Aareo and Vaitapi valleys, where it is exceptionally dry. Similarly, the T6 haplotype is found both in areas of lowland forest in Faatoai and Urufara valleys, and in mountain scrubland on the Urufara ridge.

The argument that the mitochondrial cline is likely to have a significant historical component is compelling. Since the cline occurs in the region of a significant morphological change (from *P. t. nucleola* to *P. t. simulans*), this strongly suggests that there is a historical component to shell shape and size, despite the fact that these traits are also highly likely to be influenced by selection. Visual polymorphisms in land snails are commonly under selection exerted by bird predators (Cain & Sheppard 1954). Although many birds are now extinct on Moorea (Steadman 1989), it is likely that this has also been true for *Partula* in the recent evolutionary past.

A possible explanation for the many gradients in colour and banding patterns that do not coincide with the mitochondrial cline is that they are recent, having occurred more rapidly than changes in mitochondrial DNA as a result of selection, mediated by birds or other environmental factors. Theoretically, short-lived areas of high-frequency genotypes may also form by drift alone, when adjacent populations nearing equilibrium join to form patches that are larger than the panmictic unit (Endler 1977). However, even if this is true for *P. taeniata*, it is not expected to produce such sharp transitions as those seen in

colour and banding pattern, nor ones over such large areas relative to neighbourhood size. Selection remains a plausible explanation for the small areas of high-frequency colour morphs within the study area, although they too may have a historical component, such as has been found for *Cepaea nemoralis* shell colour 'area effects' in Britain (Cameron *et al.* 1980; Davison & Clarke 2000). Drift may contribute to establishing patches on an even smaller scale.

This work was carried out with the support of a Biotechnology and Biological Sciences Research Council studentship, and a research grant to Professor B. C. Clarke from the Natural Environment Research Council. I am very grateful to Professor Clarke for his help throughout the project, and for critically reading the manuscript. I would also like to thank Dr Angus Davison, Dr Chris Wade, Dr Diogo Thomaz, Professor Jim Murray and Dr Mike Johnson for helpful discussions and Mrs Vivien Frame and the University of Nottingham for technical support. I also thank Lorraine Southam for proofreading the manuscript.

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