

# Habitat-related genetic substructuring in a marine snail (*Littorina fabalis*) involving a tight link between an allozyme and a DNA locus

KERSTIN JOHANNESSEN<sup>1\*</sup> and NATALIA MIKHAILOVA<sup>2</sup>

<sup>1</sup>Göteborg University, Department of Marine Ecology, Tjärnö Marine Biological Laboratory, SE-452 96 Strömstad, Sweden

<sup>2</sup>Institute of Cytology RAS, St Petersburg 194064, Russia

Received 28 January 2003; accepted for publication 28 July 2003

Metapopulation structure and genetic differentiation among subpopulations will be tightly related to patterns and processes of local adaptation and microevolution. Understanding the mechanisms behind genetic substructuring will aid in the interpretation of species' ecological performances and strategies. The marine gastropod *Littorina fabalis* occurs in two size morphs – a small and a large – found in microhabitats of different wave exposure, but overlapping in distribution where wave exposure is intermediate. Earlier studies have found substantial genetic differentiation linked to morph in one allozyme locus (arginine kinase), while 29 other allozyme loci reveal no or minute differences between morphs. Here we add new results showing DNA variation in a RAPD marker being tightly linked to the allozyme variation. Indeed, 97% of the snails homozygotic for one of the *Ark* alleles had a unique DNA band, while 89% of the snails homozygotic for the other *Ark* allele lacked the marker. We discuss alternative hypotheses explaining the genetic substructure and suggest that the linkage of size, allozyme and DNA traits might be due to a paracentric chromosomal inversion involving loci coding for these traits. A genetic linkage of traits might promote microhabitat specialization of this species, and such a chromosomal transformation may therefore be adaptive. © 2004 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2004, 81, 301–306.

**ADDITIONAL KEYWORDS:** arginine kinase – differential selection – genetic linkage – microgeographical genetic variation – paracentric inversion – RAPD – size polymorphism.

## INTRODUCTION

Genetic substructuring, reflected in a patchy landscape of gene frequencies at one or more loci, will have important implications for the microevolution of a species and for the ecological performances and strategies adopted (Endler, 1977; Hedrick, 1986; Kassen, 2002). A patchy genetic landscape influences genetic variation within local populations, as well as differentiation and gene flow among populations. It might also affect local genetic adaptation and stochastic loss of genetic variation.

More than 30 years ago Ehrlich & Raven (1969) stressed that species are indeed commonly substructured and extensive evidence for this has since accu-

mulated from studies of allozyme and DNA variation on both large (e.g. Sundberg *et al.*, 1990; Shaklee *et al.*, 1991; Avise, 1992) and local geographical scales (e.g. Johannesson & Johannesson, 1989; Piertney & Carvalho, 1995; Schmidt & Rand, 1999).

Genetic substructuring at neutral loci might be a consequence of isolation by distance, and species with poor dispersal are the most likely candidates for this. Among species of marine invertebrates, benthic species that lack a larval stage and have stationary juvenile and adult stages are, as expected, often substructured over narrow distances, while species with a dispersive larva are much less so (e.g. Janson, 1987; Hellberg, 1996; Parsons, 1996). However, genetic substructuring of a species might also be a consequence of differential selection in different habitats or microhabitats (Hilbish & Koehn, 1985; Johannesson, Johannesson & Lundgren, 1995; Schmidt &

\*Corresponding author.

E-mail: Kerstin.Johannesson@tmbi.gu.se

Rand, 1999), biogeography (Parsons, 1996), founder effects (Knight, Hughes & Ward, 1987), or biased recruitment (Johnson & Black, 1984; Piertney & Carvalho, 1995).

*Littorina fabalis* (Turton), a marine intertidal gastropod, is strongly substructured over microgeographical scales at one of 30 allozyme loci (Tatarenkov & Johannesson, 1994, 1999). However, in this case it seems not to be a consequence of differential selection on the allozyme locus itself, or of any of the other mechanisms listed above. An alternative explanation is a chromosomal rearrangement, an inversion, locking several traits together in a supergene, and in this study we present support for this mechanism by showing evidence of a strong linkage between the differentiated allozyme locus and a random DNA locus (RAPD). We furthermore discuss possible consequences of a chromosomal inversion, for example, increase of fitness through gene complexes promoting microhabitat adaptation, and fitness reductions through loss of zygotes from hybrid parents.

*Littorina fabalis* is confined to littoral macroalgae in subarctic and temperate parts of the north-eastern Atlantic. It is a direct developer, and crawl-away miniature juveniles hatch from egg masses that are laid on algal fronds (Reid, 1996). Reimchen (1981) described British snails as being either dwarf (5–8 mm), with a yellow and regularly ornamented shell and present in wave-protected shore habitats, or large (11–14 mm), with a brown and irregularly ornamented shell and present in exposed habitats. The two morphs are even found in different microhabitats of the same shore. A similar association between snail size and microhabitat is found in Sweden but colour variation is not correlated with habitat (Ekendahl, 1994). Both shell colour and growth rate are under genetic control (Reimchen, 1979; Tatarenkov & Johannesson, 1998).

Of 30 allozyme loci, one, arginine kinase (*Ark*, E.C. code 2.7.3.3), shows strong habitat-related variation (Tatarenkov & Johannesson, 1994). Two alleles, *Ark*<sup>80</sup> and *Ark*<sup>100</sup>, are common in wave-exposed parts of shores, while *Ark*<sup>120</sup> dominates less exposed parts. Two rare alleles, *Ark*<sup>110</sup> and *Ark*<sup>130</sup> are also found in the protected parts. The genetic clines can be extremely sharp, and a switch from a clear dominance of exposed alleles ('E') to a dominance of sheltered alleles ('S') may occur over a few tens of metres of shore, or even less (Tatarenkov & Johannesson, 1994, 1999).

In subpopulations with both groups of alleles present, there is a marked deficiency in numbers of heterozygotic ('ES') snails (Tatarenkov & Johannesson, 1999), but this seems to be explained neither by selection against heterozygotes (Tatarenkov & Johannesson, 1998) nor by assortative mating of genotypes (Tatarenkov & Johannesson, 1999). EE and SS

homozygotes from the same sites have slightly different allele frequencies in two of three other polymorphic allozyme loci (Tatarenkov & Johannesson, 1998). Thus, gene flow between the two morphs is impeded to some extent, and the question is, by what mechanism?

Growth rate and *Ark* genotype are strongly correlated (Tatarenkov & Johannesson, 1998) and a mechanism that might explain this is that these traits may be trapped in the same chromosomal inversion. Such an inversion would prevent crossing-over and all loci within the inversion would be tightly linked (Ayala & Kiger, 1980). If a chromosomal inversion is present it might involve a number of loci, and by screening random molecular loci it might be possible to find additional markers linked to the size-allozyme genotypes.

Screening 19 randomly amplified DNA loci (RAPD), we found one (OPH-11) that in a small sample (two subpopulations,  $n = 3$  in each) suggested different frequencies of a band between the two morphs of *L. fabalis* (Mikhailova & Johannesson, 1998). Here we have extended the analysis considerably and have also examined the possibility of genetic linkage between the RAPD marker and the *Ark* locus.

## MATERIAL AND METHODS

In 1997 we collected individuals of *L. fabalis* from three shores on two different islands (Jutholmen, Lökholmen-north and Lökholmen-south) on the Swedish west coast. On each shore we took three samples at sites of intermediate exposure over an exposure gradient from slightly more wave-exposed to slightly less wave-exposed. In this way we were likely to include samples with different distributions of the exposed and sheltered *Ark* alleles.

We used 109 males and 47 mature females and dissected all of them to confirm the soft part characters that discriminated them from *L. obtusata* (see Reid, 1990). We discarded snails that were infected by digenean trematodes, in order to eliminate the risk of contamination in the PCR-based RAPD analysis. Tissue from each snail was used both for extraction of DNA for RAPD analysis and for allozyme analysis. The tissue was either used fresh or stored at  $-80^{\circ}\text{C}$  for up to 1 year before analysis.

Both a modified phenol–chloroform extraction (Mikhailova & Johannesson, 1998) and the CTAB (hexadecyltrimethyl ammonium bromide) extraction (Winnepennincks, Backeljau & De Wachter, 1993) methods were used to extract high molecular weight nuclear DNA from the snails. The extracted DNA was stored either at  $-20^{\circ}\text{C}$  (weeks), or at  $-70^{\circ}\text{C}$  (months). We used an arbitrary 10-mer primer OPH-11 (CTT CCG CAG T) (Operon Tech. Inc.) to amplify the DNA of each snail, following the methods described in Mikhailova & Johannesson (1998). We

used only the OPH-11 primer, as the earlier study had shown an amplified fragment to be present in most snails of exposed habitats while being absent in snails of sheltered habitats. The amplified fragment had a size of 282 bp. (In Mikhailova & Johannesson, 1998, we suggested the fragment length to be about 240 bp, but later cloning and sequencing of the fragment has revealed the exact length to be slightly larger. The fragment sequence is indicated in the Appendix.)

The arginine kinase (*Ark*) genotype was scored in the same snails as those scored for RAPD marker using horizontal starch gel electrophoresis. The buffers and staining techniques used were those described by Tatarenkov & Johannesson (1994). No information about the presence or absence of the DNA marker was made available at time of scoring of the *Ark* genotype.

## RESULTS

All individuals scored for the DNA marker were also successfully scored for the allozyme genotype, and we found strong evidence of a genetic coupling between the RAPD and the allozyme genotypes. That is, of 103 individuals with a RAPD band at the 282-bp position of the OPH-11 primer, 100 (97%) were homozygotic for the E-allele of *Ark*, while of 35 individuals homozygotic for the S-allele, 31 (89%) had no RAPD band at the 282-bp position. Among the 15 *Ark* heterozygotes, 11 had a band and four lacked a band. This distribution did not differ significantly from an expected 50 : 50 distribution ( $\chi^2 = 3.27$ , d.f. = 1,  $P > 0.05$ ); however, the small sample size caused low statistical power, and the  $\chi^2$  value was close to the significant value of 3.84. Despite small sample sizes of individual subpopulations, significant genetic linkages between the *Ark* genotype and the DNA marker were evident in most sites, and on all shores, for pooled samples (Table 1).

## DISCUSSION

The RAPD locus was strongly linked to the *Ark* locus, and thus we have evidence of two molecular loci being linked to each other and to one or several loci influencing growth and adult size. Furthermore, the variation in all three characters (size, *Ark* and RAPD) correlates with snail microhabitat (Tatarenkov & Johannesson, 1999; this study), but the variation in these characters cannot be explained solely by differential selection over habitats. The reason is that the linkage would disappear in populations of intermediate habitats owing to recombination when the two morphs cross-bred, but this is not the case as indeed we found that the linkage persisted completely over the intermediate zones, suggesting an additional mechanism besides habitat selection.

A competing hypothesis is that the multilocus linkage is due to separate evolutionary histories of the two snail morphs. If separate lineages recently came into secondary contact and if gene flow between them was hampered, differentiation at gene loci would be expected. However, it is unlikely that strong differentiation has evolved at two molecular loci, while a large number of loci (29) remain invariant (Tatarenkov & Johannesson, 1994). Furthermore, the genetic structure of the other polymorphic allozyme loci suggests genetic coherence of populations of both morphs over geographical areas (Sweden, Wales, France) rather than a primary separation over habitats (Tatarenkov & Johannesson, 1999).

A paracentric chromosomal inversion including the linked loci would be a possible explanation of the observed genetic linkage and the habitat-related variation of the three loci. A chromosomal inversion usually suppresses crossing-over among the loci of the inversion. The reason is that in the individuals that are heterozygous for the inversion only those gametes that contain the non-crossover chromosome are intact and produce viable progeny (Ayala & Kiger, 1980).

Initially we used 19 RAPD primers to study intraspecific variation in *L. fabalis*, of which 15 revealed one or several polymorphic bands, but only one (the OPH-11 fragment) differed between morphs (Mikhailova & Johannesson, 1998). Snails of the genus *Littorina* have 17 pairs of chromosomes (Janson, 1983; Rolán-Alvarez, Buno & Gosálvez, 1996), and thus only one or perhaps two of the polymorphic RAPD markers are likely to be situated on any particular chromosome. If the inversion involves a large part of the chromosome, there is a reasonably high chance that one of 15 markers is from inside the inversion; one or more loci affecting growth and adult size being included in the inversion supports the hypothesis of a relatively large part of the chromosome being affected.

Chromosome inversions are frequently reported in various species (e.g. Dowler, 1989; Taylor, 1990; Díez & Santos, 1993). In species of *Drosophila*, distributions of inversions often seem to be related to geographical gradients and thus are probably a result of selection (Rodríguez *et al.*, 2000). In the congeneric species *L. saxatilis*, two allozyme loci are tightly linked, possibly due to a chromosomal inversion (Janson & Ward, 1984).

A chromosomal inversion in *L. fabalis* in which the inverted and the non-inverted genotypes are linked to different microhabitats (sheltered and exposed) must involve at least one or a few loci that are under differential selection. The selected loci would then tether all polymorphic (even neutral) loci of the inversion to a certain microhabitat. Preliminary results indicate

**Table 1.** Results from samples of *Littorina fabalis* from three different sites along each of three shores on two different islands, indicating for each *Ark* genotype the number of snails with or without the DNA RAPD marker band

Island and shore	Site	<i>Ark</i> genotype	Number with DNA marker band†		Significance	
			Yes	No	Per site	Per shore
Jutholmen	J1	EE	18	1	$\chi^2 = 0.055$ d.f. = 1	
		SE	1	0		
		SS	0	0		
	J2	EE	18	1	$\chi^2 = 14.7^{**}$ d.f. = 2	
		SE	6	0		
		SS	1	3		
	J3	EE	9	0	$\chi^2 = 14.4^{***}$ d.f. = 2	
		SE	1	1		
		SS	1	8		
Lökholmen North	LN1	EE	11	0	$\chi^2 = 8.6^*$ d.f. = 1	$\chi^2 = 44.7^{***}$
		SE	0	0		
		SS	1	2		
	LN2	EE	8	0	$\chi^2 = 11.0^*$ d.f. = 2	
		SE	1	0		
		SS	0	2		
	LN3	EE	7	0	$\chi^2 = 15.0^{***}$ d.f. = 2	
		SE	1	0		
		SS	0	7		
Lökholmen South	LS1	EE	11	1	$\chi^2 = 6.5$ d.f. = 2	$\chi^2 = 35.4^{***}$
		SE	1	0		
		SS	0	1		
	LS2	EE	14	0	$\chi^2 = 15.0$ d.f. = 1	
		SE	0	1		
		SS	0	0		
	LS3	EE	4	0	$\chi^2 = 11.0^{**}$ d.f. = 2	
		SE	0	2		
		SS	1	8		

Significances were tested using Monte-Carlo simulations (Zaykin & Pudovkin, 1993) when d.f. = 2 and Fisher's exact test when d.f. = 1.

†RAPD marker OPH-11, 282-bp band. E = exposed allele; S = sheltered allele.

\* $(0.01 < P < 0.05)$ ; \*\* $(0.001 < P < 0.01)$ ; \*\*\* $(P < 0.001)$ .

that size may be involved, as it affects fitness differently in sheltered and exposed environments. Thus the inversion in *L. fabalis* might have profound effects on the genetic substructuring of the species, while promoting accumulation of alleles into the inversion that increase the overall fitness in one of the habitats. In this way it is expected that gene complexes will build up where favourably interacting genes are kept unrecombined. Obviously, building gene complexes might promote microhabitat specialization in the exposed and sheltered habitats. On the other hand, heterozygotes for the inversion will lose gametes, as all cross-over chromosomes will be unviable. Indeed if heterozygotes are less fecund than are homozygotes, this might act as a weak barrier to gene flow among morphs and

such a small partial reproductive barrier is indeed observed as a small but significant differentiation in two other allozyme loci (*Pgi* and *Pgm-2*) (Tatarenkov & Johannesson, 1998).

One observation is not, however, readily explained and that is the deficiency of heterozygotes in mixed populations. (Only about half the number of heterozygotes expected from a population in Hardy-Weinberg equilibrium are observed in mixed populations, despite the fact that the two morphs mate at random, Tatarenkov & Johannesson, 1999). The loss of fecundity of the heterozygotes in itself does not predict fewer heterozygotes (F1) being produced. There is, however, a possibility that a reproductive barrier is favoured by reinforcement selection if the heterozy-

gotes are less fertile. Alternatively, the observed deficiency of heterozygotes could be a consequence of a post-zygotic barrier. Dispersal into the hybrid zone is a third possibility, although this explanation is poorly supported by dispersal data. The width of the studied hybrid zones is in the range of 20–30 m while mean dispersal distances over more than a month were in the range of 1–2 m in all habitats (Tatarenkov & Johannesson, 1998).

Overall, the chromosomal inversion hypothesis explains well observations that we failed to predict by earlier suggestions of habitat-related selection in *Ark* (Tatarenkov & Johannesson, 1994) or the presence of sibling species (Tatarenkov & Johannesson, 1998). Further tests of the predictions of the inversion hypothesis are, however, necessary to confirm its existence.

#### ACKNOWLEDGEMENTS

This work was supported by grants from the Royal Swedish Academy of Sciences and the Swedish Science Research Council.

#### REFERENCES

- Avise JC. 1992.** Molecular population structure and the biogeographic history of a regional fauna: a case history with lessons for conservation biology. *Oikos* **63**: 62–76.
- Ayala FJ, Kiger JA. 1980.** *Modern genetics*. Menlo Park, CA: Benjamin/Cummings.
- Díez M, Santos JL. 1993.** Synapsis in a paracentric inversion heterozygote of *Chorthippus jacobsi* (grasshopper). *Heredity* **70**: 231–236.
- Dowler RC. 1989.** Cytogenetic studies in three chromosomal races of pocket gophers (*Geomys bursarius* complex) at hybrid zones. *Journal of Mammalogy* **70**: 253–266.
- Ehrlich PR, Raven PH. 1969.** Differentiation of populations. *Science* **165**: 1228–1232.
- Ekendahl A. 1994.** Factors important to the distribution of colour morphs of *Littorina mariae* Sacchi & Rastelli in a non-tidal area. *Ophelia* **40**: 1–12.
- Ender JA. 1977.** *Geographic variation, speciation, and clines*. Princeton: Princeton University Press.
- Hedrick PW. 1986.** Genetic polymorphism in heterogeneous environments: a decade later. *Annual Review of Ecology and Systematics* **17**: 535–566.
- Hellberg ME. 1996.** Dependence of gene flow on geographic distance in two solitary corals with different larval dispersal capabilities. *Evolution* **50**: 1167–1175.
- Hilbish TJ, Koehn RK. 1985.** The physiological basis of natural selection at the Lap locus. *Evolution* **39**: 1302–1317.
- Janson K. 1983.** Chromosome number in two phenotypically distinct populations of *Littorina saxatilis* Olivi and in specimens of the *Littorina obtusata* (L.) species-complex. *Journal of Molluscan Studies* **49**: 224–227.
- Janson K. 1987.** Allozyme and shell variation in two marine snails (*Littorina*, Prosobranchia) with different dispersal abilities. *Biological Journal of the Linnean Society* **30**: 245–256.
- Janson K, Ward RD. 1984.** Microgeographic variation in allozyme and shell characters in *Littorina saxatilis* Olivi (Prosobranchia: Littorinidae). *Biological Journal of the Linnean Society* **22**: 289–307.
- Johannesson K, Johannesson B. 1989.** Differences in allele frequencies of *Aat* between high- and mid-rocky shore populations of *Littorina saxatilis* (Olivi) suggest selection in this enzyme locus. *Genetic Research* **54**: 7–11.
- Johannesson K, Johannesson B, Lundgren U. 1995.** Strong natural selection causes microscale allozyme variation in a marine snail. *Proceedings of the National Academy of Sciences, USA* **92**: 2602–2606.
- Johnson MS, Black R. 1984.** Pattern beneath the chaos: the effect of recruitment on genetic patchiness in an intertidal limpet. *Evolution* **38**: 1371–1383.
- Kassen R. 2002.** The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology* **15**: 173–190.
- Knight AJ, Hughes RN, Ward RD. 1987.** A striking example of the founder effect in the mollusc *Littorina saxatilis*. *Biological Journal of the Linnean Society* **32**: 417–426.
- Mikhailova N, Johannesson K. 1998.** A comparison of different protocols for RAPD analysis of *Littorina*. *Hydrobiologia* **378**: 33–42.
- Parsons KE. 1996.** The genetic effects of larval dispersal depend on spatial scale and habitat characteristics. *Marine Biology* **126**: 403–414.
- Piertney SB, Carvalho GR. 1995.** Microgeographic genetic differentiation in the intertidal isopod *Jaera albifrons* Leach. 2. Temporal variation in allele frequencies. *Journal of Experimental Marine Biology and Ecology* **188**: 277–288.
- Reid DG. 1990.** Note on the discrimination of females of *Littorina mariae* Sacchi & Rastelli and *L. obtusata* (Linnaeus). *Journal of Molluscan Studies* **56**: 113–114.
- Reid DG. 1996.** *Systematics and evolution of Littorina*. London: The Ray Society.
- Reimchen TE. 1979.** Substratum heterogeneity, crypsis, and colour polymorphism in an intertidal snail (*Littorina mariae*). *Canadian Journal of Zoology* **57**: 1070–1085.
- Reimchen TE. 1981.** Microgeographical variation in *Littorina mariae* Sacchi & Rastelli and a taxonomic consideration. *Journal of Conchology* **30**: 341–350.
- Rodriguez CR, Piccalini E, Levy E, Hasson E. 2000.** Contrasting population genetic structures using allozymes and the inversion polymorphism in *Drosophila buzzatii*. *Journal of Evolutionary Biology* **13**: 976–984.
- Rolán-Alvarez E, Buno I, Gosalvez J. 1996.** Sex is determined by sex chromosomes in *Littorina saxatilis* (Olivi) (Gastropoda, Prosobranchia). *Hereditas* **124**: 261–267.
- Sambrook J, Fritsch EF, Maniatis T. 1989.** *Molecular cloning: a laboratory manual*. New York: Cold Spring Harbor Laboratory Press.
- Schmidt PS, Rand DM. 1999.** Intertidal microhabitat and selection at *Mpi*: interlocus contrasts in the northern

- acorn barnacle, *Semibalanus balanoides*. *Evolution* **53**: 135–146.
- Shaklee JB, Klaybor DC, Young S, White BA. 1991.** Genetic stock structure of odd-year pink salmon, *Oncorhynchus gorbuscha* (Walbaum), from Washington and British Columbia and potential mixed-stock fisheries applications. *Journal of Fish Biology* **39A**: 21–34.
- Sundberg P, Knight AJ, Ward RD, Johannesson K. 1990.** Estimating the phylogeny in mollusc *Littorina saxatilis* (Oliv) from enzyme data: methodological considerations. *Hydrobiologia* **193**: 29–40.
- Tatarenkov A, Johannesson K. 1994.** Habitat related allozyme variation on a microgeographical scale in the marine snail *Littorina mariae* (Prosobranchia: Littorinacea). *Biological Journal of the Linnean Society* **53**: 105–125.
- Tatarenkov A, Johannesson K. 1998.** Evidences of a reproductive barrier between two morphs of the marine snail *Littorina fabalis* (Gastropoda). *Biological Journal of the Linnean Society* **63**: 349–365.
- Tatarenkov A, Johannesson K. 1999.** Micro- and macrogeographical allozyme variation in *Littorina fabalis*; do sheltered and exposed forms hybridize? *Biological Journal of the Linnean Society* **67**: 199–212.
- Taylor DB. 1990.** Genetics of interspecific hybridization in the triseriatus and zoosopus groups of *Aedes* (Protomacleana) (Diptera: Culicidae). *Annals of the Entomological Society of America* **83**: 1181–1191.
- Winnepennincks B, Backeljau T, De Wachter R. 1993.** Extraction of high molecular weight DNA from molluscs. *Trends in Genetics* **9**: 407.
- Zaykin DV, Pudovkin AI. 1993.** Two programs to estimate significance of  $\chi^2$ -values using pseudo-probability tests. *Journal of Heredity* **84**: 152.

## APPENDIX

### SEQUENCE OF THE 282 BP DNA FRAGMENT POLYMORPHIC IN *LITTORINA FABALIS*

Cloning of the fragment was achieved by purifying a piece of agarose gel with the band present using Sephaglas bandpreparation kit (Pharmacia Biotech, Sweden) and precipitating with ethanol. The purified fragment was ligated into a PCR 2.1 linearized vector and transformed into One Shot competent cells (TA Cloning Kit, Invitrogen). The recombinant clones were detected as white colonies in LB plates containing ampicillin, X-gal and IPTG (Sambrook, Fritsch & Maniatis, 1989). Plasmid DNA was isolated by the alkaline lysis method and purified by centrifugation in a CsCl-ethidium bromide gradient containing 0.5%

sarcosyl at 30 000 g for 24 h at room temperature. Ethidium bromide was removed by multiple isoamil alcohol extraction. DNA was precipitated by ethanol, air-dried and redissolved in sterile water. Recombinant plasmids were used as templates for sequencing by the dideoxynucleotide chain termination method. Sequencing was performed by the company Cybergene, Stockholm, Sweden.

```
CTTCCGCAGTCAGGCGGGGAATGGTAGACCAGACCGTCTGA
GAAAATCAGATCTAAAACGCAGGAAGTTATAAAATAGAAG-
TAACATTTACAGAATAAAACAGAAAATCAGGTCT-
AAAAACGCAGGAAGTTATAAAATAGAAGTAACATTTACAGA
ATAAAACACTGAGAGAACTTGAAAAAGTAAGGTCT-
TAAAAAAAAGGTGCGGGTGTGTCTGAAAATGGTGTGTCT-
TAATAGGGGGACTCCACTGTACAGAGTACTGAGGATCGAA
CTGCGGAAG
```