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THE INHERITANCE OF DIVERGENT MITOCHONDRIA IN THE LAND SNAIL, *CEPAEA NEMORALIS*

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ABSTRACT

The mitochondrial genomes of *Cepaea nemoralis* can differ widely between individuals in the same population. Various hypothesis have been proposed to account for this diversity, including unusually fast evolution and the retention of deep lineages in subdivided populations. Another possibility is that pulmonate mitochondria are inherited in the doubly uniparental mode, as in *Mytilus*, allowing separate maternal and paternal lineages to coexist. In *Mytilus*, separate lineages may differ by as much as 20% and may pre-date the origin of the species carrying them.

Until now, mitochondrial inheritance has not been studied in any molluscan group except the bivalves. I have investigated it in *C. nemoralis* through a series of matings, and assayed individuals for evidence of heteroplasmy. In five matings, mitochondrial inheritance was maternal, and no heteroplasmic individuals were detected. The maintenance of the divergent haplotypes can not be explained by doubly uniparental inheritance.

INTRODUCTION

Individuals of the land snail *Cepaea nemoralis* (L.) can contain extremely different mitochondrial genomes (Thomaz, Guiller & Clarke, 1996). At least two other pulmonate gastropods, *Helix* and *Albinaria*, appear also to have high levels of mitochondrial divergence within species and between congeners (Thomaz *et al* 1996; Douris, Cameron, Rodakis & Lecanidou, 1998). Various hypotheses have been proposed to account for this diversity. For example, mitochondrial evolution in the pulmonates could be exceptionally fast, or deep lineages could be retained because the snails have an exceptionally divided (stepping-stone) structure of populations (Slatkin, 1991). Alternatively, selection might have acted to maintain the diversity: perhaps through co-adaptation between nuclear and mitochondrial genotypes (Thomaz *et al*., 1996). Another possibility is that pulmonate mitochondria are not inherited uniparentally by conventional maternal transmission, but in the doubly uniparental mode, as found in the bivalve families Mytilidae and Unionidae (Skibinski, Gallagher & Beynon, 1994).

In the *Mytilus edulis* species-complex, female mussels usually inherit their mtDNA solely from their mothers, while males usually inherit mtDNA from both parents (Skibinski, Gallagher & Beynon, 1994; Hoeh, Stewart, Sutherland & Zouros, 1996). This doubly uniparental inheritance results in separate maternal and paternal mtDNA lineages, which may diverge from each other by as much as 20%. The coalescence of the lineages may pre-date the origin of the species carrying them. Although doubly uniparental inheritance should be easy to detect, mitochondrial inheritance has not been studied in any molluscan group apart from the bivalves. I have now investigated mitochondrial inheritance in *Cepaea nemoralis*, which is a hermaphrodite, by a series of reciprocal matings. If doubly uniparental inheritance operates, at least some individuals in some populations should be heteroplasmic for their mitochondria. Individuals of *C. nemoralis* from populations on the Marlborough Downs, Wiltshire, which contain both of the common British mitochondrial haplotypes were tested for evidence of heteroplasmy.

METHODS

The culture of snails

Juvenile (virgin) *C. nemoralis* were collected from various sites in Britain and grown to maturity in individual perspex containers (17 × 11 × 5 cm). Each container was covered with breathable cellophane film (Payne Scientific, Berkshire, UK) and moist tissue paper placed inside to maintain a high humidity.

Snails were cleaned out twice weekly and fed on each occasion with a diet of rehydrated snail food. The food was made by grinding the following ingredients to a rough powder: chalk (3 parts; 1 part = 15 ml), grass pellets (3 parts), trout pellets (1.5 parts), calcium diorthophosphate (1 part), porridge oats (3 parts), Stress (0.25 parts; Stress Pet Care for dogs) and one tablet of vitamin E (20 mg). When mature the adult snails, recognised as such by the development of a pigmented upturned lip, were transferred in pairs to large fish tanks (34 × 24 × 20 cm) containing John Innes No. 1 compost to a depth of approximately 7 cm. A high humidity was maintained by placing a large wet sponge in the tank.

Many populations of *Cepaea* in Britain have a high frequency of either the A or N mitochondrial haplotype (Thomaz *et al*., 1996), so matings were selected to maximise the probability of an informative cross between the haplotypes. Pairs of snails were observed twice daily (morning and evening) for signs of courtship and mating (presence of love darts). Usually mating occurred within a few days of transferring the snails to the large tank. Once mated, snails were separated into individual tanks, and observed daily for signs of egg laying, when the snails dig into the soil. Eggs were retrieved with a spatula and transferred to a plastic petri dish lined with damp filter paper. The petri dish was checked daily for hatching, and more water added if the filter paper dried out. Upon hatching, the snails were transferred to a perspex container and reared until they were large enough for the colour and banding patterns to be scored.

Although *C. nemoralis* is hermaphrodite, most of the initial matings were unidirectional. Therefore when a snail failed to produce a batch of eggs within about a week of mating, it was paired again with the same partner and the process repeated. Some snails produced several batches of eggs over the course of the experiment; all of them were collected. Adults and juveniles were stored for genetic analysis by freezing at −20°C.

DNA extraction

DNA was extracted from the snails using Nucleon Phytopure kits (Nucleon Biosciences). A sliver of foot tissue was cut from the snails using a sterile scalpel.

PCR and SSCP analysis

Primers for PCR were designed based upon an alignment of *C. nemoralis* and *C. hortensis* mitochondrial 16S rRNA sequences (Thomaz, Guiller & Clarke, 1996). These primers, 16S-1a (5′-GACGAGAAGACCCTAGAAGC-3′) and 16S-2a (5′-CCTAGTCCAACATCGAGGTACAC-3′) amplify a small, approximately 150 bp, fragment in which the variation is easily assayed by SSCP analysis (see below). PCR was carried out under standard conditions, including 0.4 U Thermoprime^{plus} polymerase and, 4.5 mM magnesium chloride in a 50 μl final volume. The cycling parameters were 96°C for 1 min, followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec.

The haplotype of each individual was examined by electrophoresis through an SSCP (single strand conformation polymorphism) polyacrylamide gel (Orita, Iwahana, Kanazawa, Hayashi & Sekiya, 1989). For this procedure, each 16S rRNA PCR product was precipitated with isopropanol, resuspended in a formamide/bromophenol blue loading buffer, and denatured at 98°C for 2 minutes. It was then placed on ice to minimise DNA reannealing. The PCR products were loaded on to a 0.5X MDE (FMC Bioproducts) vertical gel (20 × 20 cm) alongside suitable control haplotypes of known sequence and electrophoresed at 6°C for 15 hours at 180V. The gels were silver stained in five steps which involved: fixing (50% methanol/10% acetic acid, 10 minutes), two washes (10% ethanol, 0.5% acetic acid, 3 minutes each), soaking in silver nitrate solution (0.1% w/v, 10 minutes), and development (1.5% sodium hydroxide, 0.4% w/v formaldehyde, 0.2% w/v sodium borohydride), until bands were visible. Gels were neutralized in 0.75% sodium carbonate and dessicated in a gel drier at 80°C for 2 hours.

RESULTS

Mitochondrial Inheritance

Six matings were successful. Approximately 14 offspring from each were typed by SSCP analysis of their mitochondrial DNA. Five of the matings were between snails with different mitochondrial haplotypes (A or N, see Thomaz *et al*., 1996), as judged by SSCP (Figure 1; Table 1). Young snails were raised from reciprocal crosses in three instances (matings 4, 5, and 6 in Table 1; Figure 1b. The results of the crosses are shown in Table 1. In all five informative matings, the mitochondrial haplotype was inherited maternally, through the snail that produced the eggs. This included offspring from reciprocal crosses, and different batches of eggs from the same parent. The adult snails were confirmed as the parents by an analysis with informative microsatellite loci (Davison 1999).

SSCP analysis of polymorphic populations from the Marlborough Downs

Many populations on the Marlborough Downs, Wiltshire have roughly equal proportions of the A and N haplotypes (Thomaz *et al*., 1996). As part of a larger survey of mitochondrial variation in Britain, 459 snails from 17 mixed populations on the Marlborough Downs were typed by SSCP to identify heteroplasmic individuals. No heteroplasmic individual were observed. These findings concur with the results of Thomaz *et al*., (1996) who found no secure case of heteroplasmy in a survey of *Cepaea* mitochondrial

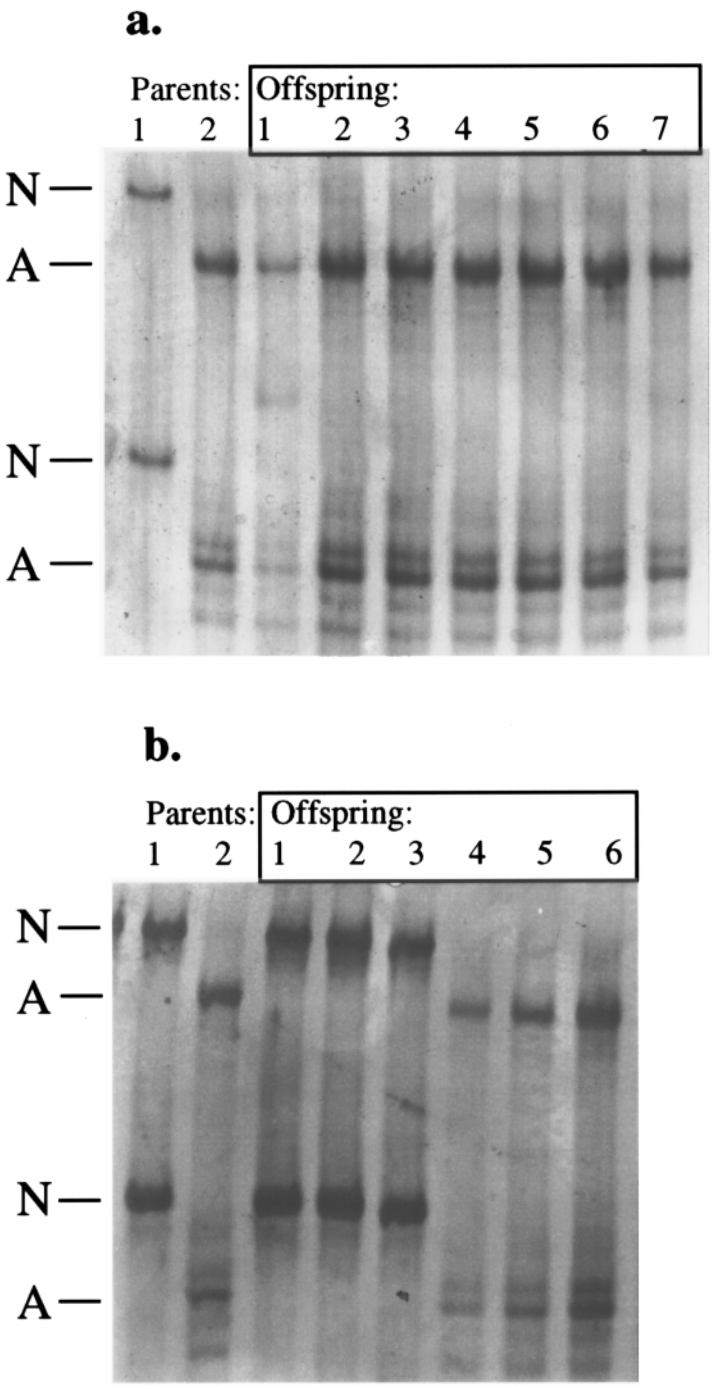

Figure 1. Two crosses of *Cepaea nemoralis* showing the maternal inheritance of mitochondrial DNA. The marked bands correspond to the denatured single-stranded PCR products of haplotypes A and N. In (a) all seven offspring came from eggs laid by parent 2 (mating 1 in Table 1). In (b) the first three offspring came from eggs laid by parent 1, the last three from eggs laid by parent 2 (mating 5 in Table 1).

Table 1. The mitochondrial haplotypes of parents and offspring in experimental crosses of *Cepaea nemoralis*.

Mating	Parental haplotypes		Offspring haplotypes:			
	Parent 1	Parent 2	Parent 1 mother		Parent 2 mother[1]	
			Haplotype	Number	Haplotype	Number
1	N	A	–	–	A	14
2	A	A	A	10	–	–
3	A	N	–	–	N	7
					N	7
4	A	N	A	3	N	4
					N	7
5	N	A	N	7	A	7
6	A	N	A	7	N	7

[1]Two consecutive lines from the same mating indicates that two batches of eggs were collected and typed.

variation in populations across Europe, albeit with much fewer snails from each population.

DISCUSSION

Until recently, clonal maternal mitochondrial inheritance has been assumed to be the rule among higher animals. However there is now indirect evidence of occasional recombination between paternal and maternal mitochondria (Eyre-Walker, Smith & Maynard Smith, 1999; Hagelberg, Goldman, Lio, Whelan, Schiefenhovel, Clegg & Bowden, 1999). A leaky transmission of paternal mitochondria (Birky, 1995), is possible because paternal mitochondria can survive for some hours in the eggs of mammals (Ankel-Simons & Cummins, 1996).

The molluscan bivalve groups Mytilidae and Unionidae are exceptional because their mitochondrial inheritance is doubly uniparental, involving divergent (10 to 20%) male and female lineages. Cytoplasmic DNA conflict is avoided because the lineages are spatially separated early in development (Hurst & Hamilton, 1992; Garrido-Ramos, Stewart, Sutherland & Zouros, 1998). A model for the maintenance of the two lineages, and sex inheritance in mussels has recently been proposed by Saavedra, Reyero & Zouros (1997). They suggest that a factor associated with the sperm mitochondria is required for development of a male gonad, and replication of the male mitochondrial DNA is controlled by a paternally coded mitochondrial factor. In mussels, rare hermaphrodites result when, during early rounds of germ cell division, a few germ cells lacking male mitochondria also produce a female gonad.

At first sight this model is not inconsistent with the presence of separate, divergent mitochondrial haplotypes in land snails. However, in five matings (70 offspring) of virgin *Cepaea nemoralis* from Britain, mitochondrial inheritance was always maternal, from the snail that produced the eggs. Moreover, no heteroplasmic individuals were detected in a survey of more than 450 snails from sites where the two most common British haplotypes are found in sympatry.

I have not specifically investigated whether there are differences in the distribution of mitochondrial types between somatic and gametic tissues, but since the DNA extractions were carried out on tissue from the foot and the SSCP analysis assayed gametic transmission, the results suggest that maternal inheritance in *Cepaea* occurs regardless of tissue type. Low levels of paternal inheritance can not be excluded. Therefore, the maintenance of the two divergent haplotypes in Britain and of the greater variation in Europe (Thomaz *et al.*, 1996), can not be explained by a present regime of doubly uniparental inheritance. It is not possible to discount the possibility that in the past there was once a 'ferminization' of paternal lineages as may have occured in *Mytilus* (Garrido-Ramos, Stewart, Sutherland, & Zouros, 1998). Alternative hypotheses, such as fast mitochondrial evolution or retention of deep lineages in structured populations are possible, but they are not supported by any evidence.

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