

Double-strand conformation polymorphism (DSCP) analysis of the mitochondrial control region generates highly variable markers for population studies in a social insect

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Abstract

Genetic markers were obtained for the termite *Nasutitermes corniger* by DSCP (double-strand conformation polymorphism) analysis of PCR-amplified mitochondrial control region DNA. This procedure revealed twenty-one haplotypes in forty-four colonies, whereas a restriction fragment length polymorphism analysis detected only nine haplotypes. Sequence analysis of DSCP fragments of contrasting mobilities suggests that the electrophoretic haplotypes are caused by DNA curvature in this highly AT-rich region. DSCP markers showed that some termite colonies contained maternally unrelated queens, each of which produced worker offspring. This pattern is consistent with nest founding by unrelated queens. Due to the availability of conserved primers for the mtDNA control region, DSCP analysis may readily reveal comparatively high levels of variation in a wide variety of organisms.

Keywords: double-strand conformation polymorphism (DSCP), mtDNA, DNA markers, DNA curvature, termites.

Introduction

A large number of genetic techniques have recently been developed for use in population biology (e.g. Hadrys *et al.*, 1992; Lessa & Applebaum, 1993; Queller *et al.*, 1993; Avise, 1994). A comparatively new technique, double-strand conformation polymorphism (DSCP) analysis (Saad *et al.*, 1994), is easily implemented and generates useful markers. Double-strand

conformation polymorphism is detected when mutations alter the curvature of the helical axis of double-stranded DNA molecules, resulting in changes in their electrophoretic mobility in nondenaturing acrylamide gels (reviewed in Hagerman, 1990). We applied DSCP analysis to PCR products from the mitochondrial DNA (mtDNA) control region (also called the A+T-rich region in invertebrates and the D-loop in vertebrates) and the 12s rRNA gene to uncover highly variable markers. Because DSCP analysis requires only basic laboratory equipment and can employ PCR products amplified with conserved primers, DSCP analysis has the additional advantage of fast and inexpensive implementation.

In this paper we demonstrate that the DSCP technique is useful for examining the breeding structure of termite colonies. In the termite *Nasutitermes corniger*, colonies often contain multiple queens and kings (polygamy). Two different origins of these multiple reproductives have been hypothesized. Multiple reproductives can be reared within the colony following the death of the original founders or during the formation of nest buds. Kings and queens produced in this manner are termed 'replacement' or 'supplementary' reproductives. Alternatively, colonies may acquire multiple reproductives by cooperative nest founding, by adoption, or by colony fusion (Roisin, 1993; Leponce *et al.*, 1996).

The two alternative causes of polygamy produce contrasting genetic structures, with different evolutionary implications. It is usually not possible to distinguish between the two classes of multiple reproductives by morphological differences, but genetic analyses employing highly variable mtDNA markers can readily discern these routes to polygamy. If multiple queens and kings arise only by replacement reproduction, then they will share the same mitochondrial DNA haplotype. If multiple reproductives are acquired by other routes, they will often have different haplotypes. Despite numerous studies on the social Hymenoptera, genetic

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evidence characterizing colony foundation processes is lacking in termites.

The application of genetic markers to assess relatedness in social insect colonies is particularly crucial because of the importance of kin selection theory in models of the evolution and maintenance of social behaviour (Crozier & Pamilo, 1996). Mitochondrial markers can be used to distinguish between alternative origins of queens and kings, to determine maternal relatedness, or to quantify reproductive skew (Reeve & Ratnieks, 1993). Although mtDNA markers do not reveal paternity, they can be used in conjunction with other markers to estimate coefficients of relatedness and other aspects of colony genetic structure.

In this paper we describe the markers used for determining maternal relatedness in multiple-queen colonies of *N. corniger* (Order Isoptera). By sequencing DNA from individuals of various haplotypes we establish the nucleotide basis for different electrophoretic haplotypes and show that the differences in electrophoretic mobility probably result from sequence-directed changes in DNA curvature (Hagerman, 1990). The variation detected by DSCP analysis is compared to that found by restriction fragment length polymorphism (RFLP) analysis. In addition, we demonstrate that DSCP analysis can be readily applied to other species, with examples from two taxonomically diverse orders (Diptera and Hymenoptera).

Results and Discussion

Characterization of *N. corniger* haplotypes

We analysed 271 queens and 147 kings from forty-four natural colonies and six additional colonies that contained replacement reproductives induced by artificial orphaning. Because reproductives within social insect colonies can be related, individual queens and kings may not always represent different matriline. To obtain an estimate of the number of independent matrilines in our sample, we assumed that the reproductives in monogamous colonies derived from different matrilines because, within pairs, the queens and kings almost always had different haplotypes. However, multiple occurrences of the same haplotype within a polygamous colony's reproductives can result from replacement reproduction. Therefore different haplotypes were counted only once in each polygamous colony and additional individuals that shared these haplotypes were assumed to have a common origin in the colony. Based on these assumptions, we sampled eighty-three independent matrilines.

A ≈ 2.1 kb mtDNA region, comprising the tRNA genes for methionine, isoleucine and glutamine, the

≈ 1500 bp control region, and 367 bases of the 5'-end of the 12s rRNA gene, was PCR-amplified with conserved primers in the 12s rRNA and methionine tRNA genes. Cytochrome *c* oxidase subunit I and II (COI/II) primers amplified ≈ 1.5 kb of the cytochrome *c* oxidase I and II genes. A restriction fragment length polymorphism (RFLP) analysis of the mtDNA control region and COI/II gene PCR products from the total sample of reproductives revealed nine haplotypes, including two common haplotypes and seven rare haplotypes, each found in only one or two colonies. The two common haplotypes (*Hinf*I/*Ddel* 'A' and 'B') differed by three *Hinf*I restriction sites in the control region and one *Ddel* restriction site in the COI/II genes. The seven rare haplotypes differed from either *Hinf*I/*Ddel* 'A' or 'B' by one additional restriction site.

Coincidentally, DSCP analysis of the uncut ≈ 2.1 kb mtDNA control region PCR products also revealed nine haplotypes. Haplotypes could be classified by electrophoretic mobility into two distinct groups corresponding to the *Hinf*I/*Ddel* 'A' or 'B' RFLP lineages, defined above. Within these two groups, additional small mobility differences were observed; however, most of the DSCP haplotypes obtained from analysis of uncut PCR product did not correspond to the rare RFLP haplotypes.

We obtained better resolution of the ≈ 2.1 kb control region PCR product by digesting it with a restriction enzyme prior to DSCP analysis, which allowed greater separation of the resulting small fragments. Restriction enzymes that cut the PCR product into ≈ 200 – 800 bp fragments produced the greatest mobility differences. The *Apo*I digest gave the best resolution in *N. corniger*. DSCP analysis using other enzymes besides *Apo*I revealed no additional haplotypes; therefore we used *Apo*I for all our analyses. DSCP analysis of the mtDNA control region PCR products digested with *Apo*I revealed a total of twenty-one haplotypes among the reproductives in 44 colonies (Fig. 1). All RFLP haplotypes appeared as distinct DSCP haplotypes; DSCP analysis distinguished twelve additional haplotypes that were not detected by RFLP analysis. The most common haplotype occurred in twenty-one of the eighty-three matrilines; ten haplotypes were seen in only one colony each. All differences between haplotypes were consistent and reproducible. MtDNA population structure will be described in future work.

The *Apo*I digest of the control region PCR product yielded five restriction fragments. Although the *Apo*I RFLP pattern from all *N. corniger* individuals appeared identical on agarose gels, restriction fragments varied in mobility when electrophoresed through acrylamide gels for the DSCP procedure, allowing the identification of haplotypes. For most haplotypes, the largest

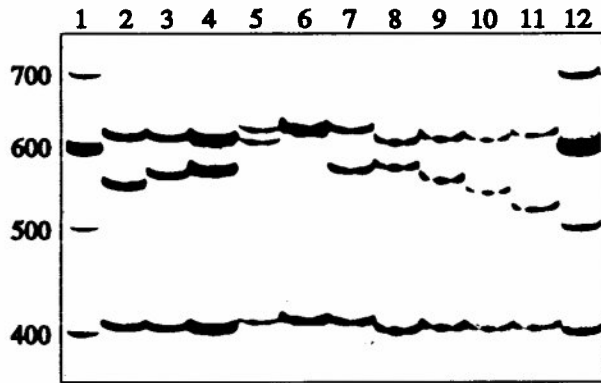


Figure 1. Ten of the control region mtDNA DSCP haplotypes found in *N. corniger* queens and kings. All PCR products were digested with *ApoI*, producing fragments of ≈ 620 bp, ≈ 558 bp and ≈ 400 bp. Smaller fragments from the restriction digests are not visible in this photograph. The ≈ 558 bp fragment shows the greatest mobility differences in most individuals. DNA sequence of this fragment for the five termites in lanes 2–6 is given in Fig. 2. Lanes 1 and 12 are 100 bp ladder size standards.

mobility differences were found in the ≈ 558 bp *ApoI* restriction fragment (Fig. 1).

Sequencing the ≈ 558 bp *ApoI* restriction fragment from five queens that displayed varying degrees of mobility difference (see Fig. 1) revealed that the electrophoretic mobility haplotypes were due to conformation rather than length differences. The fragment varied by nought to fourteen nucleotide substitutions and one to six insertions or deletions (indels) of 1–2 bp over a total length of 557–558 bp (Table 1 and Fig. 2). Double digests allowed us to locate more precisely the regions within the ≈ 558 bp *ApoI* fragment that were responsible for the observed mobility differences. The 237 bp *EcoRV*/*ApoI* fragment (Fig. 2, nucleotides 1–237) showed the same degree of mobility variation as the ≈ 558 bp *ApoI* fragment. All other double-digest restriction fragments that included this region also displayed mobility differences similar in magnitude to

Table 1. Sequence differences in the 557–558 bp *ApoI* restriction fragment from the mtDNA control region PCR product for the five *N. corniger* queens shown in Figs 1 and 2. The number of base substitutions between each queen pair are given, with the number of insertions and deletions (all limited to 1–2 bp in length) shown in parentheses. The greatest differences in base composition and mobility are between members of the 'A' and 'B' *HinfI*/*DdeI* RFLP lineages (queens 4 and 5 and queens 1–3, respectively).

Queen	1	2	3	4	5
1	–	2 (1)	5 (2)	12 (4)	12 (6)
2		–	5 (1)	14 (3)	14 (5)
3			–	14 (4)	14 (5)
4				–	0 (2)
5					–

those in the larger ≈ 558 bp *ApoI* restriction fragment. The *HaeIII* and *XbaI* digests further pinpointed the region responsible for mobility differences. By cutting next to an insertion site at nucleotide 91 (Fig. 2), these digests eliminated the mobility difference between queens 4 and 5, whereas the mobility patterns of queens 1–3 remained unchanged.

The largest mobility differences were among queens 1–3, which are *HinfI*/*DdeI* RFLP haplotype 'B', and between these queens and queens 4 and 5, which are *HinfI*/*DdeI* RFLP haplotype 'A' (see Fig. 1). These differences are caused by single base substitutions or indels at three sites (positions 182, 186 and 196; double underlined in Fig. 2) within a series of six A or T tracts (shown in bold, Fig. 2). A smaller mobility difference that differentiates queens 4 and 5 is caused by an insertion at nucleotide 91 (Fig. 2).

The electrophoretic mobility haplotypes in *N. corniger* appear to be caused by sequence-directed curvature of double-stranded DNA. As seen in the *N. corniger* DNA fragments, the electrophoretic mobilities of some double-stranded DNA fragments in polyacrylamide gels cannot be predicted solely on the basis of their size. This phenomenon has long attracted the attention of biochemists (reviewed in Trifonov, 1985; Hagerman, 1990). Often, fragments with anomalous rates of migration contain a series of oligo-(d(A).d(T)) tracts ('A-tracts') aligned approximately in phase with the DNA helix repeat (reviewed in Trifonov, 1985; Hagerman, 1990). These A-tracts cause intrinsic curving of the DNA helix; nucleotides flanking or interrupting A-tracts alter this curvature and affect the electrophoretic mobility of the DNA molecules in acrylamide gels (Koo *et al.*, 1986; Nagaich *et al.*, 1994). As shown above, a series of A-tracts spaced approximately every ten bases is found in the *N. corniger* sequence that is responsible for the greatest mobility differences, and mutations in these A-tracts account for the differences among haplotypes (Fig. 2). A-tracts and loci of curvature have been found in the mtDNA origins of replication of several other organisms (e.g. Marini *et al.*, 1982; Singh *et al.*, 1987; Welter *et al.*, 1989). In addition to A-tracts, other dinucleotide 'wedges' that occur with a period close to the helical repeat of DNA can also produce DNA curvature (Bolshoy *et al.*, 1991).

Besides the few indels or substitutions that affect the electrophoretic mobility of fragments in *N. corniger*, sequencing revealed other nucleotide substitutions and indels associated with each haplotype that appear to have no effect on conformation. Although it is therefore likely that DSCP analysis will miss some mutations, sequencing ≈ 350 bp of the 12s rRNA gene and control region from two to three termites from each of six DSCP haplotypes collected in different geographic

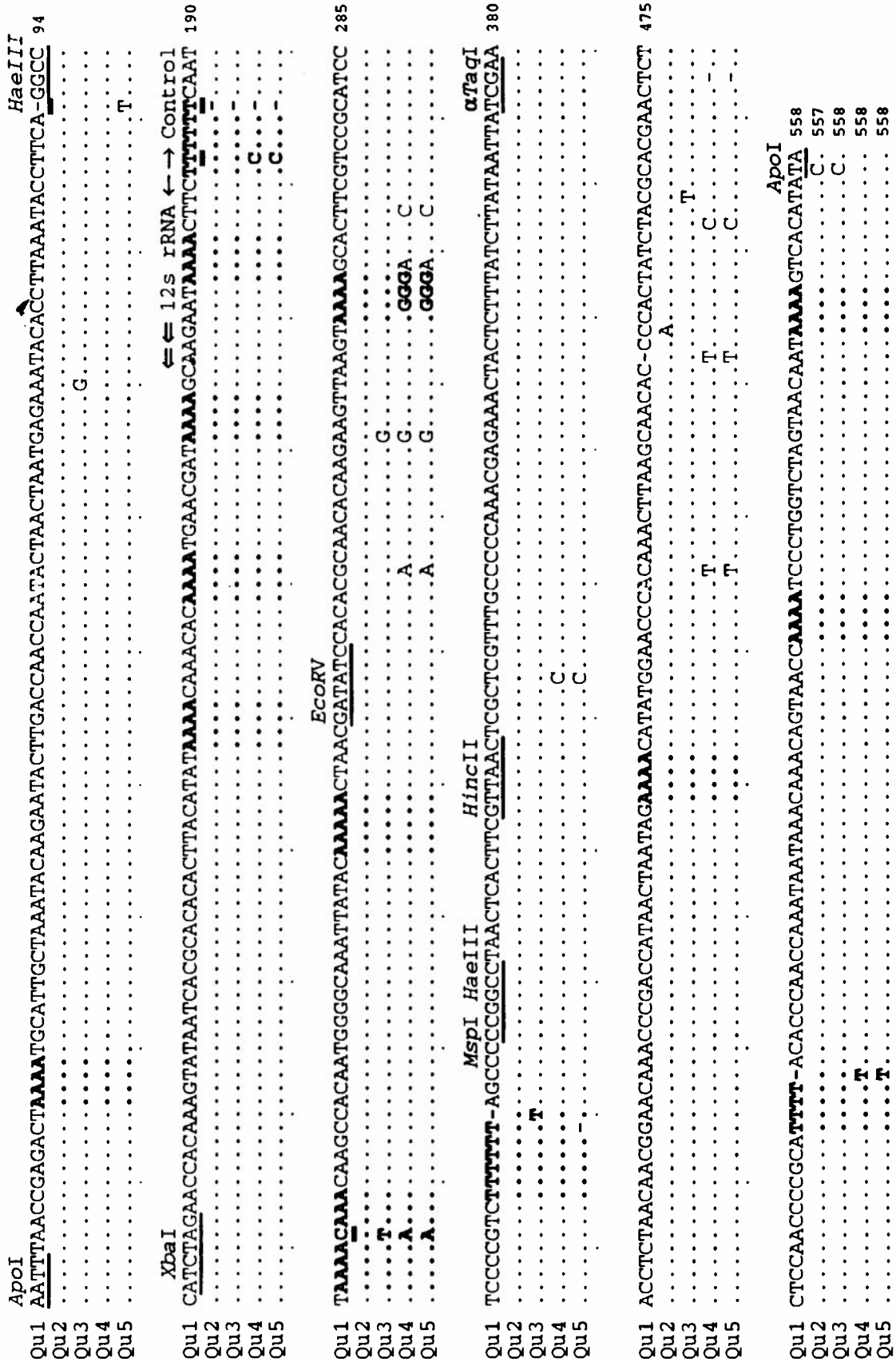


Figure 2. Alignment of the 557–558 bp *ApoI* restriction fragment DNA sequence from five *N. corrigiger* queens. Sequences encompass the 5' end of the 12s rRNA gene and the adjacent portion of the control region. A dot indicates a base identical to that of queen 1; a dash indicates that the nucleotide is absent. Restriction sites are underlined. The A and T tracts are in bold. Double underlining indicates the nucleotide positions responsible for mobility differences among haplotypes. The RFLP haplotypes of queens 1–3 are *HinfI/DdeI* 'B'; queens 4 and 5 are *HinfI/DdeI* 'A'. Differences between sequences are summarised in Table 1 and DSCP haplotypes are shown in Fig. 1, lanes 2–6. GenBank accession numbers for the sequences of queens 1–5 are U71288–U71292.

areas (5–60 km apart) failed to distinguish additional haplotypes.

Double-strand conformation polymorphisms (DSCPs) as genetic markers

The high haplotype diversity revealed in *N. corniger* by DSCP analysis is useful for examining this termite's breeding structure and the mechanisms by which multiple reproductives arise in colonies. In six of forty-four naturally occurring colonies, we obtained clear evidence of maternally unrelated queens within the same colony. In several of the colonies the queens were similar in size and coloration, indicating they were probably the same age. In at least these cases it appears that colonies were founded by two or more unrelated queens. Each queen produced offspring. The DSCP haplotypes of the queens and some of the workers from a two-queen colony are shown in Fig. 3. Detailed description of the termite study and the colony structures revealed by DSCP haplotype patterns are given elsewhere (Atkinson & Adams, 1997). The apportionment of reproduction among queens will be described in future work.

In our study DSCP analysis detected as many haplotypes as were obtained by sequencing several hundred bp per individual, at a much lower cost. DSCP markers were 2.3 times more variable than conventional RFLP markers. Furthermore, DSCP analysis offers a fast way to obtain markers. Its utility can be

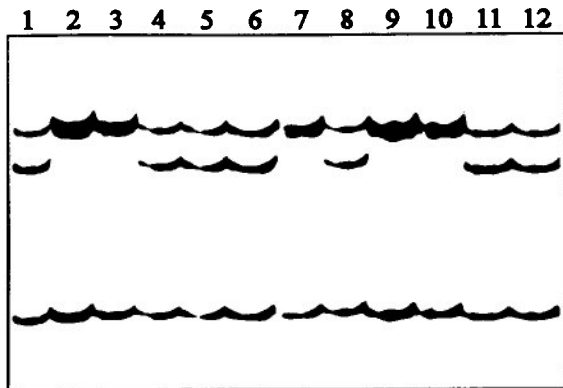


Figure 3. DSCP haplotypes for the queens and ten workers from a two-queen colony. Lanes 1 and 2 are queens 1 and 2, respectively. Lanes 3–12 are workers 1–10. The ≈ 620 bp and ≈ 558 bp *ApoI* restriction fragments of Queen 2 and her offspring almost co-migrate and the separate fragments producing the large band cannot be discerned in this photograph. The two queens were found together with one king in a royal cell at the centre of an arboreal nest. Both queens had wing scars, indicating that they developed as alate reproductives. Queen 1 (392 mg) was heavier than queen 2 (341 mg). Both queens produced offspring, but in unequal proportions ($Z = 5.65$; $n = 45$; $P < 0.001$; Dawson-Saunders & Trapp, 1994), with queen 1 producing 82% of the workers. In this figure, six workers are the offspring of queen 1.

quickly evaluated because DSCP analysis can be performed on PCR products of any length (1.6–4 kb in this study; see below). This versatility allows the use of PCR products amplified with conserved primers. To obtain markers with other equally sensitive DNA screening methods, it would be necessary to identify, and often to sequence and design primers for, a variable DNA region of < 500 bp (Lessa & Applebaum, 1993). SSCP analysis (following Hiss *et al.*, 1994) of the large PCR products used in this study did not yield any detectable polymorphism; digestion of these PCR products to obtain smaller fragments resulted in numerous poorly resolved bands.

The DSCP technique can be applied to a wide range of species. The 12s rRNA gene and control region of the mitochondrial genome harbour sequence variation detectable by DSCP analysis. Other DNA regions also display polymorphic DNA curvature (reviewed in Hagerman, 1990), but the one other region that we tested, the mtDNA cytochrome *c* oxidase subunit I and II genes in *N. corniger* (both uncut and digested with restriction enzymes), did not generate DSCP markers, even among PCR products with known restriction site differences. DSCP analysis is most likely to be useful in sequences that have A-tracts. Fortunately, the noncoding control region is highly variable and AT-rich in most insect species, and conserved PCR primers in regions flanking the control region are readily available (Simon *et al.*, 1994). We applied the DSCP technique to two other species and detected seven haplotypes among seven *Drosophila falleni* flies and four haplotypes in five colonies of the ant *Pogonomyrmex barbatus*.

As with all methods of detecting genetic variation, there are shortcomings to DSCP analysis. It is clear that some mutations do not affect DNA curving and cannot be distinguished by DSCP analysis. Therefore this technique will not work with all PCR products. In addition, the large number of haplotypes that are defined by small mobility differences requires electrophoresing some PCR products more than once to place fragments with similar mobility next to one another. Furthermore, although it is possible to make a reasonable prediction of curvature from known sequences (e.g. Bolshoy *et al.*, 1991), there are a large number of sequences that can produce any given electrophoretic pattern. Therefore, because the underlying sequence differences cannot be estimated, it is not possible to order lineages for phylogenetic purposes. However, even in phylogenetic studies, DSCP analysis may be useful in identifying haplotypes for sequencing. Also, there are potential difficulties inherent to any study that uses mtDNA markers, including biparental inheritance (Awise, 1991), heteroplasmy (Harrison, 1989; Rand,

1993), and nuclear copies of mtDNA (Zhang & Hewitt, 1996a, b).

In summary, DSCP analysis is a rapid and efficient method to detect variation in the control region of mtDNA. The level of variation detected by DSCP analysis of control region mtDNA can provide markers for examining genetic identity, maternity, kinship, family structure, and other aspects of population genetics that require variation from a rapidly evolving region.

Experimental procedures

DNA extraction and amplification

Reproductives of the termite *Nasutitermes corniger* were collected from forty-four colonies near Colon, Panama City and Portobelo in the Republic of Panama (Atkinson & Adams, 1997). Six of these colonies were resampled 1 year after removal of the original reproductives (artificial orphaning). DNA was extracted from a 1–2 mg portion of the abdominal cuticle of each termite using the Puregene DNA isolation kit (Gentra Systems Inc.). The mtDNA control region, with parts of the flanking 12s rRNA and tRNA genes, was PCR-amplified with conserved primers (Kocher *et al.*, 1989; Simon *et al.*, 1994) in the methionine tRNA and 12s rRNA genes (primers from Taylor *et al.*, 1993). PCR reactions contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 200 μM of each dNTP, 100 nM of each primer, 2.0 units of *Taq* DNA polymerase (Gibco BRL, Life Technologies Inc.) and 0.1–1 μg of template DNA in a total volume of 100 μl. Amplification conditions were twenty-five cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. In addition, portions of the cytochrome *c* oxidase subunit I and II genes were amplified using the same PCR conditions except that the annealing temperature was 37°C and the PCR was conducted for thirty-five cycles (primers C1-J-2195 and C2-N-3661, minus the 3'-base; Simon *et al.*, 1994).

RFLP analysis

To screen for restriction fragment length polymorphisms, we digested pooled PCR products (4 μl PCR product from each of five individuals) with each of twenty-one restriction enzymes (*ApoI*, *AseI*, *BamHI*, *BsrI*, *DdeI*, *DpnII*, *DraI*, *EcoRV*, *HaeIII*, *HhaI*, *HindI*, *HindIII*, *HinfI*, *MseI*, *MspI*, *PstI*, *PvuII*, *RsaI*, *SspI*, *XbaI* and α *TaqI*) following the supplier's instructions (New England Biolabs). In total, we screened mtDNA control region and COI/II gene PCR products from 271 queens and 147 kings. The digested PCR products were electrophoresed through 1.2% agarose gels, and visualized under UV light after ethidium bromide staining. Restriction enzymes that cut at polymorphic sites were subsequently used to digest PCR products from queens and kings individually.

DSCP analysis

For DSCP analysis, we used 0.4 mm, 16 × 18 cm, 6% polyacrylamide vertical gels (49:1 acrylamide:bisacrylamide) with 5% glycerol. We also tested 5% and 7% polyacrylamide gels, each with both 49:1 and 19:1 acrylamide:bisacrylamide concentrations, but these resulted in lower resolution. Protocols followed those described for SSCP analysis (Hiss *et al.*, 1994), except that we applied double-stranded PCR product to gels of

a 6% rather than 5% acrylamide concentration. Because we developed the DSCP method independently, protocols differ from those described in Saad *et al.* (1994). Uncut samples (2 μl PCR product with 1.5 μl 6 × loading buffer I; Sambrook *et al.*, 1989) or previously digested samples [2 μl PCR product, 0.4 μl enzyme buffer, 0.07 μl restriction enzyme (0.28 units; New England Biolabs), 0.07 μl BSA and 2 μl ddH₂O, with 1.5 μl 6 × loading buffer I] were loaded onto gels using a 32-well sharktooth comb. Electrophoresis was carried out in Gibco BRL model V-16 gel boxes (Life Technologies Inc.) at room temperature for 24 h at 300 V (uncut product) or 65 V (digested product). Gels were then silver stained using either a kit (Bio-Rad Laboratories Inc.) or the protocol described in Merril *et al.* (1981). To distinguish similar haplotypes, it was sometimes necessary to electrophorese PCR products through more than one gel, so that fragments of similar mobility could be placed in adjacent lanes for clarification of small differences.

Several tests were performed to examine the inheritance patterns of the mtDNA markers. Strict maternal inheritance of haplotypes was demonstrated by analysing mothers and offspring from ten single-queen colonies. Consistent inheritance over at least three generations was confirmed by analysing queens removed from field colonies, the replacement reproductives reared following the removal, and the offspring of the replacement reproductives. PCR products amplified from DNA extracted separately from termite legs and abdomens produced the same haplotype patterns, verifying that the amplified DNA did not derive from gut endosymbionts.

Sequence and fragment analysis

To examine the nucleotide basis for the DSCP haplotype differences, we sequenced the part of the control region that displayed the greatest mobility differences, the ≈558 bp *ApoI* fragment. PCR products were gel purified and cloned into an mp18 derivative designed for PCR-amplified DNA (Burke *et al.*, 1995). The cloned PCR products were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Sequenase kit (United States Biochemical) with the –40 universal primer (United States Biochemical) and internal primer 5'-CACAAAGTATAATCACGC-3' (forward). The opposite strand was sequenced using an automated sequencer with primers 5'-TAACGAAGTGAGTTAGGC-3' (reverse) and 5'-TTTGTCTCACTGG(A/G)TTCAC-3' (reverse). In addition, to examine other individuals with the same haplotypes as the five queens that were sequenced as described above, we sequenced approximately 350 bases of the same mitochondrial region from ten more queens and kings using the primer 5'-CACAAAGTATAATCACGC-3'. Sequencing reactions were performed in a Perkin-Elmer thermal cycler following the standard protocol for the ABI PRISM sequencing kit (Perkin-Elmer Corp.). Excess dye terminators were removed by extraction in Centri-sep spin columns (Princeton Separations) prior to loading on an ABI 373 automated sequencer (Applied Biosystems). All sequences were aligned by eye using the computer program ESEE (Cabot & Beckenbach, 1989).

To further isolate the region responsible for mobility differences within the ≈558 bp *ApoI* fragment, we digested mitochondrial control region PCR product with *ApoI* and each of the following restriction enzymes: *HaeIII*, *EcoRV*, *MspI*, α *TaqI*, *HincII* and *XbaI*, according to supplier's instructions (New England Biolabs Inc.). Each additional enzyme digested the

≈ 558 bp *Apol* fragment into two or three fragments, and did not cut the other *Apol* fragments. Products were resolved using the DSCP procedure.

DSCP analysis of other species

We performed DSCP analysis on digested mtDNA control region PCR product from seven individuals of the fly *Drosophila falleni* (Drosophilidae) and two workers from each of five colonies of the ant *Pogonomyrmex barbatus* (Formicidae). The protocols were as described above, except that PCR products were digested with *SspI* prior to electrophoresis, and the ant DNA was amplified with primers SR-J-14612 and C1-N-2191 (Simon *et al.*, 1994). Also, PCR reactions for the amplification of the ≈ 4 kb region of ant DNA contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3.25 mM MgCl₂, 300 μM of each dNTP, 200 nM of each primer, 0.75 units of *Taq* DNA polymerase (Gibco BRL, Life Technologies Inc.) and 0.1–1 μg of template DNA in a total volume of 25 μl. Amplification conditions were 1 min at 94°C, followed by thirty cycles of 30 s at 94°C, 1 min at 45°C, and 4.5 min at 68°C, with 5 additional minutes at 68°C after the thirty cycles.

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