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Intraspecific mitochondrial DNA variation in the migratory grasshopper, *Melanoplus sanguinipes*

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Restriction fragment analysis of mitochondrial DNA (mtDNA) was used to examine genetic variation and population structure of the migratory grasshopper, *Melanoplus sanguinipes*. The total DNA of 89 individuals was digested with 10 restriction enzymes and probed with three cloned *Eco*RI fragments representing the entire mitochondrial genome (total length 16.1 kb). Five endonucleases revealed polymorphism, yielding 12 haplotypes in combination. Nucleotide diversity indices (δ) between haplotypes ranged from 0.18 to 0.92 per cent with an average value (π) of 0.27 per cent, a comparatively low figure that possibly reflects a history of chronically small population numbers prior to the species' colonization of a suitable habitat engendered by agricultural settlement. Little, if any, differentiation was evident among locations or between prairie and parkland regions, areas spanned by a colour trait cline. Selective forces with respect to the latter must, therefore, be sufficiently powerful to withstand the attenuating effects of widespread gene flow, implied by the mtDNA data.

Keywords: grasshoppers, *Melanoplus*, mitochondrial DNA, population structure.

Introduction

The migratory grasshopper, *Melanoplus sanguinipes*, is widely distributed throughout North America, and, along with a few congeners, is regarded as a serious crop pest, especially in prairie and rangeland areas. A fundamental component of a successful pest management strategy is an appreciation of the genetic diversity and structure of targeted populations. Such information is generally lacking for most Orthopteran fauna but the situation is slowly changing, at least for *M. sanguinipes* (Chapco *et al.*, 1987). Genetic analysis of the latter has revealed a number of heritable morphological and electrophoretic polymorphisms, for which an interesting dichotomy in spacial patterns appears to exist in the province of Saskatchewan. There is a prominent two-dimensional gradient between parkland (north-east) and prairie regions with respect to a hind tibia colour gene, contrasting with the fairly homogeneous allelic distributions of a number of allozyme loci (Chapco & Bidochka, 1986). At one time, parkland and prairie populations were afforded subspecies status (Brooks, 1958) but the allozyme work would appear to confirm Vickery's (1979) suspicion that the claim is premature. Different relative strengths of gene flow and local adaptive values are possible explanations for the discrepancy in geographical distributions of the two types of traits (Chapco, 1989). A genetic marker that is ideally suited to address these and related issues is mitochondrial DNA (mtDNA). Owing to features such as maternal and non-recombinational transmission and a rapid rate of evolution (Harrison, 1989), the analysis of spatial and phylogenetic patterns associated with mtDNA has provided insights into populational structure and lineage as well as migrational and historical events for many organisms (Avise, *et al.*, 1987). On the surface, it might be expected that the amount of interpopulational differentiation is minimal, especially for a species such as *M. sanguinipes* with its almost legendary reputation for migration and outbreak (Vickery & Kevan, 1983; Pfadt, 1988). As the movement of organisms is not necessarily equivalent to gene flow (Endler, 1977), this prediction may not be realized although there is some evidence that

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migrating females do oviposit (Parker *et al.*, 1955; McAnelly & Rankin, 1986). In this paper, an analysis of restriction fragment length polymorphisms (RFLP) of grasshoppers obtained from several areas in south-central Saskatchewan and from a few areas outside the province is presented with the view to addressing the aforementioned microevolutionary issues.

Materials and methods

A total of 89 adult grasshoppers, assayed for their mtDNA profiles, were sampled during 1989 and 1990 from 14 locations in south-central Saskatchewan and from five sites outside the province (Table 1). Inter-locational distances in Saskatchewan range from 16 to 430 km. Ontario and New Mexico sites are, on average, 2,700 and 2,000 km, respectively from the main study area. A laboratory non-diapause strain of *M. sanguinipes* (Pickford & Randell, 1969) provided material from which mtDNA probes were constructed.

Isolation of mtDNA

A modification of Tamura & Aotsuka's (1988) rapid alkaline lysis method was used. Between 70 and 90 live adult grasshoppers from the non-diapause strain were gassed briefly with CO_2 before being ground in a chilled mortar with acid-washed sea sand and homogenizing buffer (0.25 M sucrose, 10 mM EDTA and 30 mM Tris–HCl, pH 7.5). Grinding was continued until there were no large tissue fragments remaining. The crude homogenate was centrifuged in a 50-ml centrifuge tube at 1,100 ***g*** for 5 min at 4°C to remove the sand. The supernatant was then transferred to a second tube and centrifuged at 3,000 ***g*** for 5 min at 4°C in order to pellet nuclei and cellular debris. The soluble cell fractions containing the mitochondria were then pooled and centrifuged at 20,400 ***g*** for 20 min at 4°C. The resulting mitochondrial pellet was resuspended in 1 ml of lysis buffer (0.15 M NaCl, 10 mM EDTA, 10 mM Tris–HCl, pH 8.0). Two volumes of a freshly prepared

Table 1 Sampling locations and mtDNA haplotypes of *Melanoplus sanguinipes*

Location	Latitude	Longitude	Haplotype* (number of individuals)	*n*†
Saskatchewan				
Prairies				
Hanley	51.4	106.3	1*(2), 3(2), 4(1), 5(2), 6(1)	8
Crestwynd	50.1	105.1	1(2), 4(1), 5(1)	4
Rowatt	50.3	104.6	1(1), 3(1), 5(2)	4
Parry	49.5	104.5	1(2), 3(1), 5(2)	4
Dilke	50.5	105.1	1(1), 2(3)	4
Avonlea	50.0	105.0	1(2), 5(1)	3
Chamberlain	50.5	105.3	1(2), 3(2), 5(1)	5
Lumsden	50.3	104.5	1(1), 2(1), 5(5), 6(1), 11(1)	9
Horizon	49.3	105.1	7(2)	7
Assiniboia	49.6	106.0	7(2), 8(1)	3
Parkland				
Springside	51.4	102.8	1(1), 9(1), 10(9)	11
Balcarres	50.5	103·3	1(1), 2(1), 3(1)	3
Hudson Bay	52.5	102.3	1(6), 10(3), 20(1)	10
Kipling	49.6	102.4	3(2), 5(3)	5
Outside Saskatchewan				
Mauston (Wisc)	43.6	89.5	3(3)	3
Alexandria (Minn)	46.0	95.2	5(4)	4
Turtle Lake (ND)	47.3	100.8	1(1)	1
Tobermory (Ont)	45.3	81.7	5(2)	2
Santa Fe (NM)	35.1	106.0	3(2), 4(2)	4

*Haplotypes: 1 = AAAAAAAAAA, 2 = AAAAAAABAA, 3 = ABAAAAABAA, 4 = ABAAAADBAA, 5 = ABAAAAAAAA, 6 = ABAAAADAAA, 7 = ABAABAABAA, 8 = AAAABAAAAA, 9 = ABAAAAGAAA, 10 = AAAAAADAAA, 11 = AAAAAADBAA, 20 = BAAAAAAAAA. Letters refer to genotypes of restriction enzymes listed in the same order as presented in Table 2. †Sample size.

solution of 1 per cent SDS in 0.2 M NaOH were added, and the mixture was vortexed briefly. Following storage on ice for 5 min, 1.5 ml of ice cold 3.0 M sodium acetate (pH 4.8) was added and the mixture was again gently vortexed before storing on ice for an additional 10 min. The mixture was then centrifuged at 20,400 ***g*** for 15 min at 4°C. The supernatant was removed and extracted with an equal volume of phenol–chloroform (one part Tris–HCl buffered phenol, pH 8.0 and one part chloroform:isoamyl alcohol, 24:1), and centrifuged at 14,600 ***g*** for 5 min at room temperature. mtDNA was precipitated from the aqueous phase by the addition of two volumes of 95 per cent ethanol. The tube was allowed to stand at room temperature for at least 15 min before being centrifuged at 20,400 ***g*** for 15 min at room temperature. The resulting mtDNA pellet was washed twice with 3 ml of 70 per cent ethanol before being dried *in vacuo*.

The dried mtDNA pellets were dissolved in 6 ml of 10 mM Tris–HCl, 1 mM EDTA, pH 8.0, to which was added 6 g CsCl and 600 μl of ethidium bromide (5 mg ml^{-1}). The solution was mixed and left at room temperature for 30 min and then centrifuged at 7,700 ***g*** for 10 min at room temperature. The supernatant was transferred to a centrifuge tube (Quick-Seal G-Max centrifuge tube, Beckman Instruments, Inc., Palo Alto, CA) sealed and centrifuged at 48,000 rpm for 20 h at 25°C in a Ti 80 rotor. A band (the lowermost) corresponding to covalently closed circular mtDNA was visualized under long-wave ultraviolet light and withdrawn by puncturing the tube from the side with an 18-gauge needle attached to a 1-ml syringe. Ethidium bromide was extracted with *n*-butanol saturated with 4 M NaCl, 10 mM Tris–HCl and 1 mM EDTA, pH 8.0. After dialysing for 16 h against two changes of dialysis buffer (4 mM NaCl, 1 mM EDTA and 10 mM Tris–HCl, pH 8.0), the purified mtDNA was stored at 4°C.

Isolation of total genomic DNA

Total genomic DNA was isolated from individual grasshoppers according to the method of Marchant (1988) with minor modifications.

Cloning of M. sanguinipes *mtDNA*

In general, the procedures described by Maniatis *et al.* (1982) were followed with respect to standard recombinant DNA techniques such as large-scale isolation of plasmid DNA, CsCl gradient centrifugation, the rapid alkaline lysis method for isolation of plasmid DNA, restriction endonuclease digestion of DNA, agarose gel electrophoresis of nucleic acids, transformation of *Escherichia coli* by the calcium chloride method, ligation reactions, dephosphorylation of linearized vectors and isolation of restriction fragments from agarose gels using a commercial electroeluter (International Biotechnologies Inc., New Haven, CT). Hexamine cobalt chloride was included in ligation reaction mixes at a final concentration of 1.2 mM (Rusche & Howard-Flanders, 1985).

Purified mtDNA from the non-diapause laboratory strain of *M. sanguinipes* was digested to completion with *Eco*RI according to the supplier's recommendations. The *Eco*RI-digested mtDNA was ligated to CIP-dephosphorylated, *Eco*RI-cleaved pUC18 vector DNA. Recombinant plasmids were selected in *E. coli* strain JM83r$^-$ using the culture conditions and screening methods described in Maniatis *et al.* (1982). Insertion of specific mtDNA fragments into the vector was confirmed by isolation of the plasmid DNA and assessing the *Eco*RI restriction pattern.

Restriction endonuclease digestion of total DNA

Total DNA from individual grasshoppers was separately digested with 10 restriction endonucleases (Bethesda Research Laboratories or New England Biolabs): *Acc*I, *Alu*I, *Bgl*II, *Dra*I, *Eco*RI, *Hin*fI, *Hpa*I, *Pst*I, *Pvu*II, and *Xba*I. All digests were carried out in 100-μl volumes in reaction conditions as specified by the supplier. Following digestion, the DNA was precipitated directly by the addition of 2.5 volumes of 95 per cent ethanol at −20°C. DNA pellets were collected by microcentrifugation for 15 min, dried in *in vacuo* and resuspended in 15 μl of 10 mM Tris–HCl, 1 mM EDTA, pH 8.0 and then electrophoresed in 1 or 1.2 per cent agarose gels.

Southern transfer procedures

Gels containing the digested total DNA were sequentially treated (30 min each) with depurination solution (0.25 M HCl), denaturation solution (0.6 M NaCl, 0.2 M NaOH) and neutralization solution (1.5 M NaCl, 0.5 M Tris–HCl, pH 7.0) with constant shaking.

Capillary transfers to nylon membranes (Hybond-N, Amersham) were set up according to Southern (1975). In certain cases double membrane capillary transfers were obtained from the same gel. Conditions were essentially the same as those used for single transfers except that gels were sandwiched between two nylon membranes.

Gels were stained with ethidium bromide to determine whether any DNA remained untransferred. Membranes were then rinsed in 2 × SSC and the bound DNA was crosslinked with an ultraviolet lamp (Stratalinker UV Crosslinker 1800, Stratagene, La Jolla, CA)

set to release 120,000 μJ. Once crosslinked, the membranes were wrapped in Saran Wrap and stored at room temperature.

Radioactive labelling of probe DNA

Recombinant plasmids pDM1, pDM2 and pDM3 (see Results) collectively containing the entire mitochondrial genome of *M. sanguinipes* were individually digested to completion with *Eco*RI. The DNA was then precipitated with 95 per cent ethanol, dried *in vacuo* and redissolved in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0. Between 100 and 200 ng of the digested plasmid DNA were used in separate random primer labelling reactions according to the method of Feinberg & Vogelstein (1983 and 1984). In general, the activity of the probes was of the order of 1.0×10^7 c.p.m. μg^{-1}. A mix containing approximately 1.0×10^6 c.p.m. from each random primer reaction was used for hybridization.

Hybridization

A hybridization oven (Model 310 Hybridization Incubator, Robbins Scientific Inc., Sunnyvale, CA) was used. Prehybridization, hybridization and wash conditions were essentially those described in the GeneScreenPlus™ instruction booklet provided by the supplier (Dupont/NEN). Membranes were washed with two changes of 2 × SSC, 1 per cent SDS for 30 min at 65°C. A final wash with two changes of 0.1 × SSC for 20 min at room temperature was used to remove excess SDS.

Data analysis

Restriction enzyme fragment profiles were designated as a sequence of 10 upper-case letters AAAAAAAAAA, BAAAAAAAAA, etc. where each letter refers to a particular banding 'morph' of *Eco*RI, *Bgl*II, *Pst*I, *Acc*I, *Pvu*II, *Xba*I, *Hin*fI, *Hpa*I, *Alu*I, and *Dra*I, respectively. Estimates of nucleotide sequence divergence, δ, based on the proportion of shared fragments, were obtained for all pairs of haplotypes according to equations 20 and 21 in Nei & Li (1979). Separate values were determined for the different classes of endonucleases and weighted according to the number of fragments revealed. Values were then used to construct a dendrogram by the unweighted pair-group method (UPGMA) employing the SPSS-X (Release 3) program, CLUSTER. A measure of the overall diversity for the species was provided by the nucleotide diversity index π (Nei, 1987) which takes into account frequencies of haplotypes.

Haplotypes were also converted into strings of 1s and 0s for a cladistic analysis using the parsimony criterion. The Phylip program, METRO provided by J. Felsenstein (1988) was used. For most enzymes exhibiting variation, the connection between one array of fragments (designated as '0') and another (designated '1') was easily visualized and required a single site change. No simple set of transformations connecting the various patterns was evident for the exception, *Hin*-fI. In this case, 1s and 0s were used to indicate the presence or absence of specific bands.

Results

Three *Eco*RI fragments of 8.0, 6.8 and 1.2 kb were cloned into pUC18 to yield recombinant plasmids pDM1, pDM2 and pDM3, respectively. When these were used collectively to probe the total DNA from individual insects, an average of about 44 scorable fragments were obtained per insect; fragment patterns and sizes are recorded in Table 2. *Alu*I, *Dra*I, and *Hin*fI produced the greatest number of bands, many of small size. As the sums of these lengths are comparatively low, it is likely that even smaller pieces were undetected. It is also possible that some bands are really collections of two or more DNA fragments of equal (or near-equal) mobilities. Totals of fragment sizes for the remaining enzymes (average

Table 2 Restriction fragment patterns and sizes (bp)

Enzyme	Pattern	Fragment sizes
*Eco*RI	A	8000, 6800, 1200
	B	8000, 3800, 3000, 1200
*Bgl*II	A	6800, 5600, 2400, 1200
	B	6800, 1200, 7800
*Pst*I	A	16300
*Acc*I	A	8800, 4800, 2600
*Pvu*II	A	13400, 2600
	B	16300
*Xba*I	A	7300, 4400, 2100, 1600, 600
*Hin*fI	A	3700, 2600, 1200, 700, 600, 500, 450
	D	3700, 2600, 1200, 900, 700, 600, 500, 450, 400
	G	2600, 1200, 900, 700, 600, 550, 450, 400
*Hpa*I	A	13300, 1700, 1000
	B	15000, 1000
*Dra*I	A	1750, 1600, 1250, 1000, 850, 700, 575, 400
*Alu*I	A	2100, 1300, 900, 800, 700, 600, 450, 400, 375

value = 16.1 ± 0.2 kb) were in close agreement with the sum of sizes for the cloned *Eco*RI pieces; it is therefore probable that this figure is a reasonable estimate of the size of the mitochondrial genome for *M. sanguinipes*.

All individuals exhibited the same patterns for *Pst*I, *Acc*I, *Xba*I, *Dra*I and *Alu*I. Two to three variant patterns were observed for each of the five remaining endonucleases. For *Eco*RI, *Bgl*II, *Pvu*II, and *Hpa*I different morphs were separable by a single loss or gain of a restriction site. Variants with respect to *Hin*fI, require at least two site changes to interconvert one to another. In total, 12 different composite genotypes (haplotypes) were detected among the 89 grasshoppers representing 19 locations (Table 1). Haplotypes 1, 3 and 5 accounted for about 75 per cent of the samples and were broadly distributed throughout the study area. Their relative frequencies were not significantly different between prairie and parkland areas (4×2 contingency $G = 1.67$, $P < 0.65$). Among the remaining genotypes, four were unique. Most haplotypes that were detected in at least two individuals were present in more than one location; in some cases, sites were far apart — e.g. haplotype 2 (Dilke and Balcarres) or proximal — e.g. haplotype 7 (Assiniboia and Horizon). Three grasshoppers in Hudson Bay possessed genotype 10, not found elsewhere. All genotypes detected outside the province also occurred in Saskatchewan. For example, of the four insects collected in Santa Fe, New Mexico, two were haplotype 3 and two were haplotype 4; grasshoppers exhibiting pattern 5 were also found in Ontario and Minnesota.

Nucleotide divergence estimates (δ in Table 3) between pairs of haplotypes ranged from 0.178 per cent (1 vs. 10, 5 vs. 6, 2 vs. 11 and 3 vs. 4) to 0.920 per cent (7 vs. 9). For Saskatchewan, the average nucleotide diversity (taking into account haplotype frequencies) was 0.265 per cent. Thirteen of 14 sites in the province were polymorphic for two or more haplotypes. Many of the latter, where they co-occur, differ by one to three restriction site changes. Crestwynd contains haplotypes 1 and 4, which differ by at least four sites taking into account the uncertainty regarding *Hin*fI. Within collection site nucleotide diversity ranged from 0 (Horizon) to 0.33 per cent (Crestwynd) with an average value of 0.194 per cent, a figure that is about 72.9 per cent of the variation among haplotypes, ignoring population structure. This translates into an interpopulational nucleotide diversity index τ_{ST} (Nei, 1982) of 27.1 per cent, signifying very little differentiation among sampled sites. The extent of differentiation between prairie and parkland regions can similarly be evaluated. The average diversity within each region is 0.256 per cent, not much different than that for the entire area when treated as a panmictic unit. There is, therefore, very little divergence ($\tau_{ST} = 3.6$ per cent) between prairie and parkland areas; this agrees with the haplotype frequency analysis presented earlier.

The UPGMA dendrogram employing interhaplotype δ values is displayed in Fig. 1. Clustered together are haplotypes 3 with 4, 5 with 6, 2 with 11, and 1 with 10. Members of each pair differ with respect to *Hin*fI cleavage sites by at least two mutational differences (A vs. D). At a higher level, there appear to be two main clusters: (3–7) and (2, 11, 1, 10, 20, 8), separated on the basis of *Bgl*II. Haplotype 9, distinguished by the *Hin*fI pattern, G, is separated from the rest. No association between the dendrogram and geographical distribution is evident. Locations involving larger sample sizes are represented by members of both major clusters.

Repeated application of the cladistics program METRO yields a myriad of equally parsimonious Wagner

Table 3 Percentage divergence (δ) between mtDNA haplotypes based on proportion of shared restriction fragments (Nei & Li, 1979)

Haplotype	20	5	2	10	8	3	6	11	7	9	4
1	0.190	0.197	0.197	0.178	0.197	0.420	0.375	0.375	0.671	0.678	0.598
20		0.403	0.403	0.367	0.403	0.641	0.580	0.580	0.912	0.883	0.819
5			0.420	0.375	0.420	0.206	0.178	0.598	0.439	0.480	0.383
2				0.375	0.420	0.206	0.598	0.178	0.439	0.901	0.383
10					0.375	0.598	0.197	0.197	0.849	0.462	0.420
8						0.671	0.598	0.598	0.439	0.901	0.849
3							0.383	0.383	0.216	0.686	0.178
6								0.420	0.617	0.265	0.206
11									0.617	0.685	0.206
7										0.920	0.393
9											0.471

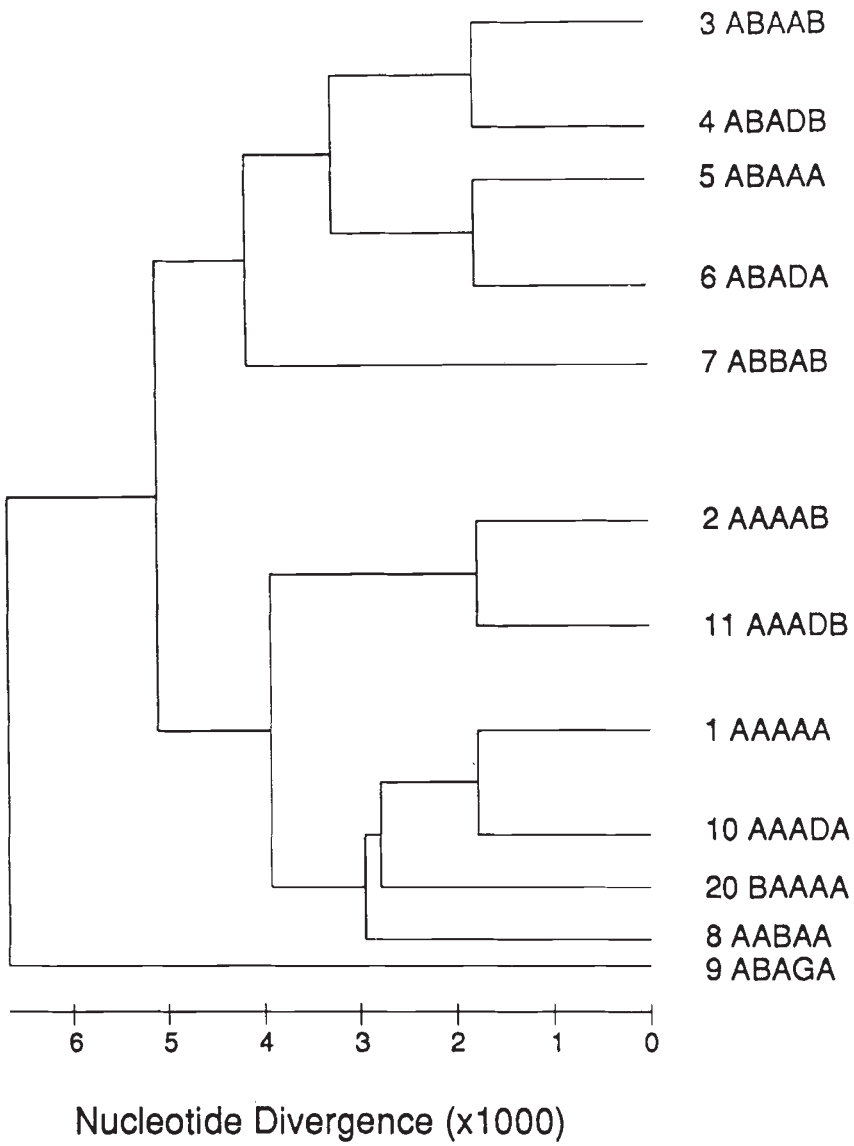

Fig. 1 Dendrogram of mtDNA haplotypes assayed in *Melanoplus sanguinipes* based on UPGMA analysis of nucleotide divergence estimates ($\delta \times 1{,}000$). Letters refer to polymorphic genotypes of restriction enzymes listed in the same order as presented in Table 1.

networks each with 14 mutational steps. Most differ in the placement of minor types usually involving *Hin*fI variants. Omitting *Hin*fI results in four equally parsimonious trees, each with six steps. To varying extents they resemble the dendrogram. One in particular is a good match if it is assumed that: (i) haplotype 5, the most frequent and widespread, is ancestral (Avise *et al.*, 1987; Excoffier, 1990); (ii) parallel losses of restriction sites are more likely than parallel gains (Templeton, 1983); and (iii) common haplotypes that differ by a single restriction site are more likely to be directly connected rather than remotely linked via rarer intermediates. In this particular cladogram, haplotypes (1, 10) constitute a hub for haplotype 8, (2, 11), 20 and (5, 6, 9). The latter group links directly with (3, 4) which in turn connects with 7.

Discussion

The estimated length of the mitochondrial genome of *Melanoplus sanguinipes* is approximately 16.1 kb, typical of most metazoans, including insects (Table 4). It is slightly larger than the mitochondrial genomes of the two acridids thus far analysed, *Locusta migratoria* and *Caledia captiva*. In other Orthopterans, individuals of two gryllid species are heteroplasmic for two of three size classes of mtDNA (Harrison *et al.*, 1985), apparently not an uncommon phenomenon in invertebrates (Boyce *et al.*, 1989). Within the limits of resolution in the present study, however, there were no arrays of banding patterns which would have suggested that individual grasshoppers possessed more than one 'type' of mtDNA.

Table 4 Mitochondrial DNA sizes and intraspecific diversity estimates for diverse insects

Order	Species	Size (kb)	Diversity*	Source†
Orthoptera	*Locusta migratoria*	15.2	—	1
	Caledia captiva	15.0	—	2
	Gryllus pennsylvanicus & *G. firmus*	15.8–16.4	—	3
	Melanoplus sanguinipes	16.1	0.27	4
Diptera	*Drosophila* spp.	15.5–18.7	0.0–4.9	5–8
	Anopheles albopictus	17.5	very low‡	9
	A. quadrimaculatus	15.3	—	10
	Glossina morsitans	—	2.0–11.1	11
	Cochliomyia hominovorax	16.0	1.1	12
Hymenoptera	*Apis mellifera*	17.0	0.33–4.1	13
	Dolichovespula spp. & *Vespula* spp.	18.0–22.7	—	14
Coleoptera	*Anthonomus grandis*	19.0	—	15
	Pissodes spp.	>30.0	—	16
	Diabrotica decimapunctata	21.0	—	12
Homoptera	*Schizaphis graminum*	—	0.17–1.2	17
	Magicicada spp.	19.6–20.0	0.01–0.12	18

*Diversities estimated in various ways. Consult sources. Includes within- and between-racial, subspecific or biotype estimates.
†Sources: 1. McCracken *et al.* (1987), 2. Marchant (1988), 3. Harrison *et al.* (1985), 4. This study, 5. Gonzalez *et al.* (1990), 6. Hale & Singh (1987), 7. Baba-Aïssa *et al.* (1988), 8. DeSalle *et al.* (1986), 9. Kambhampati & Rai (1991), 10. Cockburn *et al.* (1990), 11. Trick & Dover (1984), 12. Roehrdanz & Johnson (1988), 13. Hall & Muralidharan (1989), 14. Schmitz & Moritz (1990), 15. Roehrdanz (1990), 16. Boyce *et al.* (1989), 17. Powers *et al.* (1989), 18. Martin & Simon (1990).
‡Proportion of shared fragments, $F > 0.99$.

The extent of mtDNA variation ($\pi = 0.265$ per cent) in *M. sanguinipes* falls within the lower range of published intraspecific nucleotide diversity values for insects (Table 4) and other taxa (see summaries in Martin & Simon, 1990 and Becker *et al.*, 1988). The average index is dominated by a low δ value (0.020 per cent) between the two most common genotypes, 1 and 5, which differ from one another by a gain or loss of one restriction site. The third most frequent genotype (3) differs from these by two ($\delta = 0.420$ per cent) and one ($\delta = 0.206$ per cent) site(s), respectively, and also makes a large contribution to the overall low diversity. Many theoretical arguments (Martin & Simon, 1990) have been proposed to account for observed low levels of variation such as those based on purifying selection and low mutation rate. One of the more probable and satisfying explanations centres on the organism's biology and demographic past. Given *M. sanguinipes*' reproductive capacity (Pfadt & Smith, 1972) and preference for fields disturbed by human activities (Pfadt, 1988), and given historical accounts (see references in Chapco & Bidochka, 1986), it is reasonable to regard this insect as a recent 'colonist' of the area.

The availability of pod laying sites and introduced weeds and crops, which resulted from settlement, converted a previously innocuous and patchily distributed species to one of the major grasshopper pests on the prairies (Beirne, 1972). The situation seems to parallel Wilson *et al.*'s (1985) depiction of a population experiencing a prolonged bottleneck before expanding its numbers. The similarity is not quite exact in that there have been, at irregular intervals, population crashes as well as outbreaks in this century (Riegert, 1968). In any case, such populations are expected to exhibit low mtDNA diversity and, owing to chronically low numbers in the pre-agricultural past, low nuclear diversities. From the present study, this is clearly true for mtDNA, and from a previous investigation of isozymic variation, true for nuclear genes as well (Chapco & Bidochka, 1986). Based on neutral theory (Nei, 1987) and the assumption of an evolutionary rate of 0.01 substitutions/base pair/lineage/million years (see

below), a nucleotide diversity value of 0.265 per cent corresponds to an effective population size of $N_e = 133{,}000$. This index encapsulates previous historical events in that it is strongly influenced by small population sizes in the past. For reasons already stated, the latter were probably not large; moreover, additional depletions in population numbers must have occurred during various glaciation episodes, the last being about 15,000 years ago (Storer, 1989). Unfortunately, census records, in Saskatchewan at least, are poor prior to 1930 (Riegert, 1968). Nineteenth century accounts (see for example Smith, 1954 and Reigert, 1968) record, in qualitative terms, population sizes either for 'grasshoppers' in general of for *M. spretus*, the Rocky Mountain grasshopper, the major economic pest at that time. A conservative estimate of current population size in Saskatchewan can be obtained by multiplying a population density of three adults m^{-2} determined during non-outbreak years (Pfadt, 1988) by 16 million hectares, an estimated area of cultivated land for the province (Harris, 1987); this yields a 'guesstimate' of several billion insects. Despite the fact that *M. sanguinipes* is probably not uniformly distributed or that populations are not confined by political boundaries, it is safe to claim that the ratio of contemporary population size to N_e is astronomical, far greater than ratios estimated for vertebrates displaying similar low diversities and population structure (Avise *et al.*, 1988).

With respect to effective population size, note that, according to Pickford & Gillott (1972), mating preferences do occur in both sexes with some individuals coupling more frequently than others, some not at all. To the extent that these factors influence the number of reproducing offspring (mating frequency does influence fecundity — see Pickford & Gillott, 1972), the net result would be to increase the variance of family size, which in turn is expected to lower N_e (Nei, 1987).

The present mtDNA findings have implications for *M. sanguinipes*' population genetics. Many of the larger intraspecific diversity values in the literature (Table 4) are associated with differences between biotypes, races or subspecies, usually separated geographically; within locational variation, where estimated, is low. For example, the major portion of nucleotide diversity (1.97 per cent) within *Drosophila simulans* is ascribed to differences among three (generally) spacially isolated cytoplasmic races; only 0 to 0.046 per cent variation exists within each cytotype (Baba-Aïssa *et al.*, 1988). Non-*Drosophila* examples are *Schizaphis graminum* (Powers *et al.*, 1989) and *Glossina morsitans* (Trick & Dover, 1984). An intermediate example (Afonso *et al.*, 1990) is provided by *D. subobscura* in which 61.2 per cent of the mtDNA diversity ($\pi = 0.8$ per cent) is accounted for by population structure, with some locations possessing a fair amount of polymorphism (maximum $\pi = 0.47$ per cent in one case). At the other extreme, most (74.3 per cent) of the variation in *D. albomicans* (1.0 per cent) is represented by local polymorphism (Chang *et al.*, 1989), a situation not unlike that for *M. sanguinipes*. Some combination of an organism's historical past (see below) and gene flow could conceivably account for limited differentiation among sampled locations. Thus, if extant populations of *M. sanguinipes* are descendants of a polymorphic population that occupied a single refugium about 15,000 years ago (see below), the amount of inter-site divergence engendered up to the present would not be detectably large. This is expected to be true even in the unlikely event of no gene flow post-glacial colonization. Given the widespread occurrence of common haplotypes in Ontario and New Mexico, it seems more likely that present-day populations stemmed from more than one refugium. If ancestors occupying these regions were already genetically diverse, the present phylogeographic continuities could only be explained by gene flow. Parallel examples of organisms which lack population structure owing to recency of colonization and/or gene flow are marine fish (Becker *et al.*, 1988) and red-winged blackbirds (Ball *et al.*, 1988). Widespread panmixia should not, however, be automatically inferred on the basis of a perception of boundless movement as the biology of these organisms, including grasshoppers (Vickery & Kevan, 1983; Pfadt, 1988), would suggest. This point is nicely illustrated by studies of the cosmopolitan *D. melanogaster*, a species often regarded as a 'high migration' insect but for which populations are significantly differentiated for a variety of traits including mtDNA (Hale & Singh, 1987).

In the light of the above, the occurrence of clinal variation in hind tibia colour frequency in the region (Chapco & Bidochka, 1986) is all the more significant. Grasshoppers in parkland areas are predominantly red-legged, whereas they are predominantly blue-legged on the prairies. The cline is at least 25 years old; selective agents are unknown (Chapco & Bidochka, 1986). The absence of mtDNA differentiation between these two regions suggests that these forces must be sufficiently powerful to withstand the attenuating effects of gene flow.

Gurney & Brooks (1959) recognize three subspecies of *M. sanguinipes*. Of these, two are represented in this paper: *M. s. sanguinipes*, which occur in Canada and the northern United States, and *M. s. defectus*, which are found in the southwestern United States. *M. s. defectus* is somewhat larger than *M. s. sanguinipes* (Chapco, 1987). They interbreed freely under laboratory conditions to produce viable and fertile descend-

ants (Chapco, 1991). Haplotypes 3 and 4 are equally represented in the small New Mexico sample; both occur in Saskatchewan with haplotype 3 being the third most frequent. Further sampling is required to assess whether subspecies status is warranted. At one time (Brooks, 1958), prairie and parkland populations were regarded as possible subspecies but, as already indicated, there is very little difference between the two regions. The most northern site, Hudson Bay, possesses two haplotypes not found elsewhere. This location and more northern areas require further investigation.

To obtain a crude historical perspective of this insect, it may be useful to speculate on when pairs of haplotypes last shared a common (female) ancestor. The only insect for which evolutionary rates of substitution are available is *Drosophila*; values range from 0.12 to 3.13 per cent substitutions/base pair/lineage/million years (Solignac *et al.*, 1986). Based on the conventionally used rate of 1.0 per cent (Wilson *et al.*, 1985), which falls within this range, two randomly chosen haplotypes last shared a common ancestor about 133 thousand years ago. The two most similar and most dissimilar haplotypes last shared a common ancestor 89 thousand and 460 thousand years ago, respectively. Prior to deglaciation in Saskatchewan 10 to 17 thousand years ago, it is likely that *M. sanguinipes*, or its ancestor, occupied local (e.g Wood Mountain and Cypress Hills) or distant (e.g. Beringia or vast areas south of the ice shield; see Vickery, 1989) refugia, expanding into Saskatchewan only after prairie replaced Black Spruce forest which had perfused the area after deglaciation (Storer, 1989). Acknowledging the uncertainties that accompany such calculations (e.g. evolutionary rates, δ estimates, etc.), it is, nonetheless, probable that populations were already polymorphic or genetically differentiated with respect to two or more haplotypes before colonization and, possibly, before the last ice age.

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