Relationship Between Chromosomal Races/Subraces in the Brachypterous Grasshopper *Podisma sapporensis* (Orthoptera: Acrididae) Inferred from Mitochondrial ND2 and COI Gene Sequences

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Ann. Entomol. Soc. Am. 101(5): 837–844 (2008)

ABSTRACT *Podisma sapporensis* Shiraki, 1910 (Orthoptera: Acrididae) is distributed on the islands of northern Japan and Far East Russia (Hokkaido, Sakhalin, and Kunashiri), and it exhibits a unique diversity of chromosomal races (X0 and neo-XY) on the island of Hokkaido. To infer the history of geographical isolation and chromosome rearrangements (main races and subraces), we used mitochondrial DNA (mtDNA) sequence variation in two regions, NADH dehydrogenase subunit 2 (ND2) and cytochrome oxidase subunit II (COII), among 55 individuals representing eight X0/XX and three neo-XY/XX populations. The molecular phylogenetic tree revealed a high level of overall mitochondrial diversity, but the clustering of the examined population is in most cases closely related to their geographic distribution and associated with chromosomal races and subraces. Together with cytogenetic observations, we discuss the origin of the polymorphism within the X0 and XY races as well as differences between both northern and eastern races.

KEY WORDS mtDNA, chromosome rearrangement, phylogeography

Chromosomal rearrangements are considered to promote speciation because hybrids between different chromosomal races sometimes suffer reduced fertility due to chromosomal aberration. If the influence on hybrid fitness is less extreme, chromosomal rearrangements, jointly with genic differences, may hamper gene flow between chromosomal races and/or subraces (Searle 1998). Chromosome rearrangements and their role in speciation are well known phenomena in various organisms. Several grasshopper species are known to show complicated chromosomal polymorphism across their geographical distribution (White 1973, 1974; Shaw et al. 1976; Moran and Shaw 1977, Coates and Shaw 1982, Hewitt 1979, John 1983, Gosalvez et al. 1997).

Podisma sapporensis Shiraki, 1910 (Orthoptera: Acrididae) is distributed on the islands of northern Japan and the Russian Far East: Hokkaido, Sakhalin and Kunashiri. This species consists of two main sex chromosome systems. In one, the diploid number of chromosomes is 2n = 23 (XO) in males and 2n = 24 (XX) in females (XO/XX race). In the other, the diploid number is 2n = 22 (XY) in males and 2n = 22 (XX) in females (XY/XX race), owing to Robertsonian fusion between a M_5 autosome and the X chromosome (Bugrov et al. 2000). The X0/XX and XY/XX races are each divided into chromosomal subraces on the basis of variation in pericentric inversions (Bugrov et al. 2001, Tatsuta et al. 2006). Various levels of polymorphism resulting from the pericentric inversions, C-banding variation, and additional (B) chromosomes were revealed in some populations in both chromosome races (Bugrov et al. 2001, Warchałowska-Śliwa et al. 2001, Bugrov et al. 2003). Polymorphism in chromosomal structure can be found in some populations of each subrace, probably due to genetic drift resulting from relatively small population sizes and low levels of migration. Although a hybrid population between the two chromosomal races has not yet been found in the field (Bugrov et al. 2000), phylogenetic analysis based on mtDNA variation has implicated that genetic introgression occurs between adjacent populations of the X0/XX and XY/XX races in northeastern Hokkaido (Tatsuta et al. 2006).

Previous studies have revealed the high level of chromosome differentiation in the geographically isolated populations of brachypterous grasshopper *P. sapporensis* on the island of Hokkaido. The observed level of diversity in karyotypic structure in Hokkaido exceeds the known level of differentiation found in the *trans*-Palaearctic genus *Podisma.* This finding makes *P. sapporensis* useful for exemplifying chromosomal spe-

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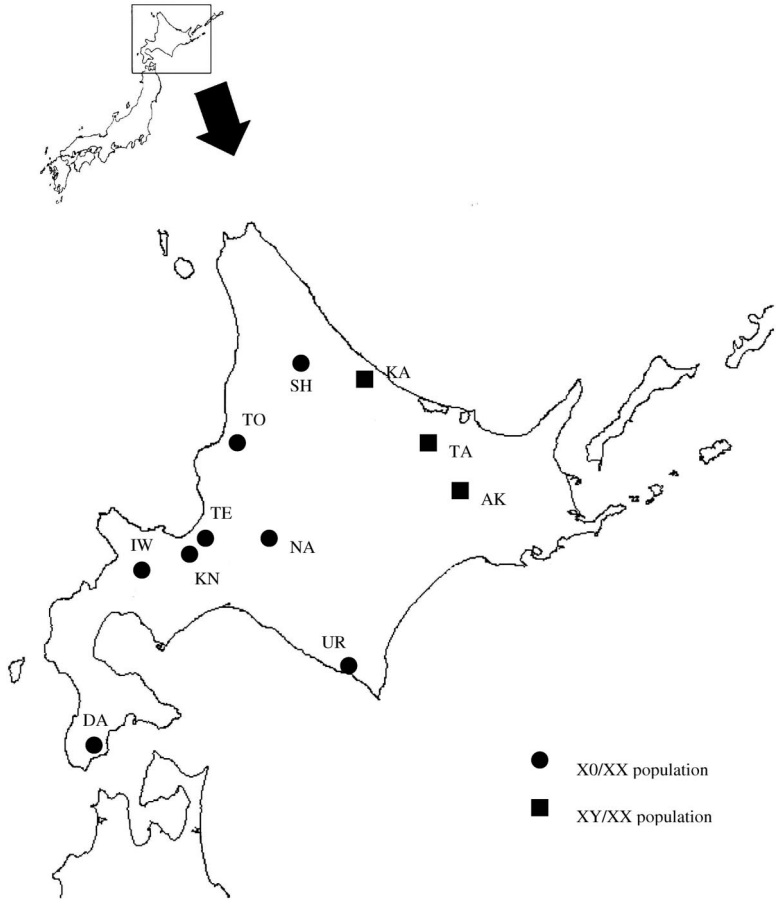

Fig. 1. Distribution of localities of *P. sapporensis*.

ciation resulting from various levels of differentiation in chromosomal structure in insects.

Mitochondrial DNA (mtDNA) is a highly informative genetic marker showing extensive sequence polymorphisms within animal species (Avise 1991). A previous study on the molecular phylogeny of *P. sapporensis* suggested the possibility of a polyphyletic origin of the XY/XX race and/or introgression of mtDNA between adjacent populations possessing different karyotypes (Tatsuta et al. 2006). In the current study, we examined whether the suggested hypotheses still held when variation in mtDNA sequences was analyzed in individuals from other populations belonging to each chromosomal race. Here, we analyzed partial sequences of NADH dehydrogenase subunit 2 (ND2) and cytochrome oxidase subunit II (COII) of *P. sapporensis* belonging to eight X0/XX and three XY/XX populations from the eastern part of Hokkaido. We used five individuals per locality for intrapopulation analysis. These populations include representatives of chromosome races/subraces found in *P. sapporensis.* According to the phylogenetic tree inferred from mtDNA sequencing data, we discuss the taxonomic status of the two main chromosomal races X0/XX and XY/ XX, and their subraces.

Materials and Methods

Sample Collection and mtDNA Sequencing. Material used in this study was collected from July to August 2001–2005. Fifty-five individuals belonging to 11 populations (five individuals from each locality) were examined in this study (Fig. 1). The collecting sites and karyotypes are listed in Table 1. Karyotypes of six populations, i.e., Shimokawa, Mt Teine, Naganuma, Mt Daisengen, Akan, and Tanno, are described in Bugrov et al. (2001), whereas the karyotypes of specimens from the other five populations, i.e., Togeshita, Iwaonobori, Kannonzawa, Urakawa, and Kamirubetsu, are reported in the current study (Table 1). On the basis of chromosome polymorphism, specimens from Urakawa and Kannonzawa are described as "hybrid" types X0/XX Naganuma × Yotei and X0/XX Standard × Yotei, respectively.

Specimens used for mtDNA analysis were preserved in 98% ethanol at −70°C. DNA was extracted from the muscle of the hind legs using the DNeasy tissue kit (QIAGEN GmbH, Hilden, Germany). mDNA sequences from a fragment of the ND2 gene were amplified using forward primer ND2A 5′-CGTTGATGATAGGAACGTACC-3′ and reverse primer

Table 1. Analyzed population, their membership to two mainly sex chromosome races and subraces

Locality name	Abbreviation of locality name	Lat (°)	Long (°)	Chromosome race/subrace
Shimokawa	SH	44.22	142.33	X0/XX-Standard
Togeshita	TO	43.85	141.82	X0/XX-Standard
Mt Teine	TE	43.10	141.22	X0/XX-Standard
Naganuma	NA	43.00	141.68	X0/XX-Naganuma
Iwaonobori	IW	42.87	140.65	X0/XX-Yotei
Kannonzawa	KN	42.97	141.27	X0/XX-Standard × Yotei
Urakawa	UR	42.18	142.77	X0/XX-Naganuma × Yotei
Mt Daisengen	DA	41.58	140.15	X0/XX-Daisengen
Akan	AK	43.45	144.05	XY/XX-Standard
Tanno	TA	43.83	143.95	XY/XX-Tanno/Oketo
Kamirubetsu	KA	44.22	143.32	XY/XX-Tanno/Oketo

ND2B 5′-GGTGTCTAATTGAATGAATATGC-3′ (Flook et al. 1995), whereas the COII region was amplified using forward primer 3′CGTTGATGATAGGAACGTACC5′ and reverse primer 3′GGRGTCTAATTGATGAATATGC5′ (Simon et al. 1994).

Each amplification was performed in a 50-μl volume containing 1.0 μl of DNA extract, 20 pmol of primers, 10 nmol of dNTPs, 1.5 U of *Taq* polymerase (QIAGEN GmbH) and 5.0 *Taq* buffer (containing mM $MgCl_2$). Amplifications were performed in a T-personal thermocycler (Biometra, Göttingen, Germany). Amplification conditions were as follows: an initial denaturation of 95°C for 5 min followed by 10 cycles of 95°C for 15 s, 45°C for 30 s, and 68°C for 50 s. For the final 25 cycles, cycling was identical to the first 10 steps except that the annealing temperature was raised to 50°C and an additional 10 s was added to the primer extension step for each cycle (Litzenberger and Chapco 2001). Polymerase chain reaction (PCR) products were resolved by electrophoresis in 1% agarose gels. The PCR product was purified using the QIAquick PCR purification kit (QIAGEN GmbH) and directly sequenced using the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, CA) and an ABI Prism 377 DNA automated sequencer (Applied Biosystems, Foster City, CA).

Data Analysis. All sequences were aligned using the Clustal W program (Thompson et al. 1994) in BioEdit version 5.0.9 (Hall 1999) computer software. Genetic polymorphism and diversity in regions and populations were quantified by haplotype diversity (h) and nucleotide diversity (π), by using the software package DNASP version 4.10.9 (Rozas et al. 2003). For other population analyses, we used the program ARLEQUIN version 3.1 (Schneider et al. 2000). Genetic differentiation between pairs of population samples was estimated by the pairwise fixation index (F_{ST}); significance of the F_{ST} values was ascertained by 10,000 random permutations. The amount of variation within and between populations was inferred using an analysis of molecular variance (AMOVA; Excoffier et al. 1992). Phylogenetic analyses were performed using PAUP 4.0b10 (Swofford 2001). Maximum parsimony (MP) was used to construct the phylogenetic trees. The most parsimonious trees were generated using heuristic search with 100 replicates of random taxon additions run with tree bisection reconnection (TBR) branch swapping, holding five trees at each step during stepwise addition. The MP analysis was performed by bootstrapping with 1,000 replicates using unweighted characters. We assessed the length differences between the ND2 and COII data sets by using the partition homogeneity test (Farris et al. 1995). We used this test to assess the possibility of simultaneous analysis of the combined data.

Results

Sequence Analysis. The ND2 and COII sequences identified in this study have been registered in DDBJ/GenBank/EMBL with accesion numbers DQ859723–DQ859733 for ND2 and EF108110–EF108120 for COII. The amplified size of the ND2 and COII gene fragments was 474 and 376 bp, respectively. In addition, all translated sequences revealed high amino acid sequences homology with *Locusta migratoria* (L.) ND2 and COII sequences. Furthermore, both of the sequenced fragments showed the typical content AT bias of insect mitochondrial DNA, i.e., 69.98% and 72.78% for COII and ND2, respectively.

Both mtDNA fragments were concatenated and analyzed together because they represent the same evolutionary history, and longer sequences provide better phylogenetic signal. This combined data set revealed 21 unique haplotypes, with 19 polymorphic nucleotide sites (Table 2). The western population belonging to the X0/XX chromosome race consisted of 17 haplotypes, and the eastern population (AK, TA, KA) belonging to the XY/XX chromosomal race contained four haplotypes. Within populations, most haplotypes were unique to their collecting locality, i.e., private alleles. However, all individuals from NA and IW populations have the same haplotype. It also should be noted that in the DA population all individuals possessed different haplotypes. Moreover, four different and unique haplotypes were found in the TO population.

Haplotype and nucleotide diversities for each population are shown in Table 3. Haplotype polymorphism was found in DA, KN, TO, and UR (the X0/XX race) populations, and also in AK and TA (the XY/XX race) (Table 3). The nucleotide diversities (π) within

Table 2. Sequence variation of the ND2 and COII partial sequence in each individuals from 11 populations

	Location	ND2 gene													COII gene					
		0	1	2	2	2	3	3	3	3	3	4	4	4	4	6	6	7	8	8
		7	9	1	2	6	1	1	3	5	7	0	2	6	9	8	9	6	0	0
		1	2	1	4	9	1	8	5	7	2	3	9	2	0	5	1	9	2	5
1	AK 1–3	A	G	C	A	T	A	C	G	G	A	C	T	A	A	T	T	A	A	A
	TA 1–3,5	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.
2	AK 2,5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
3	AK 4	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G
4	DA 5	.	.	.	.	.	T	T	A	.	.	.	.	C	.	C	C	G	G	G
5	DA 3	.	.	.	.	.	T	T	A	.	.	.	.	C	.	C	.	G	G	G
6	DA 4	.	.	.		C	T	T	A	.	.	.	.	C	.	C	C	.	G	G
7	DA 1	.	.	.	.	.	T	T	A	.	.	.	.	C	C	C	C	.	G	G
8	DA 2	.	.	.	.	.	T	T	A	.	.	.	.	C	.	C	C	.	G	G
9	KA 1–5	.	A	.	.	.	T	T	.	.	.	T	.	C	.	.	.	.	.	.
10	KN 3–4,5	.	.	.	.	.	T	.		.	C	.	.	.	.	C	.	G	.	G
11	KN 1–2	.	A	.	.		T	T	A	.	.	.	A	C	.	C	.	G	.	G
12	NA 1–5	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	G
	IW 1–5	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	G
13	SH 1–5	.	.	.	.	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.
14	TA 4	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
15	TE 1–5	.	A	.	.	.	T	T	A	.	.	.	.	C	.	C	.	G	.	G
16	TO 2	.	A	.	.	.	T	T	A	T	.	.	.	C	.	C	.	.	.	.
17	TO 3–4		A	.	.	.	T	T	A	T	.	.	.	C	.	C	.	.	.	G
18	TO 1	.	A	.	.	.	T	T	A	.	.	.	.	C	.	C	.	.	.	.
19	TO 5	.	A	.	.	.	T	T	A	.	.	.	.	C	.	C	.	.	.	G
20	UR 1,3–4	.	A	.	.	.	T	T	A	.	.	.	A	C	.	.	.	.	.	.
21	UR 2,5	C	A	.	G	.	T	T	A	.	.	.	A	C	.	.	.	.	.	.

Dots indicate nucleotides identical to the haplotype from AK1.

each population varied from 0.000 to 0.004, reflecting the high similarity of sequences within populations in both groups (X0/XX and XY/XX). Within the western part of Hokkaido, belonging to the X0/XX chromosomal race, the gene diversity varied from h = 0 in NG-IW, SH, and TE to h = 0.9 and 1.00 in TO and DA, resulting from four and five haplotypes, respectively. Within the eastern group of populations belonging to the XY/XX chromosomal race, haplotype diversity (h) varied from 0 in the KA locality (a unique haplotype), to 0.8 in Akan with three haplotypes. Thus, in the eastern group of populations belonging to the XY/XX chromosomal race, the mean nucleotide diversity ($\pi = 0.0006$) and haplotype diversity (h = 0.4) were lower than in western populations. Populations of the western part of Hokkaido belonging to the X0/XX chromosomal race showed higher nucleotide diversity than eastern populations ($\pi = 0.0011$), but the same haplotype diversity as eastern populations (h = 0,4).

Table 3. Haplotype diversity (h) and nucleotide diversity (π) for 11 localities

Location	No. haplotypes	h between pop	π between pop
AK	3	0,8	0,0012
KA	1	0	0
TA	2	0,4	0,0005
DA	5	1	0,0021
KN	2	0,6	0,0042
NA	1	0	0
IW	1	0	0
SH	1	0	0
TE	1	0	0
TO	4	0,9	0,0014
UR	2	0,6	0,0014
Mean ± SD		0.391 ± 0.387	0.001 ± 0.0013

Population Analyses. No significant difference was found in haplotype constitution between the western X0/XX and southern XY/XX chromosome races (AMOVA: $F_{CT} = 0.133$, $P = 0.149$). Populations within the western and eastern groups showed significant geographical structuring, with a higher percentage of genetic variance among populations. In the west (X0/XX race) and east (XY/XX race) groups, 80.67% and 87.41%, respectively, of genetic variance was attributed to differences among populations ($F_{ST} = 0.806$, $P < 0.001$ and $F_{ST} = 0.874$, $P < 0.001$, respectively). Pairwise comparisons of F_{ST} between all populations are shown (Table 4). Most of them were significant at the 0.05 level ($F_{ST} = 0.55–1.00$), except for the population pairs: TE-KN, TA-AK, and IW-NA. However, only one pair, DA-IW, showed significant after sequential Bonferroni adjustment (Table 4).

The haplotype diversity was used to construct an MP tree. In most cases, the haplotypes were clustered according to the population to which they originally belonged, except for the haplotypes in the KN population. Indeed, the haplotypes were split into two clades (Fig. 2). The first clade (A) was composed of specimens from two populations belonging to the XY/XX standard subrace: both AK and TA were collected in the eastern part of Hokkaido. This clade did not include the standard XY/XX subrace collected from the KA population at the northern part of the range of this chromosome subrace. The second clade (B) includes all specimens belonging to eight populations from the X0/XX chromosomal races. These localities are distributed in the western part of Hok-

Table 4. Matrix of pairwise F_{ST} values for populations (below diagonal) and probability of Fst (above diagonal)

	AK	DA	KA	KN	NA	IW	SH	TA	TE	TO	UR
AK	—	0.00781	0.00781	0.00977	0.00879	0.00781	0.00488	0.15332	0.00586	0.00879	0.00977
DA	0.820*	—	0.01465	0.00586	0.00391	0.00070	0.0104	0.00684	0.01270	0.00781	0.01074
KA	0.907*	0.882*	—	0.0087	0.00195	0.00977	0.00781	0.01270	0.00684	0.00879	0.00781
KN	0.619*	0.550*	0.750*	—	0.01172	0.00977	0.00977	0.00977	0.09277	0.00977	0.00781
NA	0.687*	0.864*	1.000*	0.609*	—	0.99902	0.01172	0.00684	0.01172	0.00977	0.00977
IW	0.687*	0.864**	1.000*	0.609*	0.000	—	0.01074	0.01172	0.00391	0.00879	0.00391
SH	0.821*	0.864*	1.000*	0.690*	1.000*	1.000*	—	0.00488	0.00488	0.01074	0.00684
TA	0.239	0.869*	0.958*	0.699*	0.909*	0.909*	0.909*	—	0.00977	0.00684	0.00684
TE	0.930*	0.763*	1.000*	0.471	1.000*	1.000*	1.000*	0.974*	—	0.00781	0.0086
TO	0.840*	0.674*	0.857*	0.556*	0.900*	0.900	0.885*	0.886*	0.700*	—	0.00586
UR	0.847*	0.797*	0.842*	0.676*	0.912*	0.912*	0.875*	0.879*	0.875*	0.700*	—

* Significant at $P < 0.05$ level, without sequential Bonferroni's correction.
** Significant after sequential Bonferroni's correction, at the 0.0007 level.

kaido. This group was poorly separated from the others (XY/XX) in the phylogenetic analysis for both genes. Additionally, this clade included the KA population belonging to the XY/XX chromosome race. In the tree topology, the northwestern population SH included all specimens from this locality (B1). The clade B2 includes populations from the X0/XX chromosome race: TO, TE, DA, and UR populations, and two individuals from the KN population. The population of Mt Daisengen (DA), which is located in the southern part of Hokkaido, formed a group supported by a bootstrap value of 63%. The clade B3 includes all specimens belonging to the KA population belonging to XY/XX-Tanno-Oketo subrace, distributed in the northeastern part of Hokkaido. The southwestern populations (clade B4) IW and NA are grouped together with the three remaining individuals from the KN population.

Discussion

The molecular results obtained in this study generally supported the patterns of chromosome divergence inferred from some cytogenetic observations. The brachypterous grasshopper *P. sapporensis* is known for its remarkable karyotypic diversity. The two geographically isolated populations belonging to the two main chromosome races are genetically and karyologically differentiated. All eastern haplotypes from the X0/XX race are grouped together (clade B). However, haplotypes belonging to the XY/XX race were grouped together in AK and TA (clade A); they were clustered within eastern haplotypes, except for the KA population (clade B_3). The KA population is very close distributed to TAN and KIM populations that did not show monophyly with other XY/XX populations (Tatsuta et al. 2006) and can be found near the contact zone of the two chromosomal races. The discrepancy in the relationship between karyotype and mtDNA haplotype could be due to multiple translocation between X chromosome and M_5 autosome or introgression of mtDNA (Tatsuta et al. 2006). However, it is less likely that translocation between X chromosome and a particular autosome has occurred multiple times. Interestingly, most F_1 hybrids from cross-breeding between the XY/XX-Tanno-Oketo and X0/XX-standard populations (Bugrov et al. 2004, Warchałłowska-Śliwa et al. 2008) have developed normally, whereas cross-experiments between the X0/XX-Sakhalin and XY/XX standard of Kunashiri races showed F_1 male sterility (Tchernykh and Bugrov 1997). These facts suggest that introgression between some particular karyotypes is possible in the wild even though they have different sex-determination system.

The sequence analyses detected polymorphism in ND2 and COII within both eastern and western populations. Indeed, a high overall mitochondrial diversity was revealed, whereas the levels of intrapopulation diversity were also high (Table 4). The phylogenetic analyses suggest that the eastern clade (B) is divided into three geographically separated subclades (B_1, B_3, and B_4) and one subclade (B_2), including individuals from five populations that did not exactly coincide with their geographical distribution. Some of the nodes defining these clades have a low bootstrap value due to few substitutional differences in the sequence data. Clade B_1, contains a basal subclade located in the northeastern part of Hokkaido, characterized by a unique haplotype, and was connected with the X0-standard chromosome subrace. These findings suggest that this region might be founder population for most of haplotypes in the eastern B_2 and western B_3 clades. Clade B_3, consisting of individuals belonging to the KA population from the XY/XX-Tanno-Oketo subrace, is distributed in the northern part of Hokkaido, near the border of two main chromosomal races. The two southeastern populations (clade B_4), IW and NA, were grouped together. From cytogenetic observations, these populations were described as X0/XX Yotei and X0/XX Naganuma subraces, respectively. The X0/XX Yotei subrace differs from other localities of *P. sapporensis* in respect of a fixed pericentric inversion with a short euchromatic arm in M_4 and the X-chromosome, as well as heterochromatic second arms in M_5 and M_8-S_{10} pairs. The samples from Naganuma were homozygous for the pericentric inversion in M_6. (Bugrov et al. 2001). The clade B_4 near the population mentioned above included three individuals from the KN locality, whereas the remaining two samples were placed in subclade B_2 together with the TE and TO (X0-Standard subrace) populations. Specimens from the KN

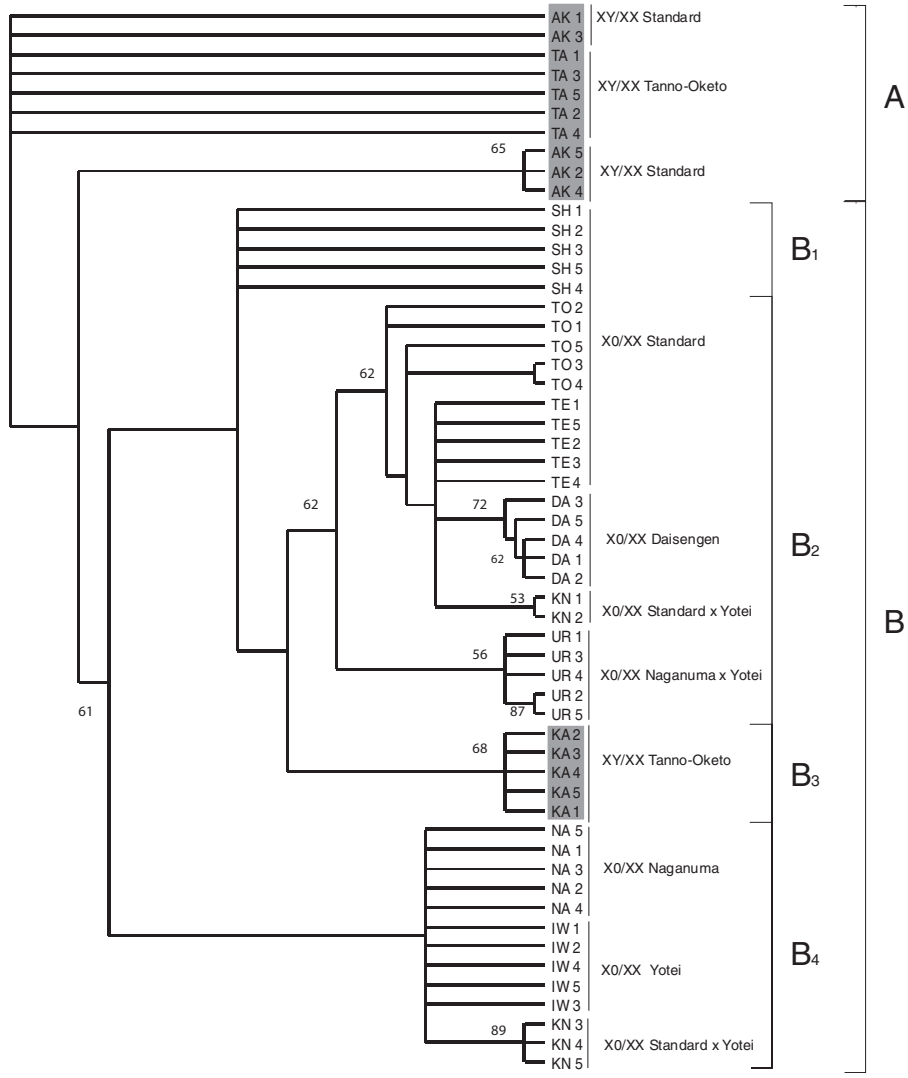

Fig. 2. Bootstrap majority rule consensus tree. Chromosomal races and subraces are also given on the right. Bootstrap percentage above 50% are provided on the side of nodes.

population are distributed on the border of the X0/XX-Standard and X0/XX-Yotei subraces and thus were putatively classified as the X0/XX Standard × Yotei hybrid. Similarly, the five individuals from the UR population formed a group that was supported by bootstrap values of 59%. This population is located in the southern part of Hokkaido and was putatively classified as the X0/XX Naganuma × Yotei hybrid.

The mtDNA analysis also suggested a high degree of concordance between sequence divergence and karyological differences. Samples from the DA population sharing five haplotypes (Table 2) were supported by a bootstrap value of 63%. Cytological observations revealed that this population, collected around the summit of Mt Daisengen (1,072 m above sea level, X0-Daisengen subrace), showed conspicuous differences in karyotypes compared with other populations. Each chromosome in the Daisengen subrace differs from the analogous chromosome in the X0-Standard subrace in terms of the C-positive short second arm (Bugrov et al. 2001, 2003). In reality, any populations have not been found except the DA population in southern Hokkaido area and therefore the DA population is considered to have been isolated for longer period compared with other populations examined in the current study. Such a unique history may have facilitated peculiar modifications of chromosomal and mtDNA features. The fact that single significant pairwise F_{ST} value after Bonferroni adjustment was found between DA and IW population underpins the perspective.

Thus, mtDNA clustering of the examined *P. sapporensis* populations was in most cases closely related to their geographic distribution and associated with chromosomal races and subraces. The low level of gene flow and modest genetic divergence between

populations probably support their subspecies designations. The mtDNA analysis implied that the karyological differences between the two main chromosome races may be an important factor in their evolutionary trajectories. Similar observations have been reported in small mammals. For example, in the Rb-chromosomal races in *M. m. domesticus* and other rodents, karyotypic variation clearly has evolutionary consequences (Hauffe et al. 2002, Zambelli et al. 2003).

Unfortunately, the current study did not give additional information to determine whether the discrepancy between chromosomal status and phylogeny based on mtDNA haplotype that is found in northeast Hokkaido is due to multiple origin of Robertsonian fusion between X chromosome and M_5 autosome or introgression of mtDNA. To elucidate this problem, it would be necessary to ascertain whether alleles of some feasible nuclear markers that are originated from either chromosomal race contain heterozygous combinations in the corresponding area.

Acknowledgments

This study was partially supported by the Polish State Committee for Scientific Research (2P04C 08826 to E.W.-S.).

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Received 6 July 2007; accepted 17 March 2008.