Theor Appl Genet (2000) 101:718–723

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Pollen flow and the intrapopulation genetic structure of *Heloniopsis orientalis* on the forest floor as determined using microsatellite markers

Received: 13 January 2000 / Accepted: 21 March 2000

Abstract Pollenflow, seedling dynamics, and the genetic structure of *Heloniopsis orientalis* (Thunb.) C. Tanaka (Liliaceae) were analyzed as part of an effort to understand the reproduction system of perennial herbs found on the forest floor by means of microsatellite markers. It was possible to assign paternity to all offspring within this population by paternity analysis. Although no ordinary pollinator visits were observed during the flowering period, effective pollen flow occurred throughout the population. It appears that low frequencies of pollinator visits nonetheless permitted adequate pollen flow. Studies of seedling dynamics suggested restricted recruitment by means of seed. Gene flow in the present population seemed to depend mainly on pollen. Local genetic structuring caused by seeds falling near the mother plant and by vegetative reproduction was detected by means of spatial autocorrelation. Three main modes of development of genetic structure were found for this species: (1) asexual reproduction by adventitious buds, which was dominant (up to 20 cm from the parent plants); (2) sexual reproduction by seeds that fell from flower stalks (up to 60 cm from the parent plants); and (3) pollen that dispersed over a wide area (throughout the population).

Key words Genetic structure · Microsatellite · Gene flow · Spatial autocorrelation · Paternity analysis

Communicated by P.M.A. Tigerstedt

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Introduction

Resources for plants are limited on the forest floor. For example, relatively low temperatures and low light intensity constrain pollinator behavior, and litter cover interferes with seedling establishment. How do plants reproduce under these limited resource conditions on forest floor? In order to understand the actual reproduction systems of plants that grow there, it is necessary to examine gene flow patterns and a plant population's genetic structure.

In flowering plants, gene flow occurs through the movement of pollen and seeds. The extent and patterns of pollen and seed dispersal directly affect changes in gene frequencies and genetic structures within populations. As well, ecological factors such as the physical distance between populations, the number of individuals flowering at any given time, the distances between flowering individuals, and the interactions among these factors may all influence pollinator behavior and, thus, gene flow in insect-pollinated plant species.

Detecting pollen dispersal by direct observation is difficult, and only a few studies on plants have directly examined pollen dispersal and gene flow patterns (e.g., Dow and Ashley 1996; Isagi et al. 2000; Richards et al. 1999; Streiff et al. 1998, 1999). For this reason, we used precise genetic markers (microsatellites) to measure gene flow between individuals as well as the population's fine-scale genetic structure. The advantages of microsatellites are that they are codominant, tend to have many alleles, and have high heterozygosity. The large number of microsatellite loci and their high variability make microsatellites potentially important tools for almost any problem that requires Mendelian markers. Spatial distributions of alleles can be used to study local gene flow, including pollen dispersal distances (Queller et al. 1993).

Heloniopsis orientalis (Thunb.) C. Tanaka (Liliaceae), an evergreen perennial herb that grows in the form of a rosette, is distributed widely throughout the temperate zone of Japan and the Korean Peninsula. Flower stalks arise from the center of the rosette of leaves and elongate

to about 15 cm in height during the flowering period. Reproduction in *H. orientalis* is either sexual or by vegetative means. This species is reported to be self-compatible and is pollinated by common insect species such as those in the Diptera and Hymenoptera (Takahashi 1988). The number of seeds produced per plant is very high in the field, and these seeds are dispersed by the wind. *H. orientalis* also reproduces vegetatively through buds that form at the tips of its leaves. When the buds on the leaf tips touch moist ground, they initiate roots and produce independent plants. *H. orientalis* grows on relatively open, moist microsites on the floor of secondary oak (*Quercus* spp.) forests, along forest edges, and on the footpaths in rice paddies. In the study area, populations of this species were primarily distributed in small valleys on the forest floor.

Materials and methods

Study site

The studies were carried out near Kyotanabe City, in Kyoto Prefecture, southwestern Japan (Fig. 1a,b; lat 35°01'N, long 135°44'E, about 100 m asl). The mean annual air temperature and precipitation in this area were 16°C and 1570 mm, respectively. Several *H. orientalis* populations along the small valleys in this area.

In order to investigate the intrapopulation genetic structure in detail, we chose an isolated population distributed within a square research plot approximately 20 m across (Fig. 1c). We mapped the locations of all individuals in this population that flowered in 1998 (Fig. 2). The study population was isolated physically, with the nearest population of the same species located more than 200 m away. This physical isolation enabled us to differentiate between gene flow within the population and gene flow between populations. The study population had sufficient flowering individuals for the purposes of this study; in particular, the resources available to pollinators did not restrict gene flow in the population. We established two subplots within the research plot to investigate the degree of vegetative reproduction and spatial autocorrelation on a fine scale (Fig. 2) and mapped the locations of all individuals in these subplots (Fig. 3a,b). Seedlings were found at few sites within the research plot; the five germination sites (labeled G1 through G5) are shown in Fig. 2. Subplot A had no germination sites, but subplot B included germination site G1.

DNA extraction and microsatellite analysis

DNA was extracted from all individuals in the research plot that flowered in 1998 (N = 196). DNA samples were also extracted from the offspring (n = 32) of one mother plant and all vegetative ramets (n = 46) in subplot A; in subplot B, the offspring (n = 92) of three mother plants and all ramets (n = 34) were assayed. The extraction, amplification, and detection procedures for microsatellites are described in Miyazaki et al. (1999). In this study, eight loci were used.

Pollen flow

Takahashi (1988) reported that many species of Diptera and Hymenoptera visit the flowers of this species. Because the anthers of this species do not open on rainy days, we observed pollinator visits to *H. orientalis* only on fine or cloudy days during the flowering period: 4 days in each of 1996 (April 3, 6, 9 and 13) and 1998 (March 3, and April 3, 7 and 12), as well as 6 days in 1999 (March 30,and April 1, 5, 11, 12, and 16).

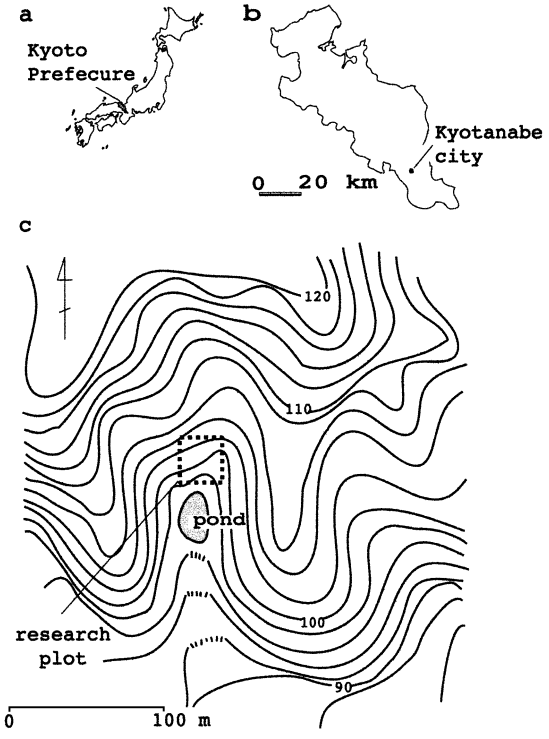

Fig. 1a–c Maps of study site. **a** Japan and Kyoto prefecture **b** Kyoto prefecture and Kyotanabe city, **c** study site

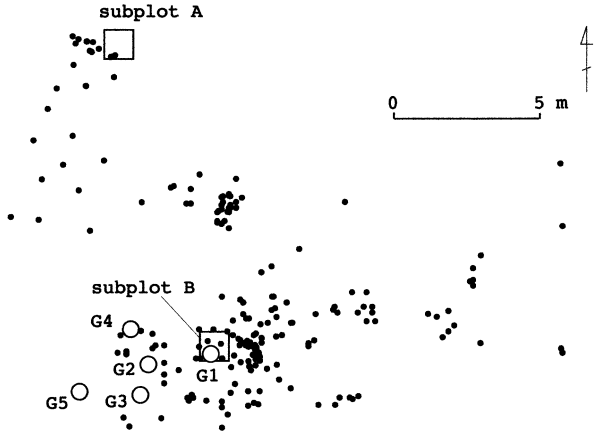

Fig. 2 Maps of all individuals that flowered in 1998 in the research plot (●). The locations of subplot A and subplot B are shown. *Open circles* (○) germination sites (*G1–G5*) in the study site

In order to estimate of the effective pollination distance, we collected seeds from one mother plant in subplot A and three mother plants in subplot B. Microsatellite analysis was conducted for the seedlings that germinated from the sample seeds and from all flowering individuals in the research plot (to assign paternity). The Microsoft Windows-based computer program CERVUS 1.0 (Marshall et al. 1998) was used to perform the paternity analysis. Paternity is assigned to a particular pollen parent if the likelihood ratio is large relative to the likelihood ratios for alternative pollen parents. The likelihood ratio is expressed in terms of LOD scores, which represent the logarithm of the likelihood ratio. The significance level of LOD scores cannot easily be derived analytically, but it has been shown that the ratio for two males provides a true

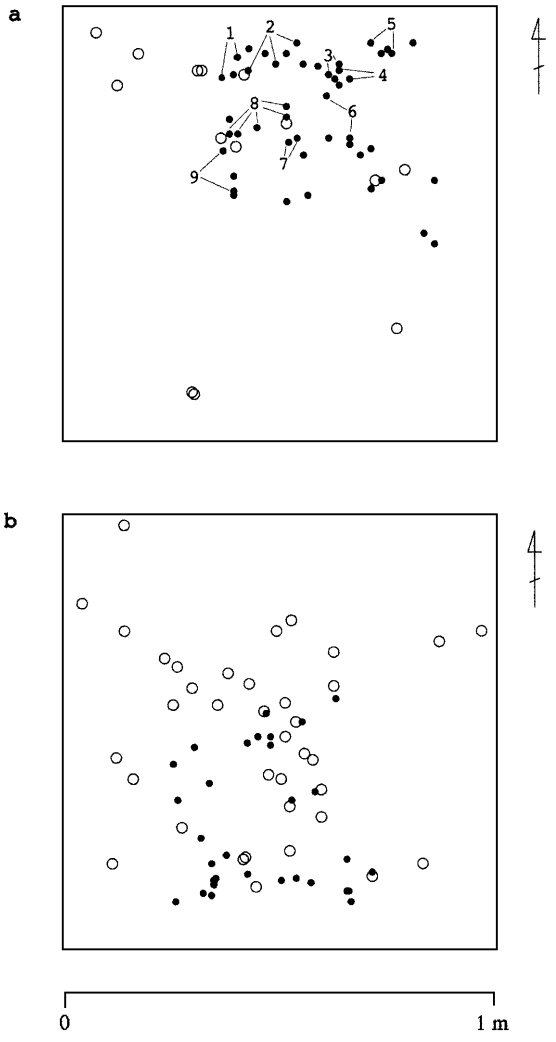

Fig. 3a, b Map of all individuals in subplot A (**a**) and subplot B (**b**). *Open circles* (○) indicate individuals that flowered in 1998. *Circles* identified by the same number (*1–9*) in subplot A represent identical clones

comparison of the various father-offspring relationships (Marshall et al. 1998).

Seedling dynamics

The seedlings that germinated in 1995 were observed at germination sites G1 through G5 from 1995 to 1997.

Genetic structure

''Coancestry'' measures the correlation between the frequencies of two homologous alleles (p_i and p_j) at a given locus for pairs of mapped individuals (i and j). We calculated the coefficient of coancestry (r) to examine the spatial autocorrelation for (1) all individuals that flowered in 1998 within the research plot, (2) all individuals in subplot A, and (3) all individuals in subplot B. The calculations used the estimation procedure of Loiselle et al. (1995). The coefficients of coancestry (r_{ij}) can be estimated as follows:

$$r_{ij} = \sum_{ij} \frac{(p_i - \bar{p})(p_j - \bar{p})}{k\bar{p}(1-\bar{p})} + \frac{2}{\sqrt{8k+1}-1} (i < j),$$

Table 1 Summary data of genetic characters

	Whole research plot	Subplot A		Subplot B			
	Individuals that flowered in 1998	All individuals	Offspring from	All individuals	Offspring from		
			parent a		parent b	parent c	parent d
Number of individuals	196	46	32	34	30	33	29
Mean number of alleles per locus	16.75	5.63	4.63	6.88	5.50	5.13	4.13
Mean expected heterozygosity	0.684	0.562	0.462	0.565	0.547	0.525	0.445

Table 2 Results of paternity analysis (*SE* standard error)

	Offspring from			
	Parent a	Parent b	Parent c	Parent d
Number of offspring	32	30	33	27
Number of self-pollination	7	4	9	9
Number of pollen parents	13	21	15	10
LOD scores:	5.427	6.651	4.757	5.705
Average				
SE	0.196	0.266	0.179	0.198
Minimum	3.946	3.806	3.330	3.949
Maximum	8.684	9.780	7.783	7.889

where the first term on the right side is the expected value of r_{ij}, and k=n(n-1)/2, which represents the total number of possible pairwise connections between *n* individuals. The second term adjusts for the bias attributable to the use of a finite sample size, and results in r_{ij} having expected values of zero for a population that is in Hardy-Weinberg equilibrium. For discrete distance intervals, the mean values of r_{ij} were obtained by summarizing over all possible pairs of individuals located that distance apart. The results were combined over all loci by weighting the result for each locus by its polymorphic index, $\Sigma p_i(1-p_i)$, to obtain a multilocus measure of spatial genetic structure.

Tests of significance for the estimated r_{ij} values were performed using randomization procedures to generate populations under the null hypothesis (i.e., that there is no spatial genetic structure). The occupied map locations were randomly assigned intact multilocus genotypes drawn at random, with replacement from the sample population. To permit the calculation of a 95% confidence interval, we required 399 simulation trials. For a given distance class, values of r_{ij} from 399 simulation trials were ordered, and the 10th and 390th r_{ij} values were used to construct a 95% confidence interval around the null hypothesis. We used $r_{(data)}$ (i.e., the estimate based on the actual data) as the 400th statistic, and the null hypothesis of $r_{ij} = 0$ was rejected at the $\alpha = 0.01$ level when $r_{(data)}$ was found to fall outside the 95% confidence interval. Significant values of r_{ij} were considered to indicate genetic structuring as a result of isolation by distance (Wright 1943,1946) due to nonrandom dispersal of localized pollen, seed movement, vegetative reproduction, or a combination of the three factors.

Results

Genetic diversity

The mean number of alleles per locus for adults was 16.75; those of their offspring ranged from 4.13 to 5.50 (Table 1).

Pollen flow

During the 4 days of observation in 1996, the 4 days in 1998, and the 6 days in 1999, no pollinator visits were observed. The weather is unstable during the flowering periods in early spring, and this might have affected the behavior of pollinators. The maximum and minimum daily temperatures for these observation dates were within the normal range recorded at the nearest weather station (in Kyotanabe) over the past 10 years. This suggests that the temperatures during our observations were normal and therefore not responsible for the lack of pollinator visits.

The LOD scores were high enough to assign the paternity of all offspring that germinated from seeds to candidate parents within the research plot. The frequency of self-pollination varied among mother plants and the number of pollen parents ranged from 10 to 21 (Table 2). The physical distances between mother plants and the pollen parents of their offspring (498.43 ± 42.48 cm) were significantly shorter than those between mothers and all flowering individuals in the research plot (608.61

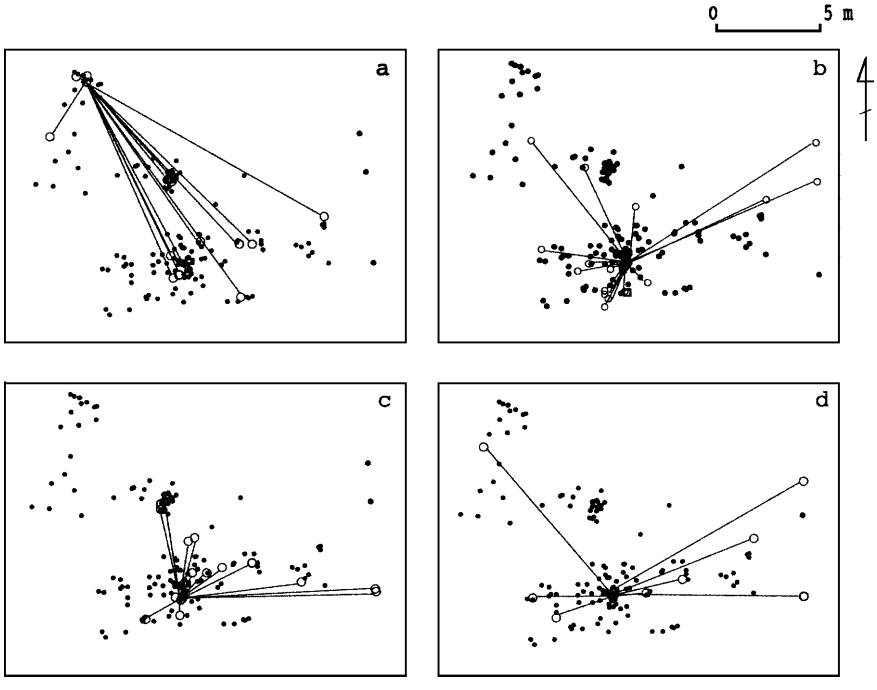

Fig. 4a–d Distribution of pollen parents. *Open circles* (○) indicate pollen parents. *Lines* indicate pollen flow to mother plant. **a** Parent a, **b** parent b, **c** parent c, **d** parent d

Table 3 Dynamics of seedling number

Germination site	Sept-95	Nov-95	Dec-95	Mar-96	May-96	Oct-96	April-97
G1	7	7	7	5	1	0	0
G2	28	23	21	19	7	5	1
G3	22	19	19	12	4	4	0
G4	17	17	11	9	1	1	0
G5	32	32	32	21	12	8	2
Total number	106	98	90	66	25	18	3
Percentage	(100.0)	(92.5)	(84.9)	(62.3)	(23.6)	(17.0)	(2.8)

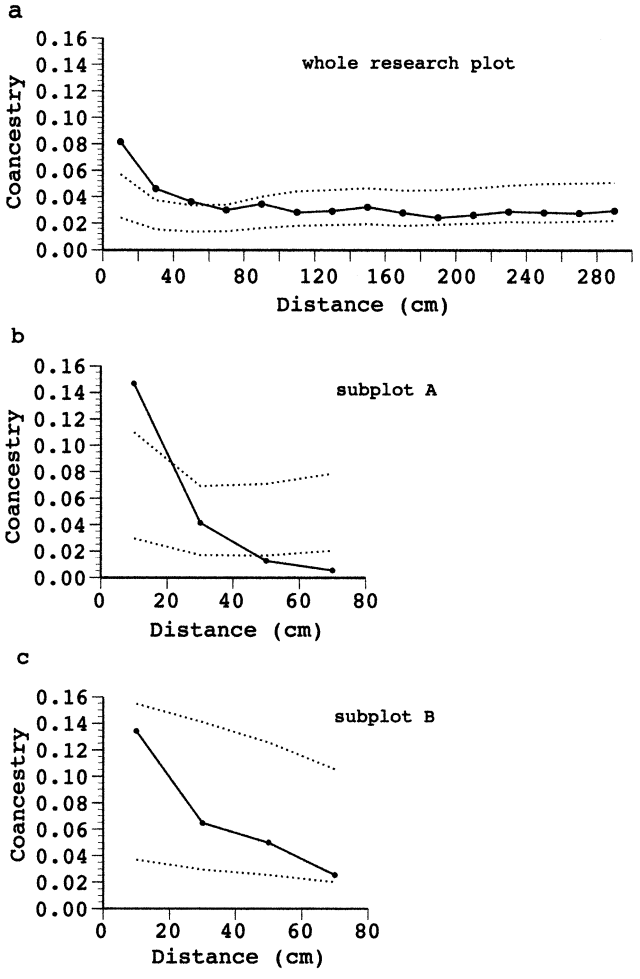

Fig. 5a–c Correlograms of estimated coancestry for pairs of *H. orientalis*. *Dotted lines* represent upper and lower 95% confidence limits. **a** All pairs of individuals that flowered in 1998 in the research plot, **b** all pairs of individuals that flowered in subplot A, **c** all pairs of individuals that flowered in subplot B

± 14.27 cm) (z=-3.921, *P*=0.0010, Mann-Whittney U-test). However, pollen parents were spatially distributed throughout the research plot (Fig. 4).

Seedling dynamics

Table 3 shows the population dynamics of surviving seedlings that germinated in 1995. Although the initial number of seedlings varied over the five germination sites, the total number of seedlings decreased dramatically within 1 year of germination, and almost every seedling was dead by April 1997.

Genetic structure

Analysis of the fine-scale genetic structure throughout the research plot indicated a significant positive autocorrelation among individuals located up to 60 cm apart, and coancestry values were not significant beyond this distance (Fig. 5a). In subplot A, the genetic structure indicated a significant positive autocorrelation among individuals located up to 20 cm apart (Fig. 5b), but in subplot B, no significant autocorrelation was detected (Fig. 5c).

We analyzed multilocus genotypes for all individuals in subplots A and B, and found nine clones in subplot A (Fig. 3a).

Discussion

The study population was spatially isolated from other populations, and pollen flow was found to be restricted to within the population. The absence of pollinators during our study may suggest that the pollinator species reported for *H. orientalis* (Takahashi 1988) only visits at very low frequencies in the present research plot. Despite the apparent lack of pollinator activity, effective pollen flow occurred throughout the research plot.

Studies of actual pollen flow using microsatellite markers (e.g., Chase et al. 1996; Dow and Ashley 1996; Isagi et al. 2000; Streieff et al. 1999) have shown that long-distance pollen flow is relatively common. However, most such studies have focused on tree species, and few have focused on perennials growing on the forest floor. Note that active gene flow occurred in the study population (Fig. 4) even though the expected pollinators of *H. orientalis* (Diptera and Hymenoptera) appeared to visit the plants in the study at very low frequencies. This active pollen movement seems to have led to nonsignificant autocorrelations between individuals located more than 60 cm apart (Fig. 5a).

There may be two possible causes for the discrepancy between the apparently infrequent pollinator visits and the effective pollen movement for the present *H. orien-*

talis population (as indicated by our analysis of microsatellite markers). First, even the low frequencies of pollinator visits may have been sufficient to provide adequate pollen flow for *H. orientalis*. Second, other unknown pollinator species may have performed this task for *H. orientalis* (e.g., nocturnally, when we were not present to observe them). These hypotheses should be examined by amplifying the microsatellite loci of pollen grains found on pollinating organisms collected around *H. orientalis*.

It is possible to assess recruitment to this population by examining the individual size distribution. In the research plot, seedlings were observed only on the limited number of suitable microsites such as bare ground or ground covered by mosses, and most seedlings survived less than 1 year (Table 3). This shows that recruitment by means of seeds occurs infrequently. One suitable microsite for seedlings was found in subplot B, but none were observed in subplot A. In subplot B, individuals of various sizes and many flowering individuals were present, which suggests continuous recruitment by means of seeds. In contrast, vegetative reproduction was predominant over sexual reproduction in subplot A, where 22 of 46 individuals belonged to nine clones (Fig. 3). These clones were close to each other (up to 15 cm apart; Fig. 3), which suggests they originated from adventitious buds on leaf tips. The absence of suitable microsites in subplot A favored an asexual reproductive mode and caused more significant local genetic structuring. Conversely, no genetically identical plants (i.e., ramets of a clone) were found in subplot B; we found all the germination sites in the study plot near this subplot.

Vegetative reproduction had the strongest influence on the local genetic structure, followed by seed dispersal and pollen flow. As we mentioned above, vegetative reproduction in subplot A caused obvious genetic structuring. However, the genetic structure in the research plot as a whole (Fig. 5a) could not explained solely by the existence of vegetative reproduction because no flowering plants sampled from throughout the research plot were ramets of the same clone. Given that the overall genetic structure represents the sum of local genetic structures, it appears reasonable to suggest that the observed genetic structure for the research plot as a whole resulted from a combination of vegetative reproduction, restricted seed dispersal, and pollen flow.

We found three main modes of development of genetic structure for this species: (1) asexual reproduction by means of adventitious buds was dominant (up to 20 cm from parent plants), (2) seeds fallen from flower stalks (up to 60 cm from parent plants), and (3) widespread pollen dispersal (to the whole population). Plants living on the forest floor might maintain their population levels by dominating suitable microsites by means of vegetative reproduction and seed dispersal while maintaining genetic diversity by exchanging genes through pollen dispersal.

Acknowledgments We thank Dr. K. Kitamura and Dr. K. Yamanoi for their analysis of coancestry. We also thank Dr. H. Tabata and Dr. K. Odani for valuable comments and encouragement.

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