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ORIGINAL ARTICLE

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Genetic diversity and multilocus genetic structure in the relictual endemic herb *Japonolirion osense* (Petrosaviaceae)

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Abstract Plant clonality may greatly reduce effective population size and influence management strategies of rare and endangered species. We examined genetic diversity and the extent of clonality in four populations of the monotypic herbaceous perennial *Japonolirion osense*, which is one of the most rare flowering plants in Japan. Allozyme analysis revealed moderate levels of genetic variation, and the proportion of polymorphic loci ($P = 66.7\%$) was higher than the value for species with similar life-history traits. With four polymorphic loci, 19 multilocus genotypes were observed among 433 aerial shoot samples and 10 (52%) were found only in single populations. The proportion of distinguishable genotypes ($PD = 0.10$) and Simpson's index of diversity ($D = 0.52$) also exhibited moderate levels of genotypic diversity compared to other clonal plants, with genotype frequencies at Hardy-Weinberg equilibrium. The distributions of genotypes were often localized and they were mostly found within a radius of 5 m. Spatial autocorrelation analysis showed that shoot samples located 4 m apart were expected to be genetically independent. The results suggest that the spatial extent of genets was relatively narrow and thus the clonality was not extensive.

Key words Allozyme · Clonal structure · Genetic diversity · *Japonolirion osense* · Rare and endemic species · Spatial autocorrelation analysis

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Introduction

Japonolirion is a monotypic and endemic genus of Japan, and *J. osense* is a relictual species that is endemic to serpentine areas in Honshu and Hokkaido, Japan (Nakai 1930; Tatewaki 1931; Hara and Kanai 1959). In Honshu the plant occurs in Mt. Shibutsu and Mt. Tanigawa (Gunma Prefecture), while in Hokkaido the plant occurs only in the Teshio region. Based on this rarity and restricted disjunctive distribution, *J. osense* is listed as vulnerable (VU) under the Red Data Book of Japanese plants (Environmental Agency of Japan 2000). Despite the need for conservation, little is known of the life history characteristics of *J. osense*. Although it has been reported that this species reproduces both sexually and vegetatively (Hara 1935), there is no study on sexual reproduction. As for vegetative reproduction, Tsukui and Takahashi (1995) only describe the architecture of underground rhizomes by excavating two clonal fragments and suggest that its clonal growth forms and long-lived rhizomes may contribute to extensive vegetative spread on unstable sandy serpentine slopes. The present study focuses on the clonal diversity and structure of this species.

Clonal growth can significantly influence the conservation of rare and endangered species. First, the number of genetically distinct individuals (genets) may be much less than the ostensible number of individuals (ramets). At the extreme, each population may consist of one or several fragmented genets (e.g., Swensen et al. 1995; Sydes and Peakall 1998). Genetically small populations are likely to be influenced by stochastic environmental changes including emergence of novel pathogens (Shaffer 1981; Schemske et al. 1994), and sexual reproduction among few genets may result in inbreeding depression (Barrett and Kohn 1991; Ellstrand and Elam 1993). If the species has strong self-incompatibility, restricted pollen transfer within a clone may further limit seed production (Handel 1985). Therefore, genet abundance and its structure may influence population persistence. Second, the extent of clonality should be taken into account in reserve design for in situ

conservation and in sampling strategies for ex situ conservation or re-establishment projects (Frankham et al. 2002). Conservation plans should be designed to maximize genetic diversity in order to maintain the long-term evolutionary potential of the species (Frankel et al. 1995). To achieve this, knowledge of clonal diversity and its spatial arrangement within and among populations is essential.

In this study, we examined clonal diversity and structure of four populations of *Japonolirion osense* in the Teshio region, northern Hokkaido, by collecting leaf samples to be analyzed by allozyme electrophoresis. Allozyme analysis requires only a small amount of plant material and is therefore appropriate for studies on rare and endangered species where extensive survey and sampling are impractical.

Materials and methods

Species and study sites

Japonolirion osense Nakai (Petrosaviaceae) is a diploid ($2n = 24$; Sato 1942), long-lived herbaceous perennial, which is one of the most rare flowering plants in Japan. *Japonolirion* had been classified as Liliaceae thus far, but recent molecular phylogenetic analysis suggested that the species should be placed in Petrosaviaceae along with *Petrosavia* (Fuse and Tamura 2000). Aerial shoots often aggregate (10–200 shoots·m^{-2}) and form patches, which can easily be distinguished in space. Rhizomes creep underground with an annual elongation of ≤90 mm and persist for at least 24 years (Tsukui and Takahashi 1995). Erect aerial shoots sprout from the tips of the rhizomes of the previous year and grow for only one season. One to five new buds are formed at the base of the shoots and some of them elongate as rhizomes (Tsukui and Takahashi 1995) so that the plants can acquire horizontal space effectively via frequent rhizome branching. Flowering scapes sprout from the tips of some of the previous year's shoots (Hara 1935; Tsukui and Takahashi 1995). The density of flowering scapes is ≤10 m^{-2}. The inflorescence is an erect raceme with many yellowish, hermaphrodite flowers. The fruit is a greenish capsule with small (~0.8 mm), wingless seeds (Utech 1984).

The study was conducted in four populations (Table 1). In these sites, the species occurs as small populations and grows in exposed riverside slopes. Because the sites are moist, serpentine sands with a steep slope, they are susceptible to landslide. The Kikusui-A population is close to the Kikusui-B population with a separation of several hundred meters, while the other populations were separated by 3–10 km.

Leaf sampling and allozyme electrophoresis

In 1999, young leaves were sampled from 433 aerial shoots in the four populations (Table 1). In each population, leaf samples were collected from almost all the shoot patches. Within each patch, between 1 and 21 samples were randomly collected and the positions sampled were recorded. Sample sizes depended on the actual density of plants and the size of patches. Leaf samples were transported in ice to the laboratory and kept at –80°C until allozyme electrophoresis. Enzyme extraction and polyacrylamide vertical gel electrophoresis were carried out as described by Tomimatsu and Ohara (2003). We preliminarily tested 16 enzymes; subsequently a total of six enzyme systems that consistently showed interpretable banding patterns were examined: aspartate aminotransferase (AAT; E.C. 2.6.1.1; 1 locus), leucyl aminopeptidase (LAP; E.C. 3.4.11.1; 1 locus), phosphoglucomutase (PGM; E.C. 5.4.2.2; 1 locus), 6-phosphogluconate dehydrogenase (6PGDH; E.C. 1.1.1.44; 1 locus), alcohol dehydrogenase (ADH; E.C. 1.1.1.1; 1 locus), and sorbitol dehydrogenase (SODH; E.C. 1.1.1.14; 1 locus). We resolved six putative loci and assumed Mendelian inheritance of all loci based on banding patterns given the expected enzyme unit substructures.

Analysis

Standard measures were used to quantify genetic variation: proportion of polymorphic loci (P) and mean number of alleles per locus (A). Genotypic diversity within populations was evaluated using the following measures: the number of detected genotypes (G), the proportion of distinguishable genotypes (PD), and Simpson's index of genotypic diversity (D). PD was calculated as G/N, where N is the number of samples (Ellstrand and Roose 1987). This measure corresponds to the probability that the next sample will be a different genotype. Simpson's index of diversity was calculated as:

$$D = 1 - \sum_i \frac{n_i(n_i - 1)}{N(N - 1)} \quad (1)$$

Table 1. Locations and genetic diversity of four populations of *Japonolirion osense*. *N* Sample size, *P* proportion of polymorphic loci (%), *A* mean number of alleles per locus, *G* number of multilocus genotypes, *PD* proportion of distinguishable genotypes, *D* Simpson's index of genotypic diversity

Population	Latitude	Longitude	Area (m^2)	*N*	*P*	*A*	*G*	*PD*	*D*
Kikusui-A	44°56′N	142°11′E	500	168	66.7	1.83	11	0.07	0.78
Kikusui-B	44°56′N	142°11′E	120	55	33.3	1.33	4	0.07	0.39
Shiunbashi	44°57′N	142°14′E	10	11	16.7	1.17	2	0.18	0.18
Hassenzawa	45°00′N	142°02′E	700	199	66.7	1.83	16	0.08	0.72
Mean					45.9	1.54	8.3	0.10	0.52
Overall					66.7	1.83	19		

where n_i is the number of sampled shoots of the ith genotype (Pielou 1969). Geneotypic diversity among populations was measured as the number of local genotypes (genotypes found only in a single population) and the number of widespread genotypes (genotypes found in 75% or more of the populations) (Ellstrand and Roose 1987). These measures can be compared to other studies of clonal plants (Ellstrand and Roose 1987; Widén et al. 1994).

Observed heterozygosity (H_o) and gene diversity (H_e) were calculated with each multilocus genotype treated as an individual. Because there were few observed genotypes in each population, these indices were determined only at the overall population level. Mating system was assessed by calculating the inbreeding coefficient (F_{IS}) only for populations where greater than ten multilocus genotypes were found, using the estimate of Weir and Cockerham (1984). Other fixation indices (F_{IT} and F_{ST}) were not calculated due to the low number of genotypes. The analysis was performed using the program FSTAT version 2.9.1 (Goudet 2000), and the level of significance was obtained by randomization procedures (see Goudet 2000 for details). The probability that identical genotypes arise through sexual reproduction by chance was determined within each population for each genotype assuming random mating and linkage equilibrium among loci:

$$P_{gen} = \left(\prod_i p_i q_i \right) 2^h \quad (2)$$

where p_i and q_i are the frequencies of the two alleles at the ith locus and h is the number of loci that are heterozygous (e.g., Parks and Werth 1993).

To investigate fine-scale spatial genetic structure, we conducted spatial autocorrelation analyses (Sokal and Oden 1978; Smouse and Peakall 1999; Kalisz et al. 2001) for the Kikusui-A and Hassenzawa populations where a large number of samples were obtained. In the Hassenzawa population, the site consisted of two subpopulations divided by a small stream. Thus the data of the larger subpopulation were used for analysis. We followed the method of Smouse and Peakall (1999), who developed a multilocus estimate of autocorrelation coefficient (r). Distance classes of 1 m were used and significance was tested for each distance class by comparing the observed value of r with those obtained from 999 randomizations. The significance of the correlogram as a whole was then assessed by T^2-test (see Smouse and Peakall 1999 for details). Analysis was conducted using the program written by H. Tomimatsu in S language (Lucent Technologies, Murray Hill, N.J.) on the software S-PLUS version 3.3 (Insightful, Seattle, Wash.).

Results

Genetic and genotypic diversity

Two of the six loci resolved (*Aat* and *Lap*) were monomorphic. Four loci were polymorphic in at least one of the populations examined: *Pgm* exhibited three alleles and *6Pgdh*, *Adh*, and *Sodh* exhibited two alleles. *P* and *A* at the species level were 66.7% and 1.83, respectively (Table 1). A total of 19 multilocus genotypes were found (Table 2); *G* per population averaged 8.3, ranging from 2 (Shiunbashi) to 16 (Hassenzawa) (Table 1). Although 162 genotypes ($= 6 \times 3 \times 3 \times 3$) were possible with the four polymorphic loci, a much lower number of genotypes was observed. *PD* exhibited relatively low values, with a mean of 0.10. Large populations (Kikusui-A and Hassenzawa) were more genotypically diverse, with relatively large numbers of genotypes and high *D* values. At the overall population level, H_o was 0.133 and H_e was 0.247. Inbreeding coefficients in two large populations were somewhat elevated but did not differ significantly from Hardy-Weinberg expectations ($F_{IS} = 0.211$ for Kikusui-A; $F_{IS} = 0.207$ for Hassenzawa). Among the 19 genotypes observed, 10 (52%) were local genotypes and found only in single populations. Only four (21%) were widespread genotypes (genotypes 8, 13, 15, and 18; Table 2). This was partly explained by the fact that two alleles (*Adh-a* and *Sodh-a*; Table 3) were common in the Hassenzawa population, while in the other populations only a single genet hold the alleles as heterozygotes (genotype 7; Table 2). These results suggest that the populations were genetically differentiated.

In the case of the local genotypes, P_{gen} values were low enough to reject the null hypothesis ($P_{gen} < 0.05$) that identical genotypes were generated through sexual reproduction by chance, indicating that aerial shoots with the same multilocus genotype were highly likely to be members of the same clone. However, P_{gen} of the other genotypes including those that were widespread were sometimes not significant ($P_{gen} > 0.05$) and exceeded 0.1 in six cases (data not shown). In such cases, it is probable that two or more genets with the same multilocus genotype arose through sexual reproduction.

Table 2. Nineteen multilocus genotypes with the four polymorphic loci detected for four populations of *Japonolirion osense*. *Pgm* Phosphoglucomutase, *6Pgdh* 6-phosphogluconate dehydrogenase, *Adh* alcohol dehydrogenase, *Sodh* sorbitol dehydrogenase

Multilocus genotype	*Pgm*	*6Pgdh*	*Adh*	*Sodh*
1	aa	bb	bb	bb
2	ab	aa	bb	bb
3	ab	ab	bb	bb
4	ab	bb	ab	ab
5	ab	bb	bb	bb
6	bb	aa	aa	aa
7	bb	aa	ab	ab
8	bb	aa	bb	bb
9	bb	ab	ab	ab
10	bb	ab	bb	bb
11	bb	bb	bb	bb
12	bc	aa	ab	ab
13	bc	aa	bb	bb
14	bc	ab	ab	ab
15	bc	ab	bb	bb
16	bc	bb	bb	bb
17	cc	aa	ab	ab
18	cc	aa	bb	bb
19	cc	bb	bb	bb

Table 3. Allele frequencies at four polymorphic loci in four populations of *Japonolirion osense*, calculated with each multilocus genotype treated as an individual

Locus	Populations				Overall
	Kikusui-A	Kikusui-B	Shiunbashi	Hassenzawa	
Pgm					
a	0.227	0.000	0.000	0.063	0.106
b	0.636	0.500	0.500	0.594	0.591
c	0.136	0.500	0.500	0.344	0.303
6Pgdh					
a	0.500	0.875	1.000	0.625	0.636
b	0.500	0.125	0.000	0.375	0.364
Adh					
a	0.045	0.000	0.000	0.250	0.136
b	0.955	1.000	1.000	0.750	0.864
Sodh					
a	0.045	0.000	0.000	0.250	0.136
b	0.955	1.000	1.000	0.750	0.864

Multilocus genetic structure

The distribution of multilocus genotypes is shown in Fig. 1. The results for the Kikusui-A and Kikusui-B populations are representatively included in the figure. Many shoot patches possessed multiple genotypes. In Kikusui-A, for example, the distributions of genotypes were often localized and the majority of genotypes were found within a radius of 5 m. Some genotypes were widely distributed across the populations (e.g., genotypes 8 and 10; Fig. 1), although these were widespread genotypes that may consist of several genets (e.g., $P_{gen} = 0.09$ for genotype 8, $P_{gen} = 0.17$ for genotype 10). It is also noteworthy that although the Kikusui-A and Kikusui-B populations are closely located only several hundred meters apart along the Kikusui River, their composition of multilocus genotypes was largely different.

Figure 2 shows the correlograms of spatial autocorrelation analyses for two large populations, Kikusui-A and Hassenzawa. Both populations showed significant multilocus genetic structure ($P < 0.01$); r remained significantly positive out to 4 m in the Kikusui-A and 2 m in the Hassenzawa populations. The coefficients were significantly negative at around 10 m.

Discussion

Genetic diversity and the extent of clonality

Although rare species typically show low levels of genetic variation (Hamrick and Godt 1996; Gitzendanner and Soltis 2000), *Japonolirion osense* maintained moderate allozyme variation (Table 1). The proportion of polymorphic loci at the species level ($P_s = 66.7\%$) was larger than the value (48.1%) for endemic long-lived perennial species (Hamrick and Godt 1996). The measures of genotypic diversity (G, PD, D) were slightly lower than values for 45 clonal species in which sexual recruitment is rare ($G = 11.1$, $PD = 0.27$, $D = 0.75$ at the population level; Ellstrand and Roose 1987; Widén et al. 1994). However, genotypic diversity is sensitive to the number of diagnostic loci (Widén et al. 1994), and therefore the estimates of *J. osense* with only four polymorphic loci may be underrated. Substantial genetic variations may be those among populations because over half of the genotypes were population-specific. The result also infers low levels of pollen and seed dispersal. But within populations the species are not severely inbred, as suggested by the genotype frequencies in Hardy-Weinberg expectations.

The clonal habit can be advantageous in the unstable and generally oligotrophic serpentine slopes where *Japonolirion osense* grows, as plants can acquire space horizontally and minimize the effect of disturbances such as landslides (Tsukui and Takahashi 1995). Since a genet is physiologically integrated, plants can also buffer the effect of habitat heterogeneity by sharing resources such as water and nutrients among ramets within a genet (e.g., Stuefer et al. 1996). On the other hand, extensive clonality reduces effective population size and may threaten its long-term persistence. In general, the extent of clonality greatly differs among species and therefore cannot be simply predicted by the level of sexual reproduction or spatial patterns of aboveground aerial shoots (reviewed by Ellstrand and Roose 1987; Widén et al. 1994; Hangelbroek et al. 2002). Hence, genetic analyses with molecular markers are essential to clarify the present status of endangered clonal plants and to design suitable management strategies.

In *Japonolirion osense*, the distribution of multilocus genotypes revealed that the spatial extent of genets may be relatively narrow, mostly a radius of <5 m (Fig. 1). This view was also supported by significant positive autocorrelations of genotypes only within small distance classes (<4 m; Fig. 2). That is, overall, aerial shoots located 4 m apart were expected to be different genotypes. Although some widespread genotypes covered wide areas (Fig. 1), these seemed to comprise multiple genets that were generated by sexual reproduction. Therefore, the spatial extent of these genets would be more restricted.

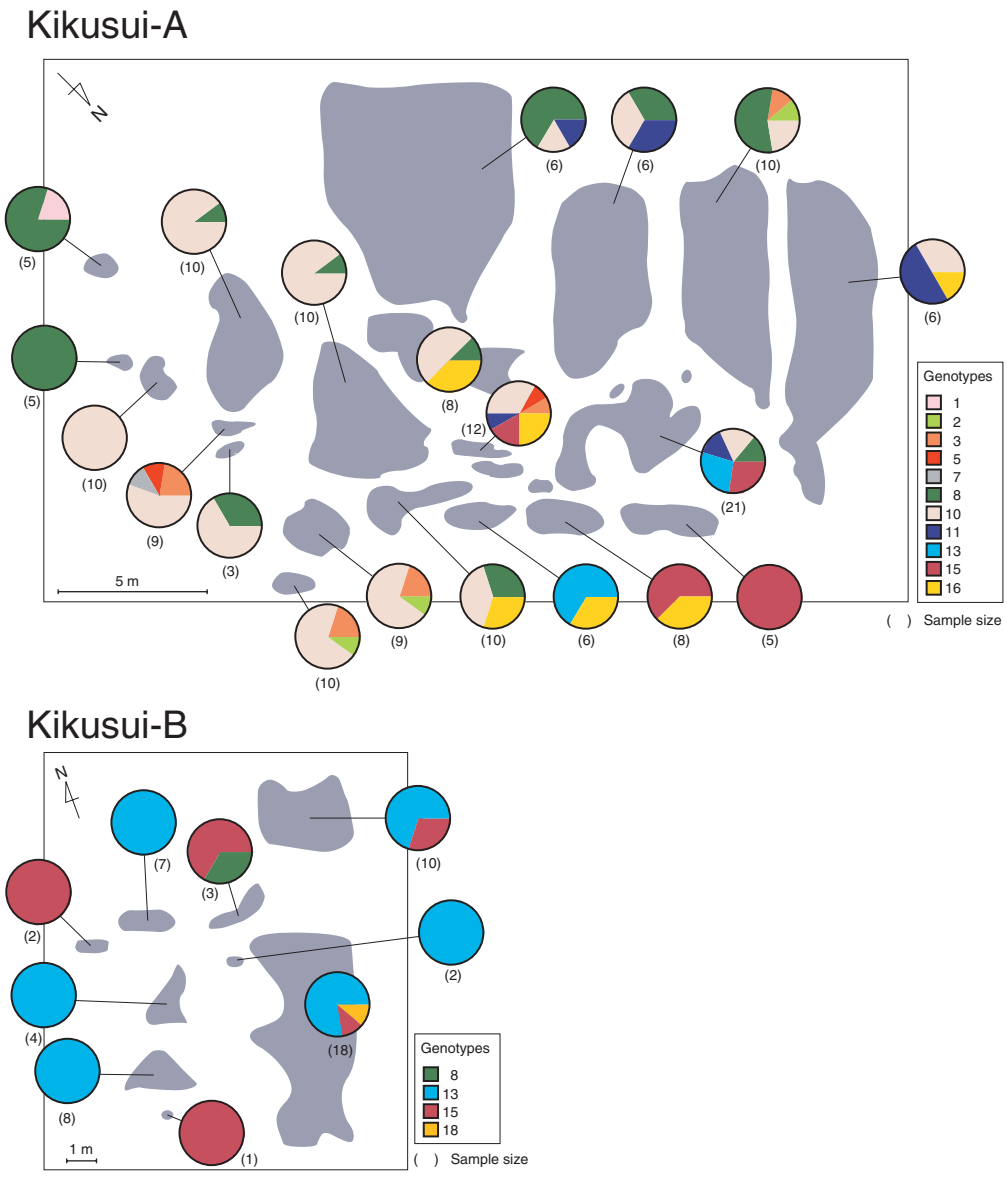

Fig. 1. Map showing the distribution of multilocus genotypes at Kikusui-A and Kikusui-B. The patches of *Japonolirion osense* are indicated in *gray*. Numbers in parentheses are sample sizes

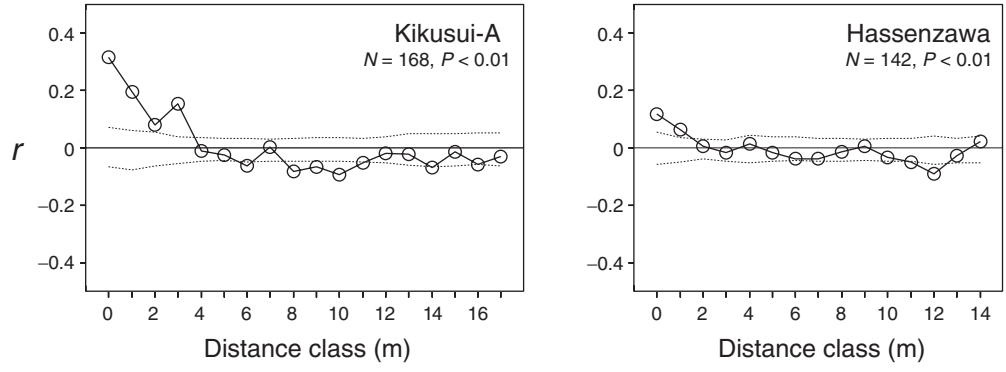

Fig. 2. Correlograms (*solid lines*) resulting from spatial autocorrelation analyses based on six allozyme loci for two populations of *J. osense*. The *dotted lines* represent a 95% null hypothesis confidence region with no genetic structure based on 1,000 randomizations

Implications for conservation

Genotypic diversity of *Japonolirion osense* was not low compared to other clonal plants, and the spatial extent of genets was relatively narrow. These results suggest that the clonality was not so extensive as to extremely limit the number of genets within populations. To preserve the populations, long-term monitoring of population sizes is necessary, because the habitat of *J. osense* is restricted to small serpentine areas and disturbances such as landslides can

further reduce population size. The results of this study also emphasize the role of sexual reproduction in the dynamics of populations. The frequency of successful seedling recruitment needs to be quantified and the factors limiting recruitment should be considered if this frequency is extremely low. Unfortunately, almost no information is available for sexual reproduction, and even the level of seed production and the breeding system (whether the plant is self-compatible or incompatible) are not known for this species. The substantial levels of genotypic diversity among populations suggest that efforts should be made to conserve as many populations as possible across the species' distribution range.

This species was roughly estimated to have declined by 20% during the past decade owing to damage by mountain climbers and collection for horticultural purposes (Environmental Agency of Japan 2000). Under the circumstances, ex situ conservation and re-establishment of populations may be needed in the future. The intercept of the correlograms can also be used to establish efficient sampling strategies for these purposes (Diniz-Filho and Telles 2002). The results indicate that samples at a distance equal or greater than the intercept (2–4 m; Fig. 2) are genetically independent, and collection of plants or seeds at intervals of this distance can sample most of the genetic variation (including clonal variation) with less replications of samples of correlated genotypes.

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