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SHORT COMMUNICATION

Polymorphic chloroplast simple sequence repeat primers for systematic and population studies in the genus *Hordeum*

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Abstract

In this study we report the development of primers to amplify polymorphic chloroplast simple sequence repeats in the genus *Hordeum*, which includes cultivated barley (*H. vulgare* ssp. *vulgare*) and its wild progenitor *H. vulgare* ssp. *spontaneum*. Polymorphic products were amplified in a wide range of *Hordeum* spp. and intraspecific variation was detected in both cultivated and wild barley. A decrease in cytoplasmic diversity was observed between sspp. *spontaneum* and *vulgare* as well as between ssp. *vulgare* landraces and cultivars, which is characteristic of domestication processes in many crop species. We also observed possible evidence for reticulate evolution of *H. brachyantherum* polyploids, with apparent multiple cytoplasmic introgressions during successive polyploidization events.

Keywords: barley, chloroplast, *Hordeum*, microsatellite, simple sequence repeat

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Introduction

The analysis of animal mitochondrial DNA polymorphism is now a firmly established technique in both population and phylogenetic studies due to its uniparental mode of inheritance, lack of recombination, and high level of sequence variation, particularly within the D-loop control region (Avise *et al*. 1987, 1989; Moritz *et al*. 1987; Harrison 1989). However, in plant mitochondrial genomes, the extremely low levels of nucleotide substitution, coupled with a high frequency of intramolecular recombination, have restricted the use of mitochondrial DNA in plant phylogeographic and systematic studies (Palmer *et al*. 1992). Chloroplast DNA does not undergo recombination but low levels of nucleotide substitution in the chloroplast genome have limited the scope of chloroplast-based studies below the species level (for reviews see Soltis *et al*. 1992 and McCauley 1995), and the need for new techniques to detect intraspecific variation has been highlighted (Palmer *et al*. 1988; Schaal *et al*. 1998).

The discovery of highly polymorphic simple sequence repeats (SSRs) in the chloroplast genomes of plants has provided new opportunities for high-resolution cytoplasmic analysis in population and phylogenetic studies (Powell *et al*. 1995, 1996; Provan *et al*. 1998a). Studies using chloroplast simple sequence repeats (cpSSRs) have reported higher levels of chloroplast variation than had previously been detected using restriction fragment length polymorphisms (RFLPs) (Provan *et al*. 1996, 1997) and this allowed the analysis of intraspecific chloroplast diversity in natural plant populations (Powell *et al*. 1995; Provan *et al*. 1998b). In general, primers to amplify polymorphic cpSSRs have previously been designed from those species whose chloroplast genomes have been completely sequenced (Powell *et al*. 1995), and partial chloroplast sequences of individual genes or groups of contiguous genes represent a hitherto untapped source of cpSSR sequences in a wide range of plant species. Furthermore, identification of SSR regions in chloroplast genomes has generally been limited to mononucleotide repeats of 10 or more bases, although SSRs with shorter repeat motifs have been found to be polymorphic in other species (J. Provan *et al*., unpublished observations).

In this study we report the development of cpSSR markers in the genus *Hordeum*, which includes cultivated barley (*H. vulgare* ssp. *vulgare*) and its wild progenitor, *H. vulgare* ssp. *spontaneum*. The cpSSR primers described amplified polymorphic products in a wide range of *Hordeum* spp., as well as displaying intraspecific polymorphism in both cultivated and wild barley.

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Materials and methods

Genetic material

We analysed 31 accessions from 24 wild *Hordeum* species, 51 accessions of *H. vulgare* ssp. *spontaneum*, 125 accessions of *H. vulgare* ssp. *vulgare* landraces and 101 *H. vulgare* ssp. *vulgare* cultivars. The 31 accessions representing the genus *Hordeum*, including diploid, tetraploid and hexaploid species, are given in Table 1. The 51 *H. vulgare* ssp. *spontaneum* accessions were collected from the primary centres of diversity of wild barley and include those described in Pakniyat *et al*. (1997), while the 125 *H. vulgare* ssp. *vulgare* landraces were a subset of those described in Ceccarelli *et al*. (1987). The cultivars were 101 accessions representing the majority of spring barley varieties grown in Europe this century. A complete list of material is available from the authors on request. DNA was extracted from leaf tissue using the method of Saghai-Maroof *et al*. (1984).

Identification of SSR regions in the barley chloroplast genome and polymerase chain reaction

Barley chloroplast sequences in the EMBL database were searched for all mononucleotide repeats of 8 bp or more using the STRINGSEARCH and FINDPATTERNS programs (Genetics Computer Group). Primers were designed to amplify mononucleotide SSRs in noncoding regions using the program PRIMER (version 0.5) and are given in Table 2. PCR was carried out in a total volume of 10 μL containing 1× PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 200 μM dNTPs, 10 pmol [^{32}P]-ATP endlabelled forward primer (Sambrook *et al*. 1989), 10 pmol reverse primer, 0.5 U *Taq* polymerase (Promega) and 50 ng of genomic DNA. Reactions were carried out on a Perkin-Elmer 9600 thermal cycler using the following parameters: (i) initial denaturation at 94 °C for 3 min; (ii) 30 cycles of denaturation at 94 °C for 15 s, annealing at T_m for 15 s and extension at 72 °C for 60 s; (iii) final extension at 72 °C

Table 1 List of wild *Hordeum* species used in this study

Species		Ploidy	No.	Origin	Collector*
H. arizonicum		6*x*	2313	USA	Bo,Ja
H. bogdanii		2*x*	4014	Pakistan	Bo
H. brachyantherum	ssp. *brachyantherum*	4*x*	2360	Canada	Bo,Ja
		6*x*	2001	USA	Ba,Bo,Ja
	ssp. *californicum*	2*x*	2401	USA	Bo
H. capense		4*x*	334	South Africa	An
H. chilense		2*x*	1819	Chile	Bo
H. cordobense		2*x*	6429	Argentina	Se
H. depressum		4*x*	2306	USA	Bo,Ja
H. erectifolium		2*x*	1150	Argentina	Ja
H. euclaston		2*x*	1263	Argentina	Ja
H. flexuosum		2*x*	1133	Argentina	Ja
H. guatemalense		4*x*	2299	Guatemala	Unknown
H. intercedens		2*x*	1940	USA	Ba,Ja
H. marinum	ssp. *marinum*	2*x*	546	Spain	Ja
	ssp. *marinum* var. *gussoneanum*	2*x*	155	Greece	Ha
		4*x*	834	Iran	Bo
H. murinum	ssp. *murinum*	4*x*	721	Denmark	LL
	ssp. *glaucum*	2*x*	219	Tunisia	Sc
	ssp. *leporinum*	4*x*	591	Greece	Ja
		6*x*	796	Iran	Bo
H. muticum		2*x*	958	Bolivia	VS
H. parodii		6*x*	6328	Argentina	Unknown
H. patagonicum	ssp. *setifolium*	2*x*	1357	Argentina	Ja
H. procerum		6*x*	1781	Argentina	Bo
H. pubiflorum	ssp. *halophilum*	2*x*	1244	Argentina	Ja
H. pusillum		2*x*	2038	USA	Bo,Ba
H. roshevitzii		2*x*	7202	China	Bo
H. secalinum		4*x*	231	Sweden	Ni
H. stenostachys		2*x*	1783	Argentina	Bo
H. tetraploidum		4*x*	1466	Argentina	Baum

*Bo, Bothmer; Ja, Jacobsen; Ba, Bailey; An, Andersson; Se, Seberg; Ha, Hansen; LL, Linde-Laursen; Sc, Scholtz; VS, van Soest; Ni, Nilsson.

Table 2 Barley cpSSR primers used in this study, numbers of alleles (*n*) and diversity values (*H*)

Locus	Repeat	Location	Primers (5' – 3')	T_m (°C)	Size (bp)	Hordeum		Spont.		Landraces		Cultivars	
						n	*H*	*n*	*H*	*n*	*H*	*n*	*H*
hvcppsbK	$(A)_{11}$	*psbK/ORF174* intergenic region	TAGCCTTTGTTTGGCAAGCT TAAAACTTCTCGGCTTTTACCC	60	123	3	0.18	2	0.11	1	0.00	1	0.00
hvcppsbA	$(T)_8$	Downstream of *psbA*	AATGGATAAGGTTTTTCTG CTGAATAGAAAGATTAAGAAGA	50	130	2	0.04	2	0.21	1	0.00	1	0.00
hvcprpoA	$(T)_8(CTT)_3$	Downstream of *rpoA*	CTCTCGTTTTAAATCCATTGCA TGATCCATTTCGCGAAAATA	60	122	4	0.47	1	0.00	1	0.00	1	0.00
hvcprps12	$(T)_8$	rpS12 intron I	AAGAAAGGGCTCCGGTGTAT CCACGATTTTTTATTCCACTCC	60	149	2	0.11	1	0.00	1	0.00	1	0.00
hvcptrnS1	$(A)_7CGC(T)_{13}$	Downstream of *trnS*	CTTTAGCGGGCATTTCCATA ATGGTGGATTTGATAAGAACCC	60	130	2	0.11	2	0.11	1	0.00	1	0.00
hvcptrnS2	$(T)_{10}$	Downstream of *trnS*	CAACTCCTTTGCGCTACACA ACCCCTTTTTTCCCATTCC	60	113	7	0.83	3	0.25	1	0.00	1	0.00
hvcptrnLF	$(C)_9$	*trnL/trnF* intergenic region	GAGTATCGGCAAGAAATCTTGG TCAAAATTTGAAAGGGGGG	60	101	6	0.76	3	0.22	2	0.51	1	0.00
Haplotype*						25	0.95	11	0.75	2	0.47	1	0.00

* Values based on haplotype are calculated by combining data from all seven loci.

for 5 min (for T_m values see Table 1). After addition of 10 μL of loading buffer (95% formamide), products were resolved on 6% denaturing polyacrylamide gels containing 1× TBE buffer and 8 M urea at 80 W constant power for 2–4 h. Gels were transferred onto 3 mm blotting paper (Whatman), dried and exposed to X-ray film for 24 h without intensification screens.

Phenetic analysis of relationships within the genus Hordeum

Genetic distances between individuals were calculated based on the proportion of shared alleles between cpSSR haplotypes using the computer program MICROSAT (version 1.5, Eric Minch, Stanford University, USA) and a neighbour-joining tree was constructed using the NEIGHBOR and DRAWGRAM options in the PHYLIP package (version 3.57c: Felsenstein 1995).

Results and Discussion

We have used partial chloroplast DNA sequences from the EMBL database to design seven pairs of primers for the amplification of mononucleotide repeats in the barley chloroplast genome. All loci were polymorphic in the wild *Hordeum* spp. with between two and seven alleles detected at individual loci. Diversity values based on allele frequencies were calculated for each locus using Nei's unbiased statistic (1987) and ranged from 0.04 to 0.83 (Table 2). As there is no recombination in the chloroplast molecule, data from multiple, physically linked loci were combined to give haplotypes and the diversity value based on haplotype frequency was 0.95. Twenty-one of the 31 *Hordeum* spp. could be uniquely genotyped and 25 haplotypes were found in all, highlighting the high resolving power of cpSSRs (Table 3). Within *H. vulgare* ssp. *spontaneum*, five of the loci were polymorphic with two or three alleles present at each locus and 11 haplotypes were found in the 51 accessions studied, giving an overall diversity value of 0.75 (Table 2). Only locus hvcptrnLF was polymorphic in the *H. vulgare* ssp. *vulgare* landraces, giving two haplotypes and a diversity value of 0.471. These two haplotypes were a subset of the 11 haplotypes found in ssp. *spontaneum* and one of these was found in all the European cultivars (Table 3). This bottleneck effect resulting from the domestication of a species from its wild progenitor is consistent with the processes described by Tanksley & McCouch (1997) where genetic diversity is dramatically reduced during the domestication process and further depleted in the breeding of varieties due to strong selection for a few agriculturally desirable traits. In a previous population study using cpRFLPs in barley, Neale *et al*. (1988) found three haplotypes in a sample of 245 *H. vulgare* ssp. *spontaneum* accessions from Israel. We detected six haplotypes in the 12 ssp. *spontaneum* accessions in our study which originated from Israel, further highlighting the higher resolving power of cpSSRs compared with cpRFLPs. In common with the cpRFLP study, we found much lower levels of diversity in cultivated barley, with only two haplotypes present in the ssp. *vulgare* landraces and a single haplotype in the European cultivars.

The phylogenetic tree showing the relationships between the accessions studied is shown in Fig. 1. There is a large split between the wild *Hordeum* spp. and the *H. vulgare* ssps. *vulgare* and *spontaneum* accessions which is in accordance with previous phylogenetic studies of the genus using RFLPs (Baum & Bailey 1991; Molnar *et al*. 1992) and random amplified polymorphic DNA fragments (RAPDs) (Svitashev *et al*. 1994). Within the *Hordeum* spp., the accessions tended to be resolved into groups containing either American and Asian (two groups) or European and African species. One group contained the *H. murinum* accessions, all but one of the *H. marinum* accessions, *H. secalinum* from Sweden, and *H. capense* from South Africa. *H. cordobense*, a diploid species from Argentina, seems out of place in this group. Similarly, *H. marinum* ssp. *marinum* var. *gussoneanum* (4*x*) appears with the American/Asian species, despite being native to the eastern Mediterrannean.

The distribution of haplotypes across different *Hordeum* species gave rise to some interesting observations. A single cpSSR haplotype was shared by *H. erectifolium*, *H. euclaston*, *H. flexuosum* and *H. stenostachys*, all of which are diploids indigenous to Argentina/southeast South America. The latter three species are generally morphologically similar, although *H. erectifolium* is very distinct and unlike other South American diploids. Conversely, diploid, tetraploid and hexaploid accessions of *H. brachyantherum* exhibited three very different haplotypes. This may be evidence of reticulate evolution of the species, with multiple cytoplasmic introgressions during polyploidization events. Similar observations in the genus *Helianthus* have been documented by Rieseberg and coworkers and other examples of chloroplast 'capture' have been reported in *Brassica*, *Pisum* and *Zea* amongst other species (for review see Rieseberg & Soltis 1991). *H. marinum* ssp. *marinum* also displayed different haplotypes at differing ploidy levels but, in contrast, both tetraploid and hexaploid *H. murinum* ssp. *leporinum* share the same cpSSR haplotype, with no evidence of cytoplasmic introgression between 4*x* and 6*x* ploidy levels.

The development of primers to amplify polymorphic mononucleotide repeats in the chloroplast genome of barley provides new opportunities for high-resolution

Table 3 Haplotypes detected in the genus *Hordeum* using seven cpSSRs

	Locus						
Haplotype	hvcppsbK	hvcppsbA	hvcprpoA	hvcprps12	hvcptrnS1	hvcptrnS2	hvcptrnLF
*H. arizonicum**	121	146	122	148	128	115	101
H. bogdanii	121	146	122	148	135	102	99
H. bra ssp. *bra* 4*x*	122	146	122	148	128	112	101
H. bra ssp. *bra* 6*x*	121	146	116	148	128	116	99
H. bra ssp. *cal* 2*x*	121	146	122	148	128	114	99
H. capense	121	146	118	148	135	113	101
H. chilense†	121	146	122	152	128	114	99
H. cordobense	121	146	126	148	128	114	103
H. depressum	121	146	122	148	128	116	99
H. erectifolium‡	121	146	122	148	128	114	100
H. guatemalense	121	146	122	148	128	113	99
H. intercedens	121	146	122	148	128	116	101
H. mar ssp. *mar*	121	146	118	148	128	113	101
H. mar var. *gus* 2*x*	121	146	118	148	128	112	102
H. mar var. *gus* 4*x*	120	146	122	148	128	116	97
H. mur ssp. *mur*	120	146	118	148	128	112	101
H. mur ssp. *gla*	121	146	118	148	128	115	101
H. mur ssp. *lep* 4*x*§	121	146	118	148	128	114	101
H. parodii	121	146	122	148	128	115	102
H. pat ssp. *set*	121	146	122	148	128	116	102
H. procerum	121	146	122	152	128	114	99
H. pub ssp. *hal*	121	145	122	148	128	115	102
H. pusillum	121	146	122	148	128	113	101
H. roshevitzii	121	146	122	148	128	103	100
H. secalinum	121	146	118	148	128	112	101
H. stenostachys	121	146	122	148	128	114	100
H. tetraploidum	121	146	122	148	128	115	99
H. vulgare ssps.¶							
A	122	146	121	149	130	113	101
B	122	146	122	149	130	114	100
C	122	146	122	149	130	113	100
D	122	145	122	149	130	114	100
E	122	146	122	149	130	113	99
F	122	146	122	149	127	112	100
G	122	146	122	149	130	113	101
H	122	146	122	149	130	112	101
I	122	146	122	149	130	113	100
J	122	146	122	149	126	114	101
K	122	146	122	149	130	114	100

* *H. arizonicum* shares a haplotype with *H. muticum*.
† *H. chilense* shares a haplotype with *H. procerum*.
‡ *H. erectifolium* shares a haplotype with *H. euclaston*, *H. flexuosum* and *H. stenostachys*.
§ *H. murinum* ssp. *leporinum* 4x and 6x share the same haplotype
¶ Haplotypes A–K were found in *H. vulgare* ssp. *spontaneum*. Haplotypes C and G were found in the *H. vulgare* ssp. *vulgare* landraces and, of these, haplotype G was the sole haplotype found in the ssp. *vulgare* cultivars.

analysis of the genus. Doebley (1992) and Soltis *et al*. (1992) have highlighted the importance of being able to describe and quantify intraspecific variation in the chloroplast genomes of plants. The higher levels of intraspecific variation detected by maternally inherited cpSSRs compared with RFLPs will allow the determination of the relative contributions of seed and pollen flow to the genetic structure of natural populations (McCauley 1995), as well as offering new insights into short-term evolutionary processes and systematics.

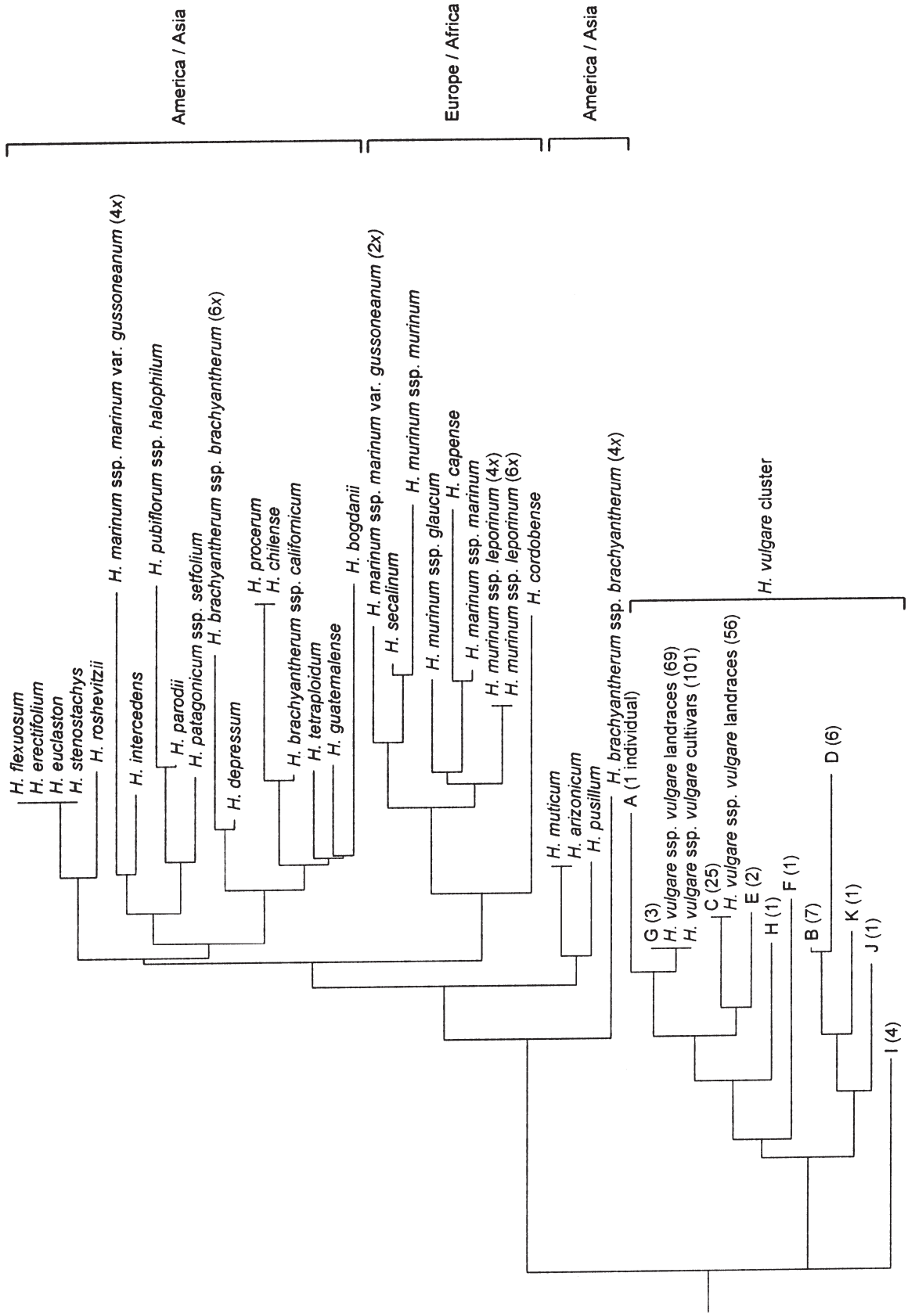

Fig. 1 Phylogenetic tree showing relationships between *Hordeum* accessions revealed by cpSSRs. All haplotypes in the lower cluster represent *H. vulgare* ssp. *spontaneum* accessions, including two which are shared with ssp. *vulgare* accessions. One of these is found in all European cultivars. Numbers represent absolute frequency of the haplotype in the total sample ($n = 51$). Haplotypes are described in Table 3.

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