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Primer Note

Polymorphic microsatellite markers in *Anthoxanthum* (Poaceae) and cross-amplification in the Eurasian complex of the genus[1]

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- *Premise of the study:* Nonplastid microsatellite primers were developed for the first time in the Euro-Siberian complex of *Anthoxanthum* (Poaceae), a genus of temperate grasses in which reticulate evolution is common.
- *Methods and Results:* A microsatellite-enriched genomic DNA library allowed the detection of 500 fragments containing a microsatellite motif. Fifteen primer pairs were selected for an extended primer test. A preliminary analysis was conducted on the Eurasian diploid lineages of *Anthoxanthum*, with special emphasis on three populations of the Mediterranean *A. aristatum–A. ovatum* complex. Thirteen out of 15 markers tested were polymorphic in the complex, with successful cross-amplification in *A. odoratum* (93% polymorphic loci), *A. amarum* (73% polymorphic), *A. alpinum* (73% polymorphic), and *A. maderense* (60% polymorphic).
- *Conclusions:* These microsatellite markers will enable the analysis of evolution and phylogeography in diploid and polyploid lineages of this important genus.

Key words: *Anthoxanthum aristatum–Anthoxanthum ovatum*; microsatellites; Poaceae; polyploidy; simple sequence repeat (SSR); transferability.

Next-generation sequencing (NGS)–based methods have allowed the quick development of microsatellite primers specific to nonmodel organisms (e.g., Duwe et al., 2015; González et al., 2015). Here, microsatellite markers are presented for the grass genus *Anthoxanthum* L., comprising around 20 species often affected by reticulation (Pimentel et al., 2010, 2013). The phylogeny of *Anthoxanthum* defines a Euro-Siberian (as well as Macaronesian and Afroalpine) polyploid complex of species (Pimentel et al., 2013). It includes four diploid taxa: (i) the Mediterranean *A. aristatum* Boiss.–*A. ovatum* Lag. complex (Pimentel et al., 2010), (ii) the Macaronesian *A. maderense* Teppner, and (iii) the Arctic-alpine *A. alpinum* Á. Löve & D. Löve (Pimentel et al., 2013). The clade also includes at least three polyploid lineages (Chumová et al., 2015): the Iberian endemic *A. amarum* Brot. (16*x*–18*x*); the East African *A. nivale* K. Schum. (4*x*, 6*x*), and the Eurasian *A. odoratum* L. (4*x*).

Fifteen microsatellite markers that can be applied to the Euro-Siberian complex of *Anthoxanthum* are presented here. These markers will be used to determine the geographic patterns of gene flow within and among the different diploid lineages in the complex, as well as to unravel the origin of its polyploid groups.

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METHODS AND RESULTS

***Microsatellite development*—**A microsatellite-enriched genomic library (motifs AC, AG, ACC, AGG, and ACG) was constructed at AllGenetics & Biology SL (A Coruña, Spain) from an equimolar mix of DNA extracts from the diploid *A. aristatum–A. ovatum* (two individuals) and the tetraploid *A. odoratum* (one individual; Appendix 1) using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, California, USA). Given the difficulty in morphologically distinguishing *Anthoxanthum* cytotypes (Chumová et al., 2015), between one and five individuals per population were assessed using flow cytometry following Galbraith et al. (1983). DNA was extracted from silica-dried leaves using the DNAeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The enriched genomic library was sequenced in a fraction of an Illumina MiSeq PE300 run (Illumina), and the reads were processed using the software Geneious 7.1.5 (Biomatters, Auckland, New Zealand). Five hundred loci were detected containing a microsatellite and flanked by regions adequate to design PCR primers using Primer3 (Untergasser et al., 2012).

Primer pairs were multiplexed with Multiplex Manager 1.0 (Holleley and Geerts, 2009). Forty microsatellite loci were combined so that differences in annealing temperatures were minimized and spacing between markers was maximized. Primers were tested for polymorphism on six diploid and two tetraploid samples (Appendix 1) that belonged to the different *Anthoxanthum* lineages and came from geographically distant populations. Each PCR reaction was performed following Schuelke (2000) with three primers (one of them fluorescently labeled using FAM or HEX; Table 1). PCR reactions were conducted in a final volume of 12.5 µL, containing 1 µL of DNA (10 ng/µL), 6.25 µL Type-it Microsatellite PCR Kit (QIAGEN), 4 µL PCR-grade water, and 1.25 µL of the primer mix (Schuelke, 2000). The optimal PCR protocol consisted of an initial denaturation step at 95°C for 5 min; followed by 30 cycles of 95°C for 30 s, 56°C for 90 s, and 72°C for 30 s; eight cycles of 95°C for 30 s, 52°C for 90 s, and 72°C for 30 s; and a final extension step at 68°C for 30 min. Labeled PCR products were then subjected to fragment analysis by Macrogen (Seoul, Republic of Korea). The resulting .fsa files were manually analyzed using Geneious 7.1.5 (Biomatters). Fifteen primers were selected based on amplification success and the number of alleles generated (Table 1).

TABLE 1. Characterization of 15 microsatellite loci obtained from *Anthoxanthum aristatum–A. ovatum* and test of cross-amplification in different Eurasian diploid and polyploid *Anthoxanthum* spp.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Allele size (bp)	Successful cross-amplification[a]	Fluorescent dye	GenBank accession no.
AG_AX_232[b]	F: AGTACAACAGCACTGGAGCATC R: CAGGTTGTCAGCGAACTGAG	$(AGC)_6$	59	112	*A. made*, *A. alpi*, *A. ama*, *A. odo*	FAM	KU883614
AG_AX_24[b]	F: CTTCCGGATCGAGAAACTGA R: AGAACATGGGAAGCAACCAG	$(AGC)_5$	60	112	*A. made*, *A. alpi*, *A. ama*, *A. odo*	HEX	KU883615
AG_AX_402[c]	F: GGTGGCCGTCAAACAAA R: CGTCTGCCACCTCCCAT	$(AG)_5$	59	106	*A. made*, *A. alpi*, *A. ama*	FAM	KU883616
AG_AX_01[c]	F: TCACGTGGTCCAGGTAAACA R: TGCTCGAGGAAGAACTCGAT	$(AG)_8$	60	107	*A. made*, *A. alpi*, *A. ama*, *A. odo*	HEX	KU883617
AG_AX_07[d]	F: GCTTGTTCCTGTTCGACTCC R: GCGTGAATTTGACCATTCCT	$(AC)_7$	60	230	*A. made*, *A. alpi*, *A. ama*, *A. odo*	FAM	KU883618
AG_AX_390[d]	F: TGGTCTCCTCGTCAGCG R: AAGTGTATAAGAATGCACTCGGC	$(AGG)_8$	59	149	*A. ama*, *A. odo*	HEX	KU883619
AG_AX_29[d]	F: TCTTGAGAGGTGGATTTCCG R: GAGGATGCAGTGAAGGAGGA	$(AG)_6$	60	245	*A. ama*, *A. odo*	HEX	KU883620
AG_AX_39[e]	F: ACGACAGGACTTTCACCTGG R: TGTAGCATAGCATCCGGGTT	$(AG)_5$	60	307	*A. made*, *A. alpi*, *A. ama*, *A. odo*	FAM	KU883621
AG_AX_159[e]	F: CAGTGCTCAGTTACATCGGG R: GGCCACCACTCATATGTGAAC	$(AG)_5$	60	131	*A. alpi*, *A. odo*	HEX	KU883622
AG_AX_472[e]	F: CTTGTAACCTGCGCGACAAT R: ATCGGTTCTTGGTCGGATTA	$(AG)_6$	60	295	*A. made*, *A. alpi*, *A. ama*, *A. odo*	HEX	KU883623
AG_AX_17[f]	F: TGTTGAGGTAGGCACTGACG R: CCACCTAGCTTCCAGGACAA	$(ATC)_5$	60	106	*A. made*, *A. alpi*, *A. ama*, *A. odo*	FAM	KU883624
AG_AX_08[f]	F: GAGTAGCGACTCGTGGAAGC R: AGGGAGAAGAAGGGCTTGAG	$(CCG)_5$	60	371	*A. made*, *A. alpi*, *A. ama*, *A. odo*	HEX	KU883625
AG_AX_55	F: TTGCCTGTTTGAGAGTCACG R: CATGAAGGGAGCACATGAAG	$(AG)_7$	60	222	*A. odo*	HEX	KU883626
AG_AX_476	F: AAGGATGAGCACCCAGAGC R: AGTCGTCTCCTCGAATCCTG	$(AC)_5$	60	117	*A. odo*	FAM	KU883627
AG_AX_177	F: CAATCGTGCCTTGTATCGC R: GGATTTGAGGGAGGAGATGA	$(AC)_5$	60	328	*A. alpi*, *A. odo*	HEX	KU883628

Note: *A. made* = *Anthoxanthum maderense*; *A. alpi* = *A. alpinum*; *A. ama* = *A. amarum*; *A. odo* = *A. odoratum*; T_a = annealing temperature.

[a]All loci are polymorphic for these species, with a minimum of two (*A. maderense*, *A. amarum*) or three alleles (*A. alpinum*, *A. odoratum*).

[b,c,d,e,f]Indicate the primers that were coamplified in multiplex reactions. The primers AG_AX_177, AG_AX_55, and AG_AX_476 were amplified in singleplex reactions.

TABLE 2. Genetic properties of the developed microsatellites of specimens from three populations of the Eurasian diploid *Anthoxanthum aristatum–A. ovatum* lineage.[a]

Locus	Fluminimaggiore (n = 21)				Omalos Plain (n = 20)				Cabo Silleiro (n = 20)				Average values (±SEM)		
	A	H_e	H_o	PIC	A	H_e	H_o	PIC	A	H_e	H_o	PIC	Total A	H_e	H_o	PIC
AG_AX_232	4	0.52	0.52	0.47	3	0.52	0.60	0.46	5	0.64	0.70	0.59	6	0.56 ± 0.04	0.60 ± 0.05	0.50 ± 0.04
AG_AX_24	2	0.32	0.30	0.26	1	0.00	0.00	0.00	3	0.14	0.06	0.14	4	0.17 ± 0.10	0.12 ± 0.09	0.13 ± 0.07
AG_AX_402	1	0.00	0.00	0.00	1	0.00	0.00	0.00	1	0.00	0.00	0.00	1	0.00	0.00	0.00
AG_AX_01*	6	0.73	0.56	0.69*	4	0.62	0.75	0.57	7	0.76	0.76	0.73*	10	0.70 ± 0.04	0.69 ± 0.06	0.66 ± 0.05
AG_AX_07	1	0.00	0.00	0.00	2	0.47	0.45	0.36	4	0.67	0.60	0.60	4	0.38 ± 0.19	0.35 ± 0.18	0.32 ± 0.17
AG_AX_390	6	0.57	0.60	0.55	4	0.30	0.35	0.28	4	0.60	0.83	0.52	7	0.49 ± 0.09	0.59 ± 0.14	0.48 ± 0.08
AG_AX_29	3	0.51	0.50	0.43	4	0.65	0.47	0.60	1	0.00	0.00	0.00	5	0.38 ± 0.19	0.32 ± 0.16	0.34 ± 0.20
AG_AX_39	4	0.44	0.45	0.40	2	0.5	1.00	0.37*	3	0.52	0.10	0.46*	4	0.48 ± 0.02	0.51 ± 0.26	0.41 ± 0.03
AG_AX_159*	5	0.63	1.00	0.57*	2	0.31	0.23	0.26	5	0.60	0.53	0.56	10	0.51 ± 0.10	0.58 ± 0.22	0.41 ± 0.10
AG_AX_472*	3	0.46	0.14	0.37*	3	0.55	0.20	0.50*	3	0.54	0.55	0.44	7	0.51 ± 0.03	0.26 ± 0.09	0.43 ± 0.04
AG_AX_17	3	0.52	0.63	0.45	4	0.60	0.75	0.52	5	0.62	0.67	0.57	5	0.58 ± 0.03	0.68 ± 0.04	0.51 ± 0.03
AG_AX_08	6	0.43	0.49	0.42	2	0.27	0.33	0.24	4	0.56	0.56	0.51	6	0.42 ± 0.08	0.46 ± 0.06	0.39 ± 0.08
AG_AX_177	2	0.01	0.10	0.10	1	0.00	0.00	0.00	2	0.46	0.72	0.35	3	0.16 ± 0.15	0.27 ± 0.22	0.15 ± 0.10
AG_AX_476	1	0.00	0.00	0.00	1	0.00	0.00	0.00	1	0.00	0.00	0.00	1	0.00	0.00	0.00
AG_AX_55	1	0.00	0.00	0.00	1	0.00	0.00	0.00	1	0.00	0.00	0.00	1	0.00	0.00	0.00

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals used per population; PIC = polymorphism information content.

[a] Voucher and locality information are provided in Appendix 1.

* Primers that were not in Hardy–Weinberg equilibrium in all populations.

Polymorphism assessment: Amplification in Eurasian taxa—Analyses were conducted on 61 *A. aristatum–A. ovatum* individuals (three populations, population size: 20–21; Appendix 1). Descriptive statistics (number of alleles, observed [H_o] and expected heterozygosities [H_e], and polymorphism information content [PIC]) and departure from Hardy–Weinberg equilibrium (HWE) were estimated per population using GenAlEx 6.5 (Peakall and Smouse, 2006) and GENEPOP (Raymond and Rousset, 1995). Twelve out of 15 candidate microsatellite primers used in the test were polymorphic at least in two of the analyzed populations (Table 2), whereas the remaining three primers were monomorphic. Across these populations, mean H_o and H_e in polymorphic markers were 0.364 (0.117–0.692 per locus, standard error of the mean [SEM] = 0.04) and 0.359 (0.154–0.705 per locus; SEM = 0.04), respectively (Table 2). Mean PIC was 0.452 (0.160–0.792 per locus; SEM = 0.04), and the number of alleles per locus across populations ranged from three to 10. All polymorphic loci but four (AG_AX_01, AG_AX_39, AG_AX_159, and AG_AX_472; $P < 0.01$) were in HWE in all surveyed populations (Table 2).

An extended polymorphism test was conducted in 80 individuals (15 populations; Appendix 1, Table 3) belonging to the different diploid taxa included in the Euro-Siberian clade of *Anthoxanthum*. This extended analysis was limited to diploids due to the uncertainty of allele dosage in polyploids (Servick et al., 2011). Thirteen out of 15 microsatellite primers used were polymorphic in *A. aristatum–A. ovatum* individuals (nine populations, 50 specimens; Table 3; locus AG_AX_472, monomorphic in the first test, was polymorphic in this extended analysis). The number of alleles ranged between three and 10. H_o and H_e were 0.385 (0.063–0.731 per locus; SEM = 0.05) and 0.630 (0.363–0.815 per locus; SEM = 0.04), respectively. PIC ranged between 0.331 and 0.8 (SEM = 0.04). The number of alleles recovered in *A. maderense* (one population, five specimens) ranged between two and three, with only nine out of 15 primers showing amplification and polymorphism. H_o and H_e were 0.41 (SEM = 0.12) and 0.407 (SEM = 0.09), respectively (0.2–1.0 per locus in both parameters; Table 3). PIC values were between 0.160 and 0.470 (SEM = 0.04). In *A. alpinum* (five populations, 25 specimens), the number of alleles per locus ranged between two and 10, with 11 out of 15 primers showing polymorphism. Overall H_o and H_e for *A. alpinum* was 0.27, showing a greater variation across loci (H_o = 0.07–0.80, SEM = 0.07; H_e = 0.07–0.85, SEM = 0.07). PIC values ranged between 0.062 and 0.80 (SEM = 0.07; Table 3).

Amplification was successfully conducted in two polyploid lineages in the complex (Table 1). Eighty specimens (10 populations) of the widespread tetraploid *A. odoratum* and 15 plants of the narrow endemic polyploid *A. amarum* (16*x*–18*x*, three populations) were used. Eleven and 14 primers out of 15 were polymorphic in *A. amarum* and *A. odoratum*, respectively. The number of alleles obtained for each species ranged between two and six for *A. amarum* and between three and 12 in *A. odoratum*.

CONCLUSIONS

In this study, 15 novel microsatellite loci were developed for the diploid Mediterranean *A. aristatum–A. ovatum* lineage. Nine and 11 markers were polymorphic in the other Eurasian (and Macaronesian) diploid lineages of *Anthoxanthum*, *A. maderense* and *A. alpinum*, respectively. Cross-amplification in polyploid *Anthoxanthum* revealed high transferability to the highly invasive tetraploid *A. odoratum* and to the narrowly distributed polyploid Iberian endemic *A. amarum*. These markers constitute a valuable tool for biogeographic and evolutionary studies in this group of grasses.

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TABLE 3. Genetic properties of the developed microsatellites of specimens from all the diploid lineages in Eurasian *Anthoxanthum*.[a,b]

	A. aristatum–A. ovatum (9/50)				*A. alpinum* (5/24)				*A. maderense* (1/5)			
Locus	*A*	H_e	H_o	PIC	*A*	H_e	H_o	PIC	*A*	H_e	H_o	PIC
AG_AX_232	8	0.81	0.56	0.78	3	0.12	0.12	0.11	3	0.60	0.60	0.47
AG_AX_24	7	0.52	0.43	0.47	4	0.36	0.37	0.32	2	0.20	0.20	0.16
AG_AX_402	3	0.52	0.06	0.41	2	0.07	0.07	0.06	2	1.00	1.00	0.37
AG_AX_01	6	0.52	0.43	0.47	4	0.36	0.37	0.32	2	0.20	0.20	0.16
AG_AX_07	4	0.54	0.38	0.44	3	0.32	0.36	0.28	2	0.20	0.20	0.16
AG_AX_390	4	0.68	0.44	0.62	—	—	—	—	—	—	—	—
AG_AX_29	5	0.62	0.12	0.58	—	—	—	—	—	—	—	—
AG_AX_39	6	0.62	0.47	0.54	5	0.74	0.80	0.67	3	0.38	0.40	0.31
AG_AX_159	9	0.82	0.30	0.80	3	0.23	0.25	0.21	—	—	—	—
AG_AX_472	10	0.80	0.43	0.76	10	0.85	0.55	0.80	2	0.57	0.50	0.37
AG_AX_17	6	0.60	0.73	0.54	3	0.41	0.54	0.34	2	0.57	1.00	0.37
AG_AX_08	6	0.76	0.20	0.69	2	0.27	0.27	0.21	2	0.35	0.00	0.27
AG_AX_177	4	0.36	0.44	0.33	2	0.09	0.09	0.08	—	—	—	—
AG_AX_476	1	0	0	—	—	—	—	—	—	—	—	—
AG_AX_55	1	0	0	—	—	—	—	—	—	—	—	—

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; PIC = polymorphism information content.

[a] Voucher and locality information are provided in Appendix 1.

[b] The numbers of populations/specimens used per lineage are indicated in parentheses.

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Appendix 1. Voucher details for the *Anthoxanthum* samples used. All vouchers are deposited in the Universidad de Santiago de Compostela Herbarium (SANT), Santiago de Compostela, Spain.

Species (Ploidy)	Collection locality	Geographic coordinates	Collectors	*N*	Voucher no.
Anthoxanthum aristatum Boiss.–*A. ovatum* Lag. (2*x*)	[a]SPAIN: Lugo, Quiroga, Sequeiros	42°26′50.31″N, 007°13′32.04″W	M. Perille, M. Pimentel, D. Romero & E. Sahuquillo	5	SANT 52195
	[a,b]GREECE: Crete, Imbros, Imbros Gorge	35°14′49.2″N, 24°10′05.2″E	M. Pimentel & E. Sahuquillo	5	SANT 65967
	[c]MOROCCO: Boukhalef Sovahel, 15 Km SW Tanger	35°44′26.6″N, 005°54′10.5″W	M. A. Minaya	5	SANT 65971
	[c]MOROCCO: On the rd. from Tiflet to Sidi Allal El Bahraoui, Mamora Forest	34°10′19.1″N, 006°31′32.0″W	M. A. Minaya	5	SANT 65972
	[c]SPAIN: Sevilla, Alcalá de Guadaira, El Gandul	37°23′19.03″N, 005°59′13.47″W	P. Jiménez, S. Martín-Bravo, M. Luceño	5	SANT 65973
	[c]SPAIN: Santa Cruz de Tenerife, La Palma	28°40′38.86″N, 17°49′04.62″W	A. Santos-Guerra	9	—
	[c]SPAIN: Huelva, Parque Nacional de Doñana, Caño del Tío Antoñito	36°58′44.01″N, 006°28′11.17″W	M. Pimentel & E. Sahuquillo	5	SANT 53375
	[c]SPAIN: Madrid, Montejo de la Sierra	40°59′01.07″N, 003°49′00.28″W	C. Cortizo & E. Sahuquillo	5	SANT 53404
	[c]SPAIN: Ourense, Larouco	42°20′42.56″N, 007°09′40.42″W	C. Cortizo, M. Perille, M. Pimentel & E. Sahuquillo	6	SANT 53405
	[b,c]SPAIN: Pontevedra, Baiona, Cabo Silleiro	42°06′42.67″N, 008°53′53.94″W	C. Cortizo & E. Sahuquillo	20	SANT 52193
	[b]GREECE: Crete, Omalos Plain	35°19′25.0″N, 23°53′31.2″E	M. Pimentel & E. Sahuquillo	20	SANT 65966
	[b,c]ITALY: Sardinia, Iglesias, Fluminimaggiore	39°27′01.5″N, 008°28′15.7″E	M. Pimentel & E. Sahuquillo	21	SANT 65970
Anthoxanthum maderense Teppner (2*x*)	[c]PORTUGAL: Madeira, Poça da Neve, Estrada cara ao Arieiro	32°44′58.66″N, 16°58′53.09″W	M. Sequeira & P. Catalán	5	SANT 52179
Anthoxanthum alpinum Á. Löve & D. Löve (2*x*)	[c]FRANCE: Auvergne-Rhône-Alpes, Haute-Savoie, Col de Galibier	45°03′48.7″N, 006°24′29.6″E	M. Perille, M. Pimentel & D. Romero	5	SANT 52189
	[c]ITALY: Central Apennines, Gran Sasso e Monti della Laga, 2230–2240 m.s.m.	42°29′32.9″N, 13°29′48.3″E	P. Jiménez	5	SANT-72599
	[c]BULGARIA: Pirin National Park, Bangkso, path to Vihren peak from the hut, 1950–2300 m.s.m.	41°44′22.3″N, 23°25′26.9″E	P. Jiménez	5	SANT 72598
	[c]BULGARIA: Rila National Park, Seven Rila Lakes, 2100–2300 m.s.m.	42°12′07.2″N, 23°19′01.4″E	P. Jiménez	4	SANT 72597
	[b,c]SERBIA: W Balcans, path between Zarkova and Midzor, 1800–2000 m.s.m.	43°23′24.01″N, 22°40′16.60″E	P. Jiménez	5	SANT 72583
Anthoxanthum amarum Brot. (16*x*–18*x*)	SPAIN: Ourense, Montederramo, Gabín	42°15′52.71″N, 007°28′53.45″W	M. Perille, M. Pimentel & E. Sahuquillo	5	SANT 52222
	SPAIN: Asturias, Santa Eulalia de Oscos, Road Taramundi–Teixoes	43°21′42.9″N, 007°01′25.8″W	M. Pimentel & E. Sahuquillo	5	SANT 65935
	SPAIN: Pontevedra, Tomiño, Amorín	41°38′39.1″N, 008°43′54.9″W	C. Cortizo & E. Sahuquillo	5	SANT 52217
Anthoxanthum odoratum L. (4*x*)	[a,b]PORTUGAL: Guarda, Serra da Estrela, Manteigas	40°23′54.7″N, 007°32′48.3″W	M. Pimentel & E. Sahuquillo	5	SANT 72584
	ITALY: Tuscany, Alpi Apune, Vinca track to Capanna Gannerone, 1050 m.s.m.	44°08′23.7″N, 10°09′32.9″E	P. Jiménez	5	SANT 72586
	[b]SPAIN: Ourense, Rubiá, Casaio	42°20′14.15″N, 006°48′7.48″W	M. Pimentel & E. Sahuquillo	5	SANT 72596
	SWEDEN: Uppland, Alunda	60°03′46.0″N, 18°04′58.7″E	M. Pimentel	5	SANT 53396
	CHILE: X Región de los Lagos, Isla de Chiloé, Iglesia Compu	42°52.300′S, 73°42.086′W	M. Pimentel & E. Sahuquillo	10	SANT 72593
	CHILE: VIII Región del Biobío, Concepción, Coronel, Escuadrón	37°011′S, 73°08′W	M. Pimentel & E. Sahuquillo	10	SANT 72595
	CHILE: IX Región de la Araucanía, Curacautín-Lonquimay, Manzanales	38°27.676′S, 71°42.681′W	M. Pimentel & E. Sahuquillo	10	SANT 72591
	CHILE: XIV Región de Los Ríos, Valdivia, Los Liles, Castro	39°52.166′S, 73°28.348′W	M. Pimentel & E. Sahuquillo	10	SANT 72599
	CHILE: XIV Región de Los Ríos, Valdivia, Lago Ranco	40°23.348′S, 72°04.943′W	M. Pimentel & E. Sahuquillo	10	SANT 72601
	CHILE: XIV Región de Los Ríos, Valdivia, Niebla, before Caleta el Molinar	39°51.002′S, 73°23.431′W	M. Pimentel & E. Sahuquillo	10	SANT 72592

Note: *N* = number of individuals sampled.

[a] Individuals used for the construction of the microsatellite-enriched genomic library (one specimen per population).

[b] Specimens used in the first primer test (40 microsatellite loci).

[c] Individuals used in the extended primer test (two specimens per population).