Characterization and Multiplexing of 21 Microsatellite Markers for the Herb Noccaea caerulescens (Brassicaceae)

Authors: Mousset, Mathilde, Flaven, Elodie, Justy, Fabienne, Pouzadoux, Juliette, Gode, Cécile, et al.

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Primer Note

Characterization and multiplexing of 21 microsatellite markers for the herb *Noccaea caerulescens* (Brassicaceae)[1]

Mathilde Mousset[2], Elodie Flaven[2,6], Fabienne Justy[2], Juliette Pouzadoux[2], Cécile Gode[3], Maxime Pauwels[3,6], and Cédric Gonneau[4,5]

[2]Institut des Sciences de l'Évolution, Université de Montpellier, CNRS, IRD, EPHE, CC 065, Place Eugène Bataillon 34095, Montpellier CEDEX 05, France; [3]Université Lille, CNRS, UMR 8198, Evo-Eco-Paleo, F-59000 Lille, France; [4]Université de Lorraine, Laboratoire Sols et Environnement (UMR 1120), F-54518 Vandœuvre-lès-Nancy CEDEX, France; and [5]INRA, Laboratoire Sols et Environnement (UMR 1120), F-54518 Vandœuvre-lès-Nancy CEDEX, France

- *Premise of the study:* Multiplexed microsatellite markers were developed for population genetic studies in the pseudometallophyte *Noccaea caerulescens* (Brassicaceae), a model species to investigate metal tolerance and hyperaccumulation in higher plants.
- *Methods and Results:* Microsatellite loci were isolated through pyrosequencing of an enriched DNA library. Three multiplexes combining four previously published and 17 newly designed markers were developed. The new markers were screened in metallicolous and nonmetallicolous populations from southern France. The total number of alleles per locus ranged from five to 18. The observed heterozygosity per locus and per population ranged from 0 to 0.83, and expected heterozygosity ranged from 0 to 0.89.
- *Conclusions:* The investigated loci showed reasonable to high levels of polymorphism at the regional scale. The multiplex set should be helpful in investigating genetic diversity, population structure, and demographic history in *N. caerulescens* at various spatial scales.

Key words: Brassicaceae; heavy metal tolerance; microsatellite; *Noccaea caerulescens*; pseudometallophyte.

The Alpine pennycress, *Noccaea caerulescens* (J. Presl & C. Presl) F. K. Mey. (Brassicaceae), occurs over a large range in Europe. The species is particularly known for its capacity to grow on soils with a high concentration of trace elements such as zinc, cadmium, and nickel. Considering its phylogenetic proximity with *Arabidopsis thaliana*, this characteristic makes *N. caerulescens* a favorite model plant for the study of the genetic bases of metal homeostasis, as well as of the ecological and evolutionary processes involved in local adaptation to extreme environments (Assunção et al., 2003). In order to understand the effect of soil metals on the evolution of *N. caerulescens* populations, it is necessary to study the population genetics of the species. So far, however, the low number of available molecular markers (Basic and Besnard, 2006; Jiménez-Ambriz et al., 2007), low polymorphism, presence of null alleles (Basic and Besnard, 2006; Besnard et al., 2009), and low amplification rate (E. Flaven, personal observation), as well as the absence of protocols for high-throughput genotyping, has not allowed the performance of deep population genetic studies. Here, we introduce 17 new microsatellite markers organized in three multiplexes to reduce genotyping time and costs. The multiplexes also include formerly published markers, thus providing a complete resource in this species.

METHODS AND RESULTS

***Microsatellite library construction*—**Genomic DNA of three individuals from the Baraquette population (Appendix 1) was extracted using a cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1990) followed by RNase treatment, and mixed. Development of the microsatellite library was outsourced to Genoscreen (Lille, France). It involved coupling multiplex microsatellite enrichment isolation techniques with 454 GS FLX Titanium pyrosequencing of the enriched DNA, according to the protocol of Malausa et al. (2011). Enrichment was performed using probes containing the following motifs: AG_{10}, AC_{10}, AAC_8, AGG_8, ACG_8, AAG_8, $ACAT_6$, and $ATCT_6$. Sequence data were automatically screened to detect microsatellite motifs, leading to 1852 candidate loci. Primers were designed in silico by Genoscreen, using the QDD pipeline (Meglécz et al., 2010).

***Biological validation*—**Biological validation of a subset of these loci was simultaneously performed at Institut des Sciences de l'Évolution de Montpellier

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[6]Authors for correspondence: elodie.flaven-noguier@univ-montp2.fr and maxime.pauwels@univ-lille1.fr

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TABLE 1. Characterization of 21 microsatellite loci in *Noccaea caerulescens*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Ct (μM)	Multiplex	Fluorescent dye	Post-PCR dilution	Position *A.t.*[a]	Position *N.c.*[a]	Chromosomal blocks[b]	Allele size range (bp)	GenBank accession no.	Publication
Ncpm09	F: TAGACGCTGCGTTTTGAAGA	CT_{18}	58	0.4	NcM1	6-FAM	1/150	1	2	B	96–130	KR065729	
	R: CCTCTGTTGAGTGAATGGTTCTC												
Ncpm13	F: CCAAAACTATGCCGATCTCA	AG_{16}	58	0.2	NcM1	VIC	1/150	NA	NA	NA	158–166	KR065730	
	R: CCACGAGCGAATCTTCTTGT												
Ncpm21	F: GTCACCACTTGCTACGGGAT	CTT_{10}	58	0.2	NcM1	VIC	1/150	3	7	F	240–277	KR065731	
	R: CATTGGATAGCACAGAGGCA												
Ncpm23	F: TTTCATGTCTCGGATCCTCC	TTC_{11}	58	0.4	NcM1	6-FAM	1/150	NA	NA	NA	197–263	KR065732	
	R: GCAGAGCGCAATCTAAGGAC												
Ncpm31	F: GATTATCGAGCTTACTAAAAGCAGC	CTT_{12}	58	0.2	NcM1	NED	1/150	5	4	W	58–101	KR065733	
	R: GTGTTGAAGCGCAATGAAGA												
Tc-up1	F: TGCTCTGTTTCTCTCCACATTC	$(CA)_n(CT)_n$	50	0.2[d]	NcM1	NED	1/160	NA	NA	NA	132–170	AJ746212	Basic and Besnard, 2006
	R: TTCCTTGCTTCTTCTCTTCCA												
Ncpm07	F: TGGAATGGTTCTGTGGACAA	TCA_{18}	58	0.4	NcM2	6-FAM	1/150	NA	NA	NA	109–139	KR065734	
	R: TTCTGGAATTGGCTGCTTCT												
Ncpm14	F: GACCACATCTCGTCTTGCCT	CTT_{15}	58	0.4	NcM2	PET	1/150	NA	NA	NA	108–123	KR065735	
	R: GACCCTAACTACAGGCTGTGAAA												
Ncpm19	F: CCAACAATGGATTGGGAGAG	$GTGTA(TG)_{14}$	58	0.4	NcM2	VIC	1/150	NA	NA	NA	100–124	KR065736	
	R: TCCCCATCACTCCAGCTAAG												
Ncpm29	F: CTCCCTCTTCTCCTACCTACACATA	AC_{17}	58	0.4	NcM2	NED	1/150	NA	NA	NA	78–94	KR065737	
	R: GGTTGAAAGTAGGAGTGAGTCAAGA												
Tc-up4	F: GTTTTGTCCGCTTTGCTTCC	CT_{13}	50	0.2[d]	NcM2	VIC	1/140	NA	NA	NA	253–262	AJ746216.1	Basic and Besnard, 2006
	R: GCCATAGACTTTCTCATTGATTC												
9C7/Thlc3	F: GTCACGAGTTTCACCATT	AG_{13}	58	0.4	NcM2	VIC	1/150	NA	NA	NA	152–191		Jiménez-Ambriz et al., 2007
	R: ATCTTCCACAATTGTGCC												
Tc-up2	F: TGAGAAGAGGAGACACAGGAAC	$(AG)_5(AG)_5(GA)_6$	58	0.2	NcM2	PET	1/150	NA	NA	NA	232–244	AJ746213.1	Basic and Besnard, 2006
	R: CACTTACCAAATCGAAAACTGCTCC												
Nc02	F: GGAGCTGTGGTTTCTGAAGG	AGG_8	68/47[c]	0.06	NcM3	VIC	None	4	3	O	170–191	KR065738	
	R: AGCATCGTATTCCGATCCAG			0.28									
Nc03	F: TGAGCTTCTTCAGTCCCGAT	AC_{12}	68/47[c]	0.06	NcM3	NED	None	5	4	S	188–194	KR065739	
	R: TATGAGCTCGTCGCTCACAG			0.28									
Nc04	F: ACGGTCGCATACCAAAAAGT	AG_{11}	68/47[c]	0.06	NcM3	VIC	None	2	5	J	123–143	KR065740	
	R: AGGATGCACTCCTTGAGACC			0.28									
Nc06b	F: GCGTCTTCTCTCCATCCTCA	AG_{13}	68/47[c]	0.07	NcM3	PET	None	4	6	U	89–153	KR065741	
	R: GGATTTCCAATTCAATCTTCCC			0.37									
Nc07b	F: CCAGTTTCCAACGGCATAGT	AC_{10}	68/47[c]	0.06	NcM3	NED	None	3	7	F	105–112	KR065742	
	R: TTGGTTTGGTTTCTTCTTGTGA			0.28									
Nc19	F: CGGATTGTTGTGAATCCCAT	AGG_{11}	68/47[c]	0.07	NcM3	PET	None	2	5	J	199–235	KR065743	
	R: ACCTCTTCTTTCGCCCTTGT			0.28									
Nc20	F: ACACAACTCCAAAGGCTTCA	AG_{11}	68/47[c]	0.15	NcM3	6-FAM	None	4	3	O	205–234	KR065744	
	R: TTTTGTTCTAACCGTTACTCTTT			0.75									
Nc22	F: TTGCTTTCACATGTCTTGACG	AG_{10}	68/47[c]	0.11	NcM3	6-FAM	None	2	5	K	111–121	KR065745	
	R: GAACAGAACAAGAAGACATGAATGA			0.56									

Note: Ct = final concentration of primers; NA = not available; T_a = annealing temperature.

[a] Relative chromosomal position in *Arabidopsis thaliana* and *Noccaea caerulescens*, respectively (Schranz et al., 2006).

[b] Genome blocks in the "ancestral karyotype" blocks (Schranz et al., 2006).

[c] Following Godé et al. (2012), touchdown PCR was performed. During the first 10 cycles, annealing temperature was decreased from 68°C to 50°C in increments of 2°C. This was followed by 27 cycles with an annealing temperature at 47°C.

[d] Standalone PCR.

(ISEM) and at Laboratoire Évolution Écologie et Paléontologie (Evo-Eco-Paleo), with requirements for levels of genetic polymorphism at different spatial scales. At ISEM, amplification trials were performed on seven individuals from five populations from southern France (Appendix 1). DNA extraction followed a classic CTAB protocol (Doyle and Doyle, 1990). Based on type and number of repeat units, repeat structures, and amplicon size, 32 candidate loci from the library were selected and tested separately. The PCR reactions were carried out in a total volume of 10 µL, containing 1 µL of DNA template, forward and reverse primers (0.2 µM), and 1× QIAGEN Multiplex PCR Master Mix (QIAGEN, Courtaboeuf, France). Cycling conditions were: an initial denaturation step at 95°C for 15 min, then 30 cycles consisting of 30 s at 94°C, 90 s at 58°C, and 1 min at 72°C, followed by a final extension of 30 min at 60°C. PCRs were conducted on Eppendorf Mastercycler pro, Mastercycler nexus gradient (Eppendorf, Hamburg, Germany), and Techne TC-5000 (GMI, Ramsey, Minnesota, USA) machines. Amplification products were visualized with agarose gel (2%) with ethidium bromide stain. Due to inadequate amplification yield, low specificity, or unexpected size, only 15 markers were kept. Forward primers were labeled with one of the FAM, NED, VIC, or PET fluorescent dyes (Applied Biosystems, Waltham, Massachusetts, USA). PCR products were analyzed separately through electrophoresis on an ABI3130 Genetic Analyzer (Applied Biosystems). Nine loci were finally retained based on presence of polymorphism and quality of profiles.

At Evo-Eco-Paleo, 20 primer pairs corresponding to 20 additional loci from the library were selected. They were tested separately on 23 individuals scattered in the European species range (Koch and German, 2013; Appendix 1). Total DNA was extracted using the QIAGEN DNeasy kit (QIAGEN). Extraction and test PCR were performed according to Godé et al. (2012). Each primer pair was tested using FAM labeling. PCR reactions were carried out in a total volume of 10 µL, containing 1 µL of 1/20 diluted DNA template, 2 µM of forward and reverse primers, and 1× QIAGEN Multiplex PCR Master Mix. A final set of eight markers was selected based on the quality of genotyping profiles, compatibility of amplicon sizes for multiplexing, and relative positions on the genome (Table 1). Genomic positions of microsatellite loci were determined by BLASTN searches of microsatellite flanking sequences against the *Arabidopsis thaliana* genome and by using the synteny among chromosomal blocks determined for different Brassicaceae species and the ancestral karyotype (Schranz et al., 2006).

***Screening of the new microsatellite markers*—**All forward primers were labeled with fluorescent dyes, and markers were combined in multiplex PCR based on size compatibility and annealing temperatures (Table 1). Primer dimerization was checked using OligoAnalyzer 1.0.3 (Integrated DNA Technologies, Coralville, Iowa, USA). In addition to newly defined markers, four previously developed markers (Tc-up1, Tc-up2, Tc-up4, Thlc3; Basic and Besnard, 2006; Jiménez-Ambriz et al., 2007) were added to the multiplexes to increase the number of available, multiplexed markers (results not shown, see Table 1 for details). However, due to differing annealing temperatures, Tc-up1 and Tc-up4 were processed in separate PCR and added in the post-PCR steps.

Seventy-four individuals from four populations (Appendix 1, identified as "natural populations screening") were analyzed. DNA extraction followed the protocol from Doyle and Doyle (1990). The PCR reactions were carried out following the protocols described above: ISEM section for the first (NcM1) and second (NcM2) multiplexes, Evo-Eco-Paleo section for the third (NcM3), except that forward and reverse primers were mixed (concentrations in Table 1). Three microliters of diluted PCR product (dilutions in Table 1) were transferred in a mix of 15 µL of Hi-Di Formamide (Applied Biosystems) and 0.15 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems). Raw data were analyzed using GeneMapper (version 5.0; Applied Biosystems). Automatic analysis and manual check of all the peaks were performed. Detection of the presence of null alleles in populations was performed with FreeNA (Chapuis and Estoup, 2007). Two new loci (Ncpm31 and Ncpm07) harbor null alleles, with frequencies between 10% and 15%. Expected heterozygosity, intrapopulation fixation index, and linkage equilibrium tests were computed using FSTAT (version 2.9.3; Goudet, 1995), and observed heterozygosity was computed in GENETIX (version 4.05; Belkhir et al., 2004). No linkage disequilibrium was detected between loci (with Bonferroni correction). The number of alleles for each locus ranged from five to 18 (Table 2). The observed heterozygosity per locus and per population ranged from 0 to 0.83, and the expected heterozygosity ranged from 0 to 0.89. Hardy–Weinberg equilibrium was tested in GENEPOP (Rousset, 2008), and most of the loci in the four populations were at equilibrium. This result contrasts with previous studies in *N. caerulescens* (Dubois et al., 2003; Basic and Besnard, 2006; Jiménez-Ambriz et al., 2007; Besnard et al., 2009) and may be due to sample size.

TABLE 2. Statistical analysis of the 17 new microsatellite markers in four populations[a] of *Noccaea caerulescens* in southern France.

		All				Avinières (n = 18)				Saint Bresson (n = 22)				Saint Hippolyte (n = 17)				Coulet (n = 17)			
Locus	% amp	A	H_o	H_e	F_{IS}[b]	A	H_o	H_e	F_{IS}[b]	A	H_o	H_e	F_{IS}[b]	A	H_o	H_e	F_{IS}[b]	A	H_o	H_e	F_{IS}[b]
Ncpm09	100	16	0.563	0.699	0.108	8	0.722	0.794	0.091	5	0.545	0.598	0.089	10	0.823	0.893	0.078	6	0.353	0.46	0.232
Ncpm13	100	5	0.260	0.346	0.130	3	0.500	0.606	0.175	3	0.500	0.530	0.057	1	0.000	0.000	NA	4	0.176	0.224	0.213
Ncpm21	100	9	0.505	0.570	0.136	4	0.500	0.556	0.10	5	0.682	0.639	–0.068	3	0.412	0.588	0.300	3	0.294	0.449	0.344
Ncpm23	100	9	0.444	0.557	0.199	4	0.389	0.562	0.308	3	0.409	0.427	0.043	5	0.412	0.566	0.273	4	0.529	0.638	0.170
Ncpm31	98.6	9	0.493	0.694	0.396	6	0.667	0.771	0.136	4	0.111	0.645	0.828*	5	0.412	0.763	0.460	5	0.529	0.658	0.196
Ncpm07	98.6	8	0.485	0.606	0.210	6	0.833	0.725	–0.149	2	0.364	0.359	–0.012	3	0.062	0.383	0.837*	5	0.412	0.717	0.426
Ncpm14	100	5	0.254	0.405	0.357	3	0.500	0.650	0.231	2	0.091	0.089	–0.024	3	0.235	0.318	0.260	2	0.059	0.346	0.830
Ncpm19	100	11	0.544	0.647	0.130	5	0.722	0.730	0.011	4	0.591	0.639	0.075	2	0.187	0.425	0.559	7	0.706	0.774	0.088
Ncpm29	100	9	0.579	0.733	0.174	6	0.611	0.753	0.189	6	0.773	0.696	–0.110	7	0.529	0.833	0.364	5	0.529	0.746	0.291
Nc02	97.3	6	0.384	0.482	0.081	5	0.750	0.693	–0.042	4	0.273	0.290	0.060	1	0.000	0.000	NA	5	0.562	0.727	0.226
Nc03	98.6	4	0.296	0.467	0.316	3	0.350	0.292	–0.14	4	0.429	0.699	0.387	2	0.059	0.059	0.000	4	0.375	0.7	0.464
Nc04	100	9	0.528	0.618	0.125	6	0.700	0.778	0.143	4	0.318	0.290	–0.097	4	0.647	0.695	0.069	5	0.529	0.732	0.276
Nc06b	100	18	0.582	0.740	0.233	9	0.700	0.815	0.046	7	0.636	0.812	0.216	7	0.437	0.627	0.302	5	0.412	0.717	0.426
Nc07b	98.6	5	0.501	0.592	0.194	4	0.500	0.672	0.255	4	0.571	0.570	–0.002	4	0.471	0.498	0.055	3	0.294	0.57	0.484
Nc19	97.3	8	0.585	0.628	0.073	5	0.684	0.768	0.158	5	0.762	0.698	–0.092	5	0.529	0.686	0.228	4	0.562	0.575	0.022
Nc20	97.3	9	0.269	0.330	0.305	4	0.600	0.544	–0.021	6	0.619	0.664	0.068	1	0.000	0.000	NA	3	0.125	0.235	0.469
Nc22	100	6	0.347	0.522	0.305	2	0.150	0.157	–0.063	5	0.727	0.778	0.065	2	0.235	0.507	0.536	3	0.235	0.605	0.611

Note: % amp = percentage of successful amplification; A = number of alleles; F_{IS} = intrapopulation fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; NA = not available.

[a] Information on geographic locations and vouchers is provided in Appendix 1.

[b] F_{IS} values significantly different from zero after Bonferroni correction ($P < 0.0006$) are indicated with an asterisk.

CONCLUSIONS

Three multiplexes including 17 new and four published microsatellite markers were developed and validated in natural populations. These loci exhibit substantial polymorphism within and between populations. They should provide sufficient power to study population structure and mating system, and to infer demographic history at different spatial scales.

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Appendix 1. Voucher and location information for *Noccaea caerulescens* populations used in the development and testing of microsatellites.

Population	Type of population	Collection date	Locality	Geographic coordinates	Sample names and storage location[a]	Voucher no.	Use
Angleur	Metallicolous	2009	Angleur	50°36′44.61″N, 5°36′38.74″E	B02.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Anjeau	Nonmetallicolous	October 2007	Saint-Laurent-le-Minier	43°55′3.33″N, 3°37′52.52″E	AN_Z_X_2007[c]		Biological validation of primer pairs (ISEM)
Auxelles-Haut	Nonmetallicolous	2009	Auxelles-Haut	47°44′21.52″N, 06°46′35.84″E	F03.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Auxy	Nonmetallicolous	2009	Auxy	46°57′44.13″N, 04°23′47.05″E	F05.01[b,d]	503854	Biological validation of primer pairs (Evo-Eco-Paleo)
Avinières	Metallicolous	March 2013	Saint-Laurent-le-Minier	43°55′55.67″N, 3°39′46.19″E	12_AV_ID_X[c]		Natural populations screening
Baraquette	Nonmetallicolous	October 2007	Saint-Laurent-le-Minier	43°55′6.73″N, 3°36′54.82″E	BQ_Z_X_2007[c]		Library construction
Breinigerberg	Metallicolous	2009	Breining	50°44′12.73″N, 06°14′30.92″E	G01.01[b,d]	501266, 501267	Biological validation of primer pairs (Evo-Eco-Paleo)
Col du Lautaret	Nonmetallicolous	2007	Lautaret	45°02′07″N, 06°24′20″E	F01.01[b,d]	501429	Biological validation of primer pairs (Evo-Eco-Paleo)
Coulet	Nonmetallicolous	September 2013	Saint-Maurice-Navacelles	43°49′44.59″N, 3°33′41.64″E	12_CO_ID_X[c]		Natural populations screening
Coulet	Nonmetallicolous	October 2007	Saint-Maurice-Navacelles	43°49′44.59″N, 3°33′41.64″E	CO_Z_X_2007[c]		Biological validation of primer pairs (ISEM)
Fellering	Serpentine	2009	Bergenbach	47°54′22.90″N, 06°57′25.50″E	F04.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Goebelsmühle	Nonmetallicolous	2009	Goebelsmühle	49°55′22.07″N, 06°3′44.08″E	L02.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Husavik	Nonmetallicolous	2006	Husavik	66°01′38.89″N, 17°17′21.71″W	I01.01[b,d]	911812	Biological validation of primer pairs (Evo-Eco-Paleo)
Lichtenau	Metallicolous	2006	Blankenrode	51°32′0.34″N, 08°54′17.02″E	G05.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Lintich	Metallicolous	2010	Bakomi	48°26′05.48″N, 18°55′09.43″E	SK01.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Mostviertiel	Nonmetallicolous	2006	Tormäuer	47°51′08.70″N, 15°17′13.20″E	A01.01[b,d]	406733	Biological validation of primer pairs (Evo-Eco-Paleo)
Moyen Age	Metallicolous	October 2007	Saint-Laurent-le-Minier	43°55′52.16″N, 3°38′26.09″E	MG_Z_X_2007[c]		Biological validation of primer pairs (ISEM)
Oslo	Nonmetallicolous	2009	Hovedoya	59°53′39″N, 10°43′44″E	N01.01[b,d]	501275–501278	Biological validation of primer pairs (Evo-Eco-Paleo)
Papeterie	Metallicolous	2006	Ganges	43°56′10.98″N, 03°40′19.88″E	F16.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Prayon	Metallicolous	2009	Prayon	50°35′3.31″N, 5°40′23.69″E	B01.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Ramponenche	Metallicolous	2010	Florac	44°20′18.25″N, 03°40′05.45″E	F02.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Saint Baudille	Nonmetallicolous	October 2007	Montpeyroux	43°44′40.73″N, 3°29′9.96″E	BD_Z_X_2007[c]		Biological validation of primer pairs (ISEM)
Saint Bresson	Metallicolous	October 2007	Pommiers	43°56′35.89″N, 3°37′57.02″E	SB_Z_X_2007[c]		Biological validation of primer pairs (ISEM)
Saint Bresson	Metallicolous	September 2013	Pommiers	43°56′35.89″N, 3°37′57.02″E	12_SB_ID_X[c]		Natural populations screening
Saint Hippolyte	Metallicolous	April 2014	Saint-Hippolyte-du-Fort	43°58′17.07″N, 3°49′59.98″E	Zi_Qj_Hi_2014_X[c]		Natural populations screening
Saint Jost	Metallicolous	2009	Virneburg	50°21′08.95″N, 07°06′33.65″E	G02.01[b,d]	501261, 501262	Biological validation of primer pairs (Evo-Eco-Paleo)

Appendix 1. Continued.

Population	Type of population	Collection date	Locality	Geographic coordinates	Sample names and storage location[a]	Voucher no.	Use
Silberberg	Metallicolous	2009	Silberberg	52°12′38.72″N, 07°56′46.13″E	G03.01[b,d]	504547	Biological validation of primer pairs (Evo-Eco-Paleo)
Somerset	Metallicolous	2010	Priddy	51°15′39.06″N, 02°39′02.12″W	UK1.06[b,d]	501343	Biological validation of primer pairs (Evo-Eco-Paleo)
Son	Nonmetallicolous	2000	Esterri d'Aneu	42°35′51.50″N, 01°04′23.82″E	SP01.02[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Špania Dolina	Nonmetallicolous	2010	Bakomi	48°48′29.87″N, 19°08′14.65″E	SK02.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Stauffenbergallee	Nonmetallicolous	2009	Dresden	51°06′04.48″N, 13°47′17.61″E	G04.01[b,d]	280040	Biological validation of primer pairs (Evo-Eco-Paleo)
Štiavnické	Nonmetallicolous	2010	Bakomi	48°26′0.48″N, 18°51′29.86″E	SK03.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Uppsala	Nonmetallicolous	2010	Uppsala	59°50′40.68″N, 17°44′54.18″E	S01.01[b,d]	501430	Biological validation of primer pairs (Evo-Eco-Paleo)

[a] Letters in sample names are defined as: X = number of the plant on which a leaf was collected; Zi = number of the area in the population; Qj = number of the quadrat in the area; Z = plants on which seeds were collected. Several specimens were collected in each population.

[b] Vouchers of leaves were deposited at Laboratoire Évolution Écologie et Paléontologie (Evo-Eco-Paleo), Villeneuve d'Ascq, France.

[c] Vouchers of leaves were deposited at Institut des Sciences de l'Évolution (ISEM), Montpellier, France.

[d] Herbarium specimens of the corresponding population were collected and deposited by M. Koch at the Centre for Organismal Studies (COS), Biodiversity and Plant Systematics, Universität Heidelberg, Heidelberg, Germany. They are available from the BrassiBase database (Kiefer et al., 2014).