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Source: Applications in Plant Sciences, 3(12)

Published By: Botanical Society of America

URL: <https://doi.org/10.3732/apps.1500052>

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CHARACTERIZATION AND MULTIPLEXING OF 21 MICROSATELLITE MARKERS FOR THE HERB *NOCCA* *CAERULESCENS* (BRASSICACEAE)¹

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- **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: AG₁₀, AC₁₀, AAC₈, AGG₈, ACG₈, AAG₈, ACAT₆, and ATCT₆. 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 (Megléczy 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

¹Manuscript received 4 May 2015; revision accepted 4 August 2015.

The authors thank C. Hatt for her help in the sequencing of some of the individuals used in this study. Data used in this work were partly produced through the technical facilities of the LabEx Centre Méditerranéen de l'Environnement et de la Biodiversité. This work was supported by a grant from the Agence Nationale pour la Recherche (Project BIOADAPT, SEAD: ANR-13-ADAP-0011) and by funding from the Observatoire de Recherche Méditerranéen de l'Environnement (OSU-OREME) program. This is publication ISE-M 2015-208.

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doi:10.3732/apps.1500052

Applications in Plant Sciences 2015 3(12): 1500052; <http://www.bioone.org/loi/apps> © 2015 Mousset et al. Published by the Botanical Society of America. This work is licensed under a Creative Commons Attribution License (CC-BY-NC-SA).

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

| Locus | Primer sequences (5'-3') | Repeat motif | T_a (°C) | Ct (μM) | Multiplex | Fluorescent dye | Post-PCR dilution | Position A.f. ^a | Position N.c. ^a | Chromosomal blocks ^b | Allele size range (bp) | GenBank accession no. | Publication |
|-----------|---|---|--------------------|------------------|-----------|-----------------|-------------------|----------------------------|----------------------------|---------------------------------|------------------------|-----------------------|-----------------------------|
| | | | | | | | | | | | | | |
| Ncpm09 | F: TAGACGCTGCGTTTGAAGA R: CCTCTGTTGAGTGAATGGTTCTC | CT ₁₈ | 58 | 0.4 | NcM1 | 6-FAM | 1/150 | 1 | 2 | B | 96–130 | KR065729 | |
| Ncpm13 | F: CCAAAAATATGCCGATCTCA R: CCACGAGCGAAATCTTCTTGT | AG ₁₆ | 58 | 0.2 | NcM1 | VIC | 1/150 | NA | NA | NA | 158–166 | KR065730 | |
| Ncpm21 | F: GTCACCACTTGTACGGGAT R: CATTTGGATAGCACAGAGCA | CTT ₁₀ | 58 | 0.2 | NcM1 | VIC | 1/150 | 3 | 7 | F | 240–277 | KR065731 | |
| Ncpm23 | F: TTTCAITGTCGGATCTCTCC R: GCAGAGCGCAATCTAAGGAC | TTC ₁₁ | 58 | 0.4 | NcM1 | 6-FAM | 1/150 | NA | NA | NA | 197–263 | KR065732 | |
| Ncpm31 | F: GATATCGAGCTTACTAAAAGCAGC R: GTGTTGAAGCGCAATGAAGA | CTT ₁₂ | 58 | 0.2 | NcM1 | NED | 1/150 | 5 | 4 | W | 58–101 | KR065733 | |
| Tc-up1 | F: TGCTCTGTTTCTCCACATTC R: TTCCTTCTCTCTCTCTTCCA | (CA) _n (CT) _n | 50 | 0.2 ^d | NcM1 | NED | 1/160 | NA | NA | NA | 132–170 | AJ746212 | Basic and Besnard, 2006 |
| Ncpm07 | F: TGGAAATGGTCTGTGGACAA R: TTCTGGAATTTGGCTGTCTT | TCA ₁₈ | 58 | 0.4 | NcM2 | 6-FAM | 1/150 | NA | NA | NA | 109–139 | KR065734 | |
| Ncpm14 | F: GACCAATCTCGTCTTGCCCT R: GACCCATACTACAGGCTGTGAAA | CTT ₁₅ | 58 | 0.4 | NcM2 | PET | 1/150 | NA | NA | NA | 108–123 | KR065735 | |
| Ncpm19 | F: CCAACAATGGAATGGGAGAG R: TCCCATCACTCCAGCTAAG | GTGTA(TG) ₁₄ | 58 | 0.4 | NcM2 | VIC | 1/150 | NA | NA | NA | 100–124 | KR065736 | |
| Ncpm29 | F: CTCCTCTTCTCTACTACACATA R: GGTGAAAGTAGGAGTAGTCAAGA | AC ₁₇ | 58 | 0.4 | NcM2 | NED | 1/150 | NA | NA | NA | 78–94 | KR065737 | |
| Tc-up4 | F: GTTTGTCGCTTTGTCTCC R: GCCATAGACTTCTCATTTGATTC | CT ₁₃ | 50 | 0.2 ^d | NcM2 | VIC | 1/140 | NA | NA | NA | 253–262 | AJ746216.1 | Basic and Besnard, 2006 |
| 9C7/Thlc3 | F: GTCACGAGTTTCCACCAAT R: ATCTTCCACAAATGTGCC | AG ₁₃ | 58 | 0.4 | NcM2 | VIC | 1/150 | NA | NA | NA | 152–191 | | Jiménez-Ambriz et al., 2007 |
| Tc-up2 | F: TGAGAAGAGGACACAGGAAC R: CACTTACCAAAATCGAAAATCTGCTCC | (AG) ₅ (AG) ₃ (GA) ₆ | 58 | 0.2 | NcM2 | PET | 1/150 | NA | NA | NA | 232–244 | AJ746213.1 | Basic and Besnard, 2006 |
| Nc02 | F: GGAGCTGTGTTTCTGAAGG R: AGCATCTATTCGGATCCAG | AGG ₈ | 68/47 ^c | 0.06 0.28 | NcM3 | VIC | None | 4 | 3 | O | 170–191 | KR065738 | |
| Nc03 | F: TGAGCTTCTTCCAGTCCGAT R: TATGAGCTCGTCTCACAG | AC ₁₂ | 68/47 ^c | 0.06 0.28 | NcM3 | NED | None | 5 | 4 | S | 188–194 | KR065739 | |
| Nc04 | F: ACGTGCATACCAAAAAGT R: AGGATGCATCTCTTGAGACC | AG ₁₁ | 68/47 ^c | 0.06 0.28 | NcM3 | VIC | None | 2 | 5 | J | 123–143 | KR065740 | |
| Nc06b | F: GCGTCTTCTCCATCTCA R: GGATTTCCAAATCAATCTCCC | AG ₁₃ | 68/47 ^c | 0.07 0.37 | NcM3 | PET | None | 4 | 6 | U | 89–153 | KR065741 | |
| Nc07b | F: CCAGTTCCAAAGGCAATAGT R: TTGGTTTGGTTCTTCTTGTA | AC ₁₀ | 68/47 ^c | 0.06 0.28 | NcM3 | NED | None | 3 | 7 | F | 105–112 | KR065742 | |
| Nc19 | F: CGGATTTGTGAATCCCAT R: ACCCTCTTTCGCCCTTGT | AGG ₁₁ | 68/47 ^c | 0.07 0.28 | NcM3 | PET | None | 2 | 5 | J | 199–235 | KR065743 | |
| Nc20 | F: ACACAATCCAAAGGTTCA R: TTTTGTCTAAACGGTTACTTT | AG ₁₁ | 68/47 ^c | 0.15 0.75 | NcM3 | 6-FAM | None | 4 | 3 | O | 205–234 | KR065744 | |
| Nc22 | F: TTGCTTTCACATGTCTTGACG R: GAACAGAACAAAGACATGAATGA | AG ₁₀ | 68/47 ^c | 0.11 0.56 | NcM3 | 6-FAM | None | 2 | 5 | K | 111–121 | KR065745 | |

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

^aRelative chromosomal position in *Arabidopsis thaliana* and *Noccaea caerulea*, respectively (Schranz et al., 2006).

^bGenome blocks in the “ancestral karyotype” blocks (Schranz et al., 2006).

^cFollowing 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.

^dStandalone 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, Courtabouef, 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. caerulea* (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 caerulea* in southern France.

| Locus | All | | | | Avinières (n = 18) | | | | Saint Bresson (n = 22) | | | | Saint Hippolyte (n = 17) | | | | Coulter (n = 17) | | | | |
|--------|-------|----|----------------|----------------|------------------------------|---|----------------|----------------|------------------------------|---|----------------|----------------|------------------------------|----|----------------|----------------|------------------------------|---|----------------|----------------|------------------------------|
| | % 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 | 0.730 | 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.

^aInformation on geographic locations and vouchers is provided in Appendix 1.

^bF_{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 caerulea* 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 |
| Breingerberg | 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) |
| Mostviertel | 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 I. 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 | Esterrí 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).