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# Isolation and characterization of 13 microsatellite loci for *Neochamaelea pulverulenta* (Cneoraceae)

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## Abstract

**We report 13 polymorphic nuclear microsatellite loci for *Neochamaelea pulverulenta* (Cneoraceae) using an enriched-library approach. Although this plant species is tetraploid, expected patterns for tetrasomic segregation were completely absent, and all loci analysed showed a diploid pattern of inheritance. We detected a total of 102 alleles in 57 individuals genotyped (mean number of alleles per locus was 7.85). The values of observed and expected heterozygosities ranged from 0.193 to 0.737 and 0.425 to 0.812 respectively. Disomic segregation, levels of polymorphism and the exclusionary power of the developed markers render them readily applicable for parentage assignment of dispersed seeds, and for analyses of spatial genetic structure and population connectivity.**

*Keywords*: Canary Islands, diploidization, pollination, seed dispersal, seed endocarp

*Received 22 December 2008; revision accepted 12 March 2009*

We isolated and characterized microsatellite loci from the Canarian endemic plant *Neochamaelea pulverulenta* (Cneoraceae) to investigate the contemporary gene flow

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patterns via pollination by ants and solitary bees and seed dispersal by lizards. Recent advances in parentage assignment methods allow direct estimation of dispersal distances using the genotypes of both maternally derived seed endocarps contrasted with the genotype of candidate maternal plants (Godoy & Jordano 2001). Highly polymorphic levels make nuclear microsatellites ideal

markers for assignment of dispersed seeds to the source shrub. This approach could also be used to investigate the spatial genetic structure and connectivity of *N. pulverulenta* populations.

*Neochamaelea pulverulenta* (Vent.) Erdtman (Cneoraceae) is considered a palaeoendemic shrub from the Canary Islands (Gran Canary, Tenerife, La Gomera, and possibly extinct from El Hierro), and the other only member of this family being the Mediterranean *Cneorum tricoccon* L. Several authors have pointed out their status as tetraploids ($2n = 36$; ploidy level = $4x$; Suda *et al.* 2005) and it was also kindly confirmed by J. Loureiro for material collected from Teno Bajo using flow citometry (see also Loureiro *et al.* 2008). These plants are androdioecious (some individuals with only male flowers and others with both hermaphroditic—but functionally female—and male flowers), being pollinated by small solitary bees (*Lasioglossum* spp., Halictidae), ants (*Camponotus feai*, Formicidae) and introduced honeybees (*Apis mellifera*, Apidae). Its fleshy fruits are only consumed and their seeds dispersed by lizards, *Gallotia* spp., Lacertidae (Valido & Nogales 1994).

Microsatellite libraries were developed following Jones *et al.* (2002). Genomic DNA (approximately 100 μg) was extracted from silica-dried leaves from one individual shrub and using the Qiagen DNeasy Plant Extraction kit. The DNA was partially restricted with seven blunt-end restriction enzymes (*Rsa*I, *Hae*III, *Bsr*B1, *Pvu*II, *Stu*I, *Sca*I and *Eco*RV; New England Biolabs). Fragments of 300–750 bp were ligated to 20-bp oligonucleotides, which contained a *Hin*dIII site at the 5′ end. Four libraries were prepared in parallel using Biotin-CA$_{15}$, Biotin-GA$_{15}$, Biotin-ATG$_{12}$ and Biotin-AAC$_{12}$ as capture molecules (CPG Inc.). Molecules were captured with streptavidin-coated magnetic beads and the resulting repeat-enriched DNA was amplified using a primer complementary to the adaptor and digested with *Hin*dIII to remove the adapters. The resulting fragments were ligated into the *Hin*dIII site of pUC19 plasmid and introduced into *Escherichia coli* DH5α by electroporation. Recombinant clones (132) were selected at random for sequencing and 45 of them contained a microsatellite sequence. Polymerase chain reaction (PCR) primer pairs were designed for 24 clones using DESIGNER PCR 1.03 (Research Genetics Inc.).

For primer testing, DNA was isolated from silica-dried leaves of 57 individual shrubs collected in Teno Bajo, Buenavista del Norte (NW Tenerife; 28 individual plants) and Punta de Juan Centella, Icod de los Vinos (N Tenerife; 29 individual plants). We used a standard CTAB extraction method (Milligan 1998) with some minimal modifications: tissue grinding in a MM301 Retsch™ mill and TLE (10 mM Tris–HCl pH 8.0, 0.1 mM EDTA) resuspension. [Correction added after online publication 29 June 2009: in the preceeding sentence the chemical composition of TLE, (200 mM Tris-HCl pH 8.0, 70 mM EDTA) was corrected to (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA)]

Polymerase chain reaction reactions were performed in 20 μL final volume containing 1X buffer [67 mM Tris–HCl pH 8.8, 16 mM (NH$_4$)$_2$SO$_4$, 0.01% Tween-20], 2.5 mM MgCl$_2$ (2.0 mM for NpA106), 0.01% BSA (Roche Diagnostics), 0.25 mM dNTP, 0.40 μM dye-labelled M13 primer, 0.25 μM tailed-reverse primer (Table 1), 0.034 μM M13 tailed-forward primer, 0.5 U *Taq* DNA polymerase (Bioline) and 5 μL (50 ng) of genomic DNA. Samples were incubated in a 'touchdown' PCR in a Bio-Rad DNA Engine$^R$ Peltier Thermal Cycler, with an initial 2 min of denaturation at 94 °C; 17 cycles at 92 °C for 30 s, annealing at 60–44 °C (66–50 °C for NpA106) for 30 s (1 °C decrease in each cycle) and extension at 72 °C for 30 s; 25 cycles at 92 °C for 30 s, 44 °C (50 °C for NpA106) for 30 s and 72 °C for 30 s; and a final extension for 5 min at 72 °C. Amplified fragments were analysed on an ABI 3130xl Genetic Analyser and sized using GeneMapper 4.0 (Applied Biosystems) and LIZ 500 size standard.

We tested a total of 24 primer pairs. Six of them failed to amplify or showed complex amplification, five were monomorphic and 13 were polymorphic. We finally retained these 13 primers after inspecting their observed and expected heterozygosities (Cervus 3.0; Kalinowski *et al.* 2007) and testing for deviations from Hardy–Weinberg equilibrium, gametic disequilibrium (GENEPOP 4.0; Rousset 2007) and the presence of null alleles (MICRO-CHECKER 2.2.3; van Oosterhout *et al.* 2004). Besides, we specifically tested for diploidized markers in this tetraploid plant species. We used Bonferroni-corrected *P*-values to assess significance of the results obtained.

We detected a total of 102 diploidized alleles for 13 loci, corresponding to an average of 7.85 alleles per locus (range: 3–16) (Table 1). Two loci (NpA110 and NpA106) showed a significant deviation from HWE (Bonferroni-corrected $P < 0.05/13 = 0.0038$) for Teno Bajo population. Both of them plus NpB112 (Teno Bajo) and only NpB9 (Punta de Juan Centella) showed evidence for the presence of null alleles ($P < 0.0038$). Gametic disequilibrium was detected for one pair of loci (NpB125 and NpA109; $P < 0.0038$) for Teno Bajo population. Mean polymorphic information content was 0.5822. The combined nonexclusion probability across all 57 shrubs was 0.020 for the first parent and 0.0007 for the second parent. No loci yielded multiple bands (peaks) that would suggest polysomic segregation. In contrast, results were consistent with typical disomic segregation of the genome, further confirming the diploidization of these micosatellite loci. Similar results have been described, e.g. in the octoploid *Fragaria virginiana* Mill., where all microsatellite loci tested showed a diploid pattern of inheritance (Ashley *et al.* 2003).

In addition, we tested the feasibility of DNA isolation from the seed endocarp tissue (Godoy & Jordano 2001).

**Table 1** Characteristics of 13 polymorphic microsatellite markers isolated from *Neochamaelea pulverulenta* (Cneoraceae) populations at Teno Bajo (TE) and Punta de Juan Centella (CE) (Tenerife, Canary Islands)

| Locus name (GenBank ID) | Primer sequences (5'–3') | Repeat motif | Allele size range (bp) | Teno Bajo (TE) | | | | | | Punta de Juan Centella (CE) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $n$ | $K$ | $H_O$ | $H_E$ | HW | FNA | $n$ | $K$ | $H_O$ | $H_E$ | HW | FNA |
| NpA108 (FJ596185) | F: GCCATTAGGGTAAGGACACAAG R: TCGAGAGCCTCATCTGTTACAG | $(AC)_{15}$ | 127–152 | 28 | 5 | 0.786 | 0.760 | 0.601 | −0.0266 | 29 | 4 | 0.207 | 0.226 | 0.336 | 0.0624 |
| NpB125 (FJ596186) | F: CCCCATGTTAGTTCCAAATAC R: GTTTTAATCCGTCGATCAAGAG | $(CT)_{16}$ | 183–203 | 28 | 7 | 0.679 | 0.808 | 0.024 | 0.069 | 29 | 4 | 0.483 | 0.521 | 0.359 | 0.0237 |
| NpB105 (FJ596187) | F: TCCTGCTTACTGGTACGTTTAC R: CCCTCGCTTTCTAGTTTCTAAT | $(AG)_{14}$ | 243–271 | 28 | 6 | 0.607 | 0.642 | 0.535 | 0.0373 | 29 | 3 | 0.172 | 0.222 | 0.185 | 0.0791 |
| NpB115 (FJ596188) | F: TCAAAACCCAATACTCAATC R: GCAGATGGAGCCACAACT | $(TC)_{21}$ | 139–173 | 28 | 11 | 0.821 | 0.855 | 0.508 | 0.0151 | 29 | 7 | 0.655 | 0.671 | 0.302 | −0.009 |
| NpA110 (FJ596189) | F: CCAATGAACCATGTGTATATTG R: CGCTCATACTTTAGAACAAATCC | $(AC)_{13}$ | 209–231 | 28 | 7 | 0.536 | 0.766 | 0.0003 | 0.1363* | 29 | 3 | 0.552 | 0.608 | 0.051 | 0.0239 |
| NpC3 (FJ596190) | F: ACGGTCATTTGAGATTTTGAAG R: AGTGGGACCAGTACATCACAAG | $(TGT)_8$ TGG $(TTG)_5$ | 254–285 | 28 | 7 | 0.536 | 0.686 | 0.195 | 0.1148 | 29 | 1 | — | — | — | 0 |
| NpB109 (FJ596191) | F: CGTTTGACTTTCTTTCTTTACCC R: TGCCTTTACGCTTTGAGC | $(TC)_{14}$ | 170–188 | 28 | 5 | 0.571 | 0.601 | 0.530 | 0.0281 | 29 | 3 | 0.379 | 0.514 | 0.100 | 0.1172 |
| NpA106 (FJ596192) | F: CATCGGTTCAACAATCTTATG R: CGTTGGTTAGAGGATATGGTAC | $(TG)_{31}$ | 249–295 | 27 | 15 | 0.407 | 0.918 | 0 | 0.2675* | 29 | 5 | 0.448 | 0.531 | 0.237 | 0.0752 |
| NpA2 (FJ596193) | F: CCCCAAAACGACACACAG R: TGAGGACCGTGGTTAGAGG | $(TG)_{13}$ | 196–202 | 28 | 4 | 0.143 | 0.171 | 0.177 | 0.0982 | 29 | 3 | 0.621 | 0.459 | 0.989 | −0.2158 |
| NpB112 (FJ596194) | F: CACCAAGAACAAAACCATGAG R: ATCACGTCACGATATGGAGAC | $(AC)_3 (CT)_{17} (CTT)_2$ | 245–277 | 28 | 7 | 0.643 | 0.812 | 0.041 | 0.0992* | 29 | 4 | 0.172 | 0.223 | 0.171 | 0.0794 |
| NpA109 (FJ596195) | F: GCATTCTCCGTCTCACTCC R: ATGGAACACACGACACCTG | $(CA)_{18} (TA)_4$ | 322–345 | 28 | 9 | 0.607 | 0.654 | 0.418 | 0.0449 | 28 | 7 | 0.571 | 0.655 | 0.310 | 0.0671 |
| NpB9 (FJ596196) | F: TTTCTACTGGGTCTGCTACATG R: CAGTTGAATGACCTCTTCACTG | $(CT)_{13}$ | 260–272 | 28 | 4 | 0.250 | 0.318 | 0.053 | 0.1005 | 29 | 3 | 0.138 | 0.249 | 0.032 | 0.156* |
| NpA124 (FJ596197) | F: CAGCCTCTAACAAGGTCTATCC R: TGCGTCAAATGAAACACG | $(AC)_{13}$ | 279–295 | 28 | 3 | 0.571 | 0.480 | 0.919 | −0.124 | 29 | 3 | 0.552 | 0.500 | 0.768 | −0.0588 |

$n$, Number of individuals successfully genotyped; $k$, number of alleles; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity; HW, nominal *P*-values for the test of deviations from Hardy–Weinberg equilibrium; FNA, frequency of null alleles was calculated using Oosterhout method for Teno Bajo (TE) and Punta de Juan Centella (CE). PCR products were labelled using FAM, VIC, NED or PET (Applied Biosystems) dyes on an additional 19 bp M13 primer (5'-CACGACGTTGTAAAACGAC-3') according to the methods of Boutin-Ganache *et al.* (2001). Moreover, a palindromic sequence tail (5'-GTGTCTT-3') was added to the 5' end of the reverse primer to improve adenylation and facilitate genotyping (Brownstein *et al.* 1996). Forward (F) and reverse (R) primers are differentially tailed.
*Presence of null alleles.

For this purpose, the hard seeds were split open and the endocarp was separated by hand from the embryo. After different grinding tests, we used 100–140 mg (half endocarp approx.) of dry tissue, which was cut into 2–3 mm pieces with a small pliers. We followed the DNA isolation protocol for leaves with two modifications: grinding was carried out three times at 30 Hz (2.5 min each), with intermediate freezing in liquid nitrogen, and the DNA pellet was resuspended in 85 µL TLE. DNA yields ranged from 5 to 8 ng/µL. Endocarp PCR amplifications were performed with the reaction mixture described above, and reliable genotypes were obtained from dispersed (from lizard droppings) and hand-collected (from known shrubs) seeds. Besides, we confirmed the maternal derivation of the endocarp tissue from seeds collected directly from individual plants and lizard droppings, and therefore its suitability for obtaining an unequivocal maternal genetic fingerprint for the dispersed seeds. Our results indicate that polymorphism levels and exclusionary power of the markers are adequate for direct measurements of seed dispersal through parental assignment.

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# Characterization of microsatellite loci in the fungus, *Grosmannia clavigera*, a pine pathogen associated with the mountain pine beetle

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