Characterization of microsatellite markers for the endangered *Daphne rodriguezii* (Thymelaeaceae) and related species

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PREMISE: The endangered shrub *Daphne rodriguezii* (Thymelaeaceae) is endemic to the Balearic island of Menorca, where fragmentation and severe population decline are ongoing threats to this taxon. We developed a set of microsatellite markers to analyze the fine-scale genetics of its few extant populations.

METHODS AND RESULTS: Fifteen microsatellite markers were obtained through Illumina high-throughput sequencing and tested in two populations. Twelve of these loci showed no evidence of null alleles and were highly polymorphic, with a mean number of 8.3 alleles per locus. Levels of observed and expected heterozygosity ranged from 0.100 to 0.952 and from 0.095 to 0.854, respectively. Seven to nine of these loci were successfully amplified in five other *Daphne* species.

CONCLUSIONS: This set of markers provides a useful tool for investigating the factors driving fine-scale population structure in this threatened species, and it represents a novel genetic resource for other European *Daphne* species.

KEYWORDS: *Daphne rodriguezii*; fine-scale genetic structure; island genetic diversity; paternity analysis; Thymelaeaceae.
Population B (n = 20)

| Locus | Population A (n = 22) | Population B (n = 20) |
|-------|-----------------------|-----------------------|
| Dro012| 20 6 0.750 0.772     | 20 6 0.750 0.772     |
| Dro019| 20 3 0.450 0.359     | 20 3 0.450 0.359     |
| Dro028| 20 1 0.000 0.000     | 20 1 0.000 0.000     |
| Dro034| 19 8 0.700 0.791     | 19 8 0.700 0.791     |
| Dro035| 20 1 0.000 0.000     | 20 1 0.000 0.000     |
| Dro041| 20 7 0.750 0.741     | 20 7 0.750 0.741     |
| Dro042| 20 10 0.750 0.821    | 20 10 0.750 0.821    |
| Dro046| 20 3 0.450 0.563     | 20 3 0.450 0.563     |
| Dro048| 20 7 0.500 0.593     | 20 7 0.500 0.593     |
| Dro073| 21 1 0.000 0.000     | 21 1 0.000 0.000     |
| Dro078| 20 10 0.550 0.826    | 20 10 0.550 0.826    |
| Dro113| 20 5 0.600 0.728     | 20 5 0.600 0.728     |
| Dro114| 20 11 0.801 0.441    | 20 11 0.801 0.441    |

Note: A = number of alleles detected across D. rodriguezi samples; \( H_e \) = expected heterozygosity; \( H_o \) = observed heterozygosity; \( N \) = number of samples tested; \( N \) = number of samples with successful amplifications.

Local haplotypes are provided in Appendix 1.

Asterisks indicate significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction (* * * \( P < 0.05 \), *** \( P < 0.001 \)).

Presence of null alleles.

In examining the levels of variability revealed by each SSR locus, we were constrained by the conservation status (EN) of the study species. However, we were able to obtain permissions to sample leaf material from two populations representing size extremes (Calvito-Cancela et al., 2012): (1) the only population with more than 300 mature individuals (population A) and (2) a population with <50 individuals (population B) (Appendix 1).
Genomic DNA was extracted using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) following the cetyltrimethylammonium bromide (CTAB)–lysis method. PCRs for SSR amplification were set up in 10-μL reactions, including 1.5 μL of DNA (2–10 ng/μL), 5 μL of 2× Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), and 0.3 μL (0.3 μM) of each primer, with the forward primer labeled with a fluorescent dye (Table 1). Reactions were performed on a G-Storm GS2 thermal cycler (Somerton Biotechnology Centre, Somerset, United Kingdom) under the following conditions: initial denaturation at 95°C for 15 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 45 s; and a final extension at 60°C for 30 min.

To test cross-species amplification of *D. rodriguezii* primers, all 15 SSR loci were amplified in closely related *Daphne* species (Alonso and Herrera, 2011), including leaf material freshly collected from one population of *D. laureola* L. and two to three replicates from herbarium samples for *D. cneorum*, *D. mezereum*, and *D. oleoides* Schreb. (Appendix 1). Rather than collected from one population of *D. laureola* and two to three replicates from herbarium samples for *D. cneorum*, *D. mezereum*, and *D. oleoides* Schreb. (Appendix 1), including leaf material freshly collected from one population of *D. laureola* L. and two to three replicates from herbarium samples for *D. cneorum*, *D. mezereum*, and *D. oleoides* Schreb. (Appendix 1). Rather than collected from one population of *D. laureola* and two to three replicates from herbarium samples for *D. cneorum*, *D. mezereum*, and *D. oleoides* Schreb. (Appendix 1), including leaf material freshly collected from one population of *D. laureola* L. and two to three replicates from herbarium samples for *D. cneorum*, *D. mezereum*, and *D. oleoides* Schreb. (Appendix 1).

| Locus   | *D. laureola* (*N* = 5) | *D. cneorum* (*N* = 2) | *D. gnidium* (*N* = 2) | *D. mezereum* (*N* = 1) | *D. oleoides* (*N* = 2) |
|---------|------------------------|------------------------|------------------------|--------------------------|------------------------|
| Dro012  |            | 279                    | 255                    | 251                      | 257,271                |
| Dro019  |            |                   | 247                    |                |            |
| Dro025  | 156        | 136,151,163           | 152,156,168           | 156                      | 154,156,162,164        |
| Dro028  | 190        | 235,239,253,289       | 192                  | 204,206                  | 204,206                |
| Dro034  |            |                   |                       |                |            |
| Dro035  | 204,206    | 205,207               | 192                  | 204,206                  | 204,206                |
| Dro041  | 181,184    | 184                  | 184                  |                    | 184                    |
| Dro042  | 314,317,320|                     | 164                  | 290                      | 180                    |
| Dro046  | 266        |                     | 173,179             |                |            |
| Dro048  |            |                   |                       |                |            |
| Dro073  | 205,217    | 205                  | 205,217             | 205,217                  | 205                    |
| Dro078  |            | 148,157,173          |                       |                |            |
| Dro113  |            |                   |                       |                |            |
| Dro114  |            |                   |                       |                |            |
| Dro124  | 155        |                     |                       |                | 233,253                |

Note: — = unsuccessful amplification; *N* = number of samples tested for each species.

*Voucher and locality information are provided in Appendix 1.*

At the population level, the number of alleles per locus ranged from one to 11 (Table 2). The level of observed heterozygosity ranged from 0.000 to 0.952, and the level of expected heterozygosity ranged from 0.000 to 0.854 (Table 2). Three loci (Dro025, Dro035, Dro073) were fixed, or nearly so, for a single allele per population. The remaining 12 loci showed substantial levels of polymorphism, with a mean of 8.3 alleles per locus. Only one locus (Dro078) showed significant deviation from Hardy–Weinberg equilibrium after sequential Bonferroni correction in population B, most probably because this was the only combination of locus and population for which null alleles were clearly identified by MICRO-CHECKER. Significant (*P* < 0.001) linkage disequilibrium was found between loci Dro046 and Dro124, but only for population B.

In addition, this panel of microsatellites rendered positive amplifications in a minimum of seven loci per species (Table 3). The limited availability of herbarium samples per species precluded a clear assessment of the levels of polymorphism detected with these markers, but for some species (*D. laureola, D. cneorum, D. oleoides*), even relatively low sample sizes revealed that at least half of the amplified loci exhibited more than one allele (Table 3).

**CONCLUSIONS**

The set of microsatellites characterized for *D. rodriguezii* is a powerful, cost-effective tool for detecting substantial levels of genetic variation using a relatively low number of multiplexed reactions, even in small populations. Such a genetic resolution will allow us to assess population genetic studies with these markers could be easily extended to other closely related *Daphne* species.

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**AUTHOR CONTRIBUTIONS**

C.G.-V. and A.T. planned the study and collected plant tissue, J.C.I. and C.G.-V. conducted laboratory work and allele scoring, and C.G.-V. performed the analyses and wrote the manuscript, with input from J.C.I. and A.T.

**DATA ACCESSIBILITY**

The primers and microsatellite sequences developed in this study have been deposited in GenBank (accession numbers MK507747–MK507761; Table 1). Raw sequence library data were deposited in the Short Read Archive of the National Center for Biotechnology Information (NCBI) (BioProject accession number: PRJNA523502).

**LITERATURE CITED**

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**APPENDIX 1.** Voucher and location information for species and populations used in the characterization of microsatellite markers for Daphne rodriguezii and related species.

| Taxon (Population) | Voucher specimen accession no.* | Collection locality | Geographic coordinates | N |
|--------------------|---------------------------------|---------------------|------------------------|---|
| Daphne rodriguezii Texidor (popA) | JBAG8300 | Colorn, Menorca | 39°57.5’N, 04°16.9’E | 22 |
| Daphne rodriguezii (popB) | JBAG8301 | Menorca | 39°54.5’N, 04°17.0’E | 20 |
| Daphne cneorum L. | JBAG656 | Valle del Soba, Cantabria | 43°09.5’N, 03°34’1’W | 3 |
| Daphne gnidium L. | JBAG877 | Dumbria, La Coruña | 43°00.9’N, 09°07.4’W | 3 |
| Daphne lauruela L. | JBAG8299 | Ponga, Asturias | 43°12.7’N, 05°05.5’W | 5 |
| Daphne mezereum L. | JACA78470 | Canfranc, Huesca | 42°42.2’N, 00°34’1’W | 2 |
| Daphne oleoides Schreb. | JBAG384 | La Rapa, Granada | 37°20.1’N, 02°50.2’W | 2 |

Note: N = number of individuals initially assayed (some herbarium samples did not provide clear amplifications and were not used for polymorphism testing; see Table 3).

*All herbarium specimens are deposited at the Jardín Botánico Atlántico herbarium (JABG), Asturias, Spain, including one donation from the Instituto Pirenaico de Ecología herbarium (IPIE), Jaca, Spain.

**APPENDIX 2.** Optimal PCR annealing temperatures °C used for cross-species amplification of microsatellite markers developed for Daphne rodriguezii in five closely related species.

| Locus | D. cneorum | D. gnidium | D. lauruela | D. mezereum | D. oleoides |
|-------|------------|------------|-------------|-------------|------------|
| Dro012 | 55.7       | 52.6       | MB          | 52.6        | 60.2       |
| Dro019 | —          | 55.7       | —           | —           | —          |
| Dro025 | 59.0       | 59.0       | 59.0        | 59.0        | 59.0       |
| Dro028 | 50.0       | 60.0       | —           | 60.0        | 50.0       |

(Continues)
### APPENDIX 2. (Continued)

| Locus   | *D. cneorum* | *D. gnidium* | *D. laureola* | *D. mezereum* | *D. oleoides* |
|---------|--------------|--------------|---------------|---------------|---------------|
| Dro034  | —            | —            | MB            | —             | —             |
| Dro035  | 63.6         | 59.0         | 59.0          | 59.0          | 59.0          |
| Dro041  | 51.0         | 50.0         | 50.0          | —             | 50.1          |
| Dro042  | —            | 55.7         | 60.0          | 51.2          | 51.2          |
| Dro046  | —            | 51.2         | 50.1          | —             | —             |
| Dro048  | —            | —            | —             | —             | —             |
| Dro073  | 59.0         | 59.0         | 59.0          | 59.0          | 59.0          |
| Dro078  | 50.1         | —            | MB            | —             | —             |
| Dro113  | —            | —            | —             | 59.1          | 60.2          |
| Dro124  | —            | —            | 52.6          | —             | 55.7          |

Note: — = unsuccessful amplification; MB = multiple bands.