Microsatellite Loci in Two Epiphytic Lichens with Contrasting Dispersal Modes: Nephroma laevigatum and N. parile (Nephromataceae)

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MICROSATELLITE LOCI IN TWO EPIPHYTIC LICHENS WITH CONTRASTING DISPERSAL MODES: *Nephroma laevigatum* AND *N. parile* (Nephromataceae)1

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1 of 4

PRIMER NOTE

Dispersal among populations can explain gene flow patterns, can help in understanding the processes driving genetic structure and diversity, and can explain population persistence under different ecological and environmental scenarios (Lowe et al., 2004). Dispersal traits (i.e., reproductive strategy and propagule size) reflect a trade-off between dispersal ability and establishment success, and they have important consequences for population genetic structure (Hartl and Clark, 1997). In lichens, the contrast between sexual vs. asexual reproductive modes has provided a convenient system for evaluating consequent patterns of genetic structure and diversity (Werth, 2010); this is especially the case for lichen epiphytes that occur on trees mimicking discrete habitats (islands) of measurable size and age, and that are linked through dispersal dynamics to a network of surrounding trees, and at a larger scale, to adjacent woodland stands. Asexually reproducing lichen species disperse both their symbionts together (fungus and photosynthetic alga or cyanobacteria), but their propagules are largely compared to sexually reproducing species, which have a higher likelihood of long-distance dispersal (Seaward, 2008). However, this broad generalization in dispersal ability and its consequences for gene flow remain contentious owing to contradictory results (Werth et al., 2014). In particular, the majority of epiphyte studies have focused on a single model species—*Lobaria pulmonaria* (L.) Hoffm. (e.g., Scheideger and Werth, 2009)—that can reproduce both sexually and asexually, making it therefore problematic to unequivocally partition the ecological consequences of contrasting dispersal modes.

The focus of this paper is to describe microsatellite markers developed for two codistributed epiphytic lichens with different reproductive strategies and dispersal modes; i.e., *Nephroma laevigatum* Ach. and *N. parile* (Ach.) Ach. Both species are morphologically very similar and have a *Nostoc* photobiont, but they have developed contrasting reproductive traits. *Nephroma laevigatum* typically reproduces via sexual ascospores (spores = 18.5 × 6 μm), while the predominantly asexually reproducing *N. parile* normally produces marginal soredia (soredia = 150 μm) and only very infrequent apothecia (Smith et al., 2009). It has been shown using multigene phylogenies that these ecologically similar foliose lichens are each monophyletic and closely related to each other, although they are not sister species (Lohtander et al., 2002; Sérusiaux et al., 2011). Both species have oceanic to boreal-montane distributions, are common in ancient woodlands in Europe, and are sensitive to SO2 pollution (Smith et al., 2009).

METHODS AND RESULTS

For primer design, fungal genomic DNA was extracted from eight individuals per lichen species collected from four different populations across a climatic gradient in Scotland (Appendix 1). DNA was isolated from 20 mg of dried thallus material using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer’s protocol modified by increasing initial lysis incubation to 1 h at 70°C. Microsatellite sequences were isolated and primers were designed by Ecogenics GmbH (Zurich, Switzerland) using magnetic streptavidin beads and

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Applications in Plant Sciences 2014 2 (11): 1400080   Belinchón et al.—Microsatellites in Nephroma  

... Multiplex indicates loci that were mixed in the same capillary electrophoresis run. Annealing temperatures were 56 °C.

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Note: A = number of alleles.

* Multiplex indicates loci that were mixed in the same capillary electrophoresis run. Annealing temperatures were 56°C.

Table 1. Characteristics of 26 microsatellite loci developed in Nephroma laevigatum and N. parile.

| Locus  | Primer sequences (5′−3′) | Repeat motif | Multiplex* | Dye label | Allele size range (bp) | A | GenBank accession no. |
|--------|--------------------------|--------------|------------|-----------|-----------------------|---|-----------------------|
| NLa01  | F: TCCGTATGTTGCGAGAATTG  | (TCA)12      | M-NL1      | PET       | 192–231               | 8 | KM361439              |
|        | R: TCCGTATGTTGCGAGAATTG  | (TAA)3       | NED        | NED       | 198–237               | 7 | KM361440              |
|        | R: TGCTTCTTATATATATGACCTG | (TAC)12      | NED        | NED       | 230–234               | 3 | KM361441              |
| NLa02  | F: TGGTTTTCTGGAACGATATC  | (ACAT)4      | M-NL2      | PET       | 254–258               | 4 | KM361443              |
| NLa03  | F: ATGATATATATATATGAGATC | (ACAT)4      | M-NL2      | PET       | 89–129                | 8 | KM361444              |
| NLa04  | F: AGTATATATATATATGAGATC | (ACAT)4      | M-NL2      | PET       | 207–240               | 3 | KM361446              |
|        | R: AGTATATATATATATGAGATC | (ACAT)4      | M-NL2      | PET       | 126–168               | 7 | KM361447              |
| NLa05  | F: TGGTTTTCTGGAACGATATC  | (ACAT)4      | M-NL2      | PET       | 240–284               | 7 | KM361448              |
| NLa06  | F: AGTATATATATATATGAGATC | (ACAT)4      | M-NL2      | PET       | 199–229               | 4 | KM361449              |
|        | R: AGTATATATATATATGAGATC | (ACAT)4      | M-NL2      | PET       | 205–212               | 3 | KM361450              |
| NLa07  | F: TGGTTTTCTGGAACGATATC  | (ACAT)4      | M-NL2      | PET       | 170–176               | 3 | KM361454              |
|        | R: AGTATATATATATATGAGATC | (ACAT)4      | M-NL2      | PET       | 170–176               | 3 | KM361455              |
| NLa08  | F: TGGTTTTCTGGAACGATATC  | (ACAT)4      | M-NL2      | PET       | 219–241               | 3 | KM361456              |
| NLa09  | F: AGTATATATATATATGAGATC | (ACAT)4      | M-NL2      | PET       | 230–242               | 4 | KM361453              |
| NLa10  | F: TGGTTTTCTGGAACGATATC  | (ACAT)4      | M-NL2      | PET       | 240–284               | 7 | KM361454              |
| NLa11  | F: TGGTTTTCTGGAACGATATC  | (ACAT)4      | M-NL2      | PET       | 192–231               | 3 | KM361459              |
| NLa12  | F: TGGTTTTCTGGAACGATATC  | (ACAT)4      | M-NL2      | PET       | 192–231               | 3 | KM361459              |
| NPar01 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar02 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar03 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar04 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar05 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar06 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar07 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar08 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar09 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar10 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar11 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar12 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar13 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |
| NPar14 | F: CGCTATCCATTCATATTGATG | (ACAT)4      | M-NL2      | PET       | 233–244               | 3 | KM361457              |

Note: A = number of alleles.

* Multiplex indicates loci that were mixed in the same capillary electrophoresis run. Annealing temperatures were 56°C.

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were designed for 12 microsatellite inserts for *N. laevigatum* and 14 microsatellite inserts for *N. parile* and tested for polymorphism. Although few simple sequence repeats are expected in bacterial genomes, an additional BLAST search was included for all primer candidates to exclude any that matched the available *Nostoc* reference genome before testing for polymorphism.

Polymorphisms within the 26 microsatellite loci were determined by Eco
genics GmbH using a test set of 15 samples for each species collected from four Scottish populations (Appendix 1), and using M13-tailed forward primers and universal M13 fluorescent-labeled primer following Schuelke (2000). For these samples, genomic DNA was extracted from dried thallus material with the QIAGEN DNeasy Plant Mini Kit, and loci were PCR amplified in the Veriti 96-Well Thermal Cycler (Applied Biosystems, Carlsbad, California, USA). Primers that showed clear amplification profiles and reliable amplification and ties varied from 0.38 to 0.71 and 0.01 to 0.50 for *N. laevigatum* and *N. parile*, respectively (Tables 2, 3). A small sample of specimens (four per species) was repeated to check for reproducibility, and all yielded identical allele lengths.

**CONCLUSIONS**

Twelve and 14 microsatellite primers were characterized to investigate population structure and gene flow in *N. laevigatum* and *N. parile*, respectively. The markers demonstrate high-resolution variability at even a relatively small geographic

### Table 2. Results of microsatellite screening in four populations of *Nephroma laevigatum*. a,b

| Locus   | Total | Dulsie (n = 19) | Torboll (n = 14) | Glen Nant (n = 17) | Ardery (n = 16) |
|---------|-------|----------------|-----------------|-------------------|----------------|
| NLae02  | 66    | 10             | 0.71            | 14                 | 4              | 0.57           | 17            | 6              | 0.85           | 16            | 7              | 0.87           |
| NLae03  | 65    | 3              | 0.39            | 19                 | 2              | 0.35           | 13            | 1              | 0.00           | 17            | 3              | 0.69           | 16            | 3              | 0.51           |
| NLae04  | 64    | 7              | 0.50            | 18                 | 2              | 0.20           | 13            | 3              | 0.50           | 17            | 4              | 0.57           | 16            | 6              | 0.75           |
| NLae05  | 66    | 4              | 0.26            | 19                 | 2              | 0.11           | 14            | 1              | 0.00           | 17            | 3              | 0.49           | 16            | 4              | 0.44           |
| NLae07  | 66    | 13             | 0.77            | 19                 | 6              | 0.80           | 14            | 4              | 0.74           | 17            | 7              | 0.82           | 16            | 6              | 0.73           |
| NLae08  | 66    | 6              | 0.61            | 19                 | 3              | 0.51           | 14            | 3              | 0.27           | 17            | 6              | 0.83           | 16            | 6              | 0.84           |
| NLae09  | 66    | 10             | 0.83            | 19                 | 5              | 0.77           | 14            | 6              | 0.86           | 17            | 9              | 0.89           | 16            | 6              | 0.83           |
| NLae10  | 66    | 6              | 0.70            | 19                 | 4              | 0.70           | 14            | 3              | 0.60           | 17            | 5              | 0.78           | 16            | 5              | 0.73           |
| NLae11  | 66    | 5              | 0.53            | 19                 | 3              | 0.62           | 14            | 2              | 0.26           | 17            | 4              | 0.49           | 16            | 4              | 0.73           |
| NLae12  | 66    | 4              | 0.24            | 19                 | 1              | 0.00           | 14            | 1              | 0.00           | 17            | 2              | 0.31           | 16            | 4              | 0.64           |

| Mean    | 6.80  | 0.56           | 3.20            | 0.47              | 2.80           | 0.38           | 4.90           | 0.67           | 5.10           | 0.71           |
| Private alleles | 5 | 1 | 4 | 9 |

Note: A = number of alleles; Hₑ = Nei’s unbiased gene diversity; n = total number of samples analyzed.

a,b For analyses, markers with a high percentage of nonamplifications from the total number of samples tested were removed: NLae01 (9%), NLae06 (8%).

### Table 3. Results of microsatellite screening in four populations of *Nephroma parile*. a,b

| Locus   | Total | Dundonnell (n = 16) | Glen Nant (n = 16) | Dreggie (n = 14) | Dulsie (n = 15) |
|---------|-------|-------------------|-------------------|-----------------|-----------------|
| NPar01  | 59    | 3                 | 0.28              | 16               | 1              | 0.00           | 14            | 3              | 0.62           | 14            | 3              | 0.70           | 15            | 2              | 0.34           |
| NPar02  | 61    | 3                 | 0.28              | 16               | 1              | 0.00           | 16            | 2              | 0.33           | 14            | 2              | 0.44           | 15            | 2              | 0.34           |
| NPar03  | 61    | 5                 | 0.32              | 16               | 1              | 0.00           | 16            | 2              | 0.33           | 14            | 3              | 0.62           | 15            | 2              | 0.34           |
| NPar05  | 61    | 2                 | 0.23              | 16               | 1              | 0.00           | 16            | 2              | 0.13           | 14            | 2              | 0.44           | 15            | 2              | 0.34           |
| NPar06  | 61    | 6                 | 0.44              | 16               | 1              | 0.00           | 16            | 5              | 0.73           | 14            | 5              | 0.80           | 15            | 2              | 0.25           |
| NPar07  | 61    | 5                 | 0.43              | 16               | 1              | 0.00           | 16            | 4              | 0.68           | 14            | 3              | 0.70           | 15            | 2              | 0.34           |
| NPar08  | 61    | 6                 | 0.43              | 16               | 2              | 0.13           | 16            | 3              | 0.43           | 14            | 5              | 0.84           | 15            | 2              | 0.34           |
| NPar09  | 61    | 2                 | 0.06              | 16               | 1              | 0.00           | 16            | 2              | 0.23           | 14            | 1              | 0.00           | 15            | 1              | 0.00           |
| NPar10  | 61    | 2                 | 0.06              | 16               | 1              | 0.00           | 16            | 2              | 0.23           | 14            | 1              | 0.00           | 15            | 1              | 0.00           |
| NPar11  | 61    | 2                 | 0.09              | 16               | 1              | 0.00           | 16            | 1              | 0.00           | 14            | 1              | 0.00           | 15            | 2              | 0.34           |
| NPar12  | 61    | 3                 | 0.40              | 16               | 1              | 0.21           | 16            | 3              | 0.63           | 14            | 3              | 0.62           | 15            | 2              | 0.34           |
| NPar13  | 60    | 6                 | 0.43              | 16               | 1              | 0.00           | 16            | 3              | 0.58           | 13            | 4              | 0.79           | 15            | 2              | 0.34           |

| Mean    | 3.75  | 0.30           | 1.08            | 0.01            | 2.67           | 0.41           | 3.75           | 0.50           | 1.83           | 0.28           |
| Private alleles | 1 | 6 | 6 | 4 |

Note: A = number of alleles; Hₑ = Nei’s unbiased gene diversity; n = total number of samples analyzed.

a,b For analyses, markers with a high percentage of nonamplifications from the total number of samples tested were removed: NPar04 (17%), NPar14 (34%).
sampling scale, with no shared genotypes among populations for the sexual species, in contrast to the predominantly asexual *N. parile*. We conclude that the markers can potentially provide insights contributing to an improved understanding of population genetic processes, and they are currently being used to analyze population genetic structure in *N. laevigatum* and *N. parile* for variable habitats across a steep climatic gradient.

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**APPENDIX 1.** Location information, number of individuals sampled, and voucher specimens of *Nephroma* species used in this study. All specimens were sampled as epiphytes from broadleaved woodlands.

| Species             | Voucher | Locality* | Latitude   | Longitude    | n  | Collection date |
|---------------------|---------|-----------|------------|--------------|----|----------------|
| *Nephroma laevigatum* | RB1     | Ardladlech SSSI, 31 m a.s.l. | 56°33′09.8100″N | −005°05′08.1874″W | 2d | 15 Oct. 2012   |
| *Nephroma laevigatum* | RB2     | Glen Nant SSSI, 82 m a.s.l. | 56°23′46.8924″N | −005°12′38.0419″W | 2d, 17f | 20 Oct. 2012   |
| *Nephroma laevigatum* | RB3     | Birks of Aberfeldy SSSI, 241 m a.s.l. | 56°36′25.4736″N | −003°52′18.4706″W | 2d | 10 Jan. 2013   |
| *Nephroma laevigatum* | RB4     | Glen Tilt SSSI, 221 m a.s.l. | 56°48′22.2876″N | −003°50′09.0647″W | 2d | 15 Jan. 2013   |
| *Nephroma laevigatum* | RB5     | Ardura SSSI, 48 m a.s.l. | 56°23′56.9184″N | −005°45′26.3063″W | 4e | 25 Oct. 2012   |
| *Nephroma laevigatum* | RB6     | Kyles Wood, 45 m a.s.l. | 56°48′08.9964″N | −005°48′51.8989″W | 4e | 1 Nov. 2012    |
| *Nephroma laevigatum* | RB7     | Dulsie Bridge, 182 m a.s.l. | 57°26′44.6460″N | −003°47′10.0759″W | 4e, 19f | 18 Jan. 2013   |
| *Nephroma laevigatum* | RB8     | Dreggie Aspen Wood, 262 m a.s.l. | 57°20′01.7592″N | −003°37′28.3181″W | 3e | 23 Jan. 2013   |
| *Nephroma laevigatum* | RB9     | Torboll Woods SSSI, 72 m a.s.l. | 57°57′18.0036″N | −004°07′02.7178″W | 14f | 15 Apr. 2013   |
| *Nephroma laevigatum* | RB10    | Ardladlech SSSI, 31 m a.s.l. | 56°41′38.2884″N | −005°40′09.1535″W | 16f | 5 Oct. 2012    |
| *Nephroma parile*    | RB11    | Ardladlech SSSI, 31 m a.s.l. | 56°33′09.8100″N | −005°05′08.1874″W | 2d | 15 Oct. 2012   |
| *Nephroma parile*    | RB12    | Glen Nant SSSI, 82 m a.s.l. | 56°23′46.8924″N | −005°12′38.0419″W | 2d, 16f | 20 Oct. 2012   |
| *Nephroma parile*    | RB13    | Kindrogan Field Centre, 280 m a.s.l. | 56°44′50.1936″N | −003°32′41.6249″W | 2d | 15 Nov. 2013   |
| *Nephroma parile*    | RB14    | Glen Tilt SSSI, 221 m a.s.l. | 56°48′22.2876″N | −003°50′09.0647″W | 2d | 15 Jan. 2013   |
| *Nephroma parile*    | RB15    | Ardura SSSI, 48 m a.s.l. | 56°23′56.9184″N | −005°45′26.3063″W | 4e | 25 Oct. 2012   |
| *Nephroma parile*    | RB16    | Kyles Wood, 45 m a.s.l. | 56°48′08.9964″N | −005°48′51.8989″W | 3e | 1 Nov. 2012    |
| *Nephroma parile*    | RB17    | Dulsie Bridge, 182 m a.s.l. | 57°26′44.6460″N | −003°47′10.0759″W | 4e, 15f | 18 Jan. 2013   |
| *Nephroma parile*    | RB18    | Dreggie Aspen Wood, 262 m a.s.l. | 57°20′01.7592″N | −003°37′28.3181″W | 4e, 14f | 23 Jan. 2013   |
| *Nephroma parile*    | RB19    | Dundonnell Woods SSSI, 26 m a.s.l. | 57°50′18.0378″N | −005°11′16.8181″W | 16f | 1 May 2013     |

Note: n = number of samples analyzed; SSSI = Site of Special Scientific Interest.

1All specimens were collected by R.B.

2Vouchers deposited at the Herbarium of the Royal Botanic Garden Edinburgh (E).

3Locality in Scotland.

4Specimens used for shotgun sequencing.

5Specimens used for polymorphism testing.

6Specimens used for microsatellite screening.

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4 of 4

Belinchón et al.—Microsatellites in *Nephroma*