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

Authors: Belinchón, Rocío, Ellis, Christopher J., and Yahr, Rebecca

Source: Applications in Plant Sciences, 2(11)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1400080

Applications in Plant Sciences

Applications in Plant Sciences 2014 2(11): 1400080

Primer Note

Microsatellite loci in two epiphytic lichens with contrasting dispersal modes: *Nephroma laevigatum* and *N. parile* (Nephromataceae)[1]

Rocío Belinchón[2,3], Christopher J. Ellis[2], and Rebecca Yahr[2]

[2]Royal Botanic Garden Edinburgh, 20A Inverleith Row, Scotland, United Kingdom

- *Premise of the study:* Microsatellite markers were characterized for two epiphytic cyanolichens, *Nephroma laevigatum* and *N. parile* (Nephromataceae), and will be used to investigate population structure and estimate gene flow among populations of these two closely related species with contrasting dispersal modes.
- *Methods and Results:* Twelve and 14 microsatellite loci were characterized for *N. laevigatum* and *N. parile*, respectively. Allele number in *N. laevigatum* ranged from three to 13 per locus, while in *N. parile* there were from two to six alleles per locus. As expected, the sexually reproducing *N. laevigatum* had higher genetic diversity than the predominantly asexual *N. parile*.
- *Conclusions:* This new set of markers is suitable for studying population structure and providing insights into gene flow among populations and for understanding processes of diversification. Compared between the species, they will facilitate an understanding of the influence of contrasting reproductive strategies on population and community structure.

Key words: Ascomycetes; lichen-forming fungi; microsatellites; *Nephroma laevigatum*; *Nephroma parile*; Nephromataceae.

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 larger 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., Scheidegger 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 SO_2 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

[1]Manuscript received 21 August 2014; revision accepted 21 September 2014.

The authors thank Markus Ruhsam, Laura Forrest, Michelle Hollingsworth, Alfredo García-Fernández, María Prieto, and The GenePool (University of Edinburgh) for technical assistance. We also thank the Scottish Natural Heritage (SNH) and the landowners for access permission. This study was funded by a Marie Curie Intra-European Fellowship within the 7th European Community Framework Program to R.B. (PIEF-GA-2011-299330).

[3]Author for correspondence: r.belinchon@rbge.ac.uk; r.belinchon.olmeda@gmail.com

doi:10.3732/apps.1400080

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

Locus	Primer sequences (5′–3′)	Repeat motif	Multiplex[a]	Dye label	Allele size range (bp)	*A*	GenBank accession no.
NLae01	F: TCCGATACGTAATGCAAACGG	$(TCA)_{12}$	M-NL1	PET	192–231	8	KM361439
	R: TCGCTGTTGTCGCAAGAAAC						
NLae02	F: ACAACGGTAGGGCAGATCG	$(ACAT)_{8}$	M-NL2	NED	198–237	7	KM361440
	R: GTCTTGTACTCTGTAACCATGTC						
NLae03	F: CATGGATGAAACGGTCTCGG	$(AC)_{12}$	M-NL2	6-FAM	230–234	3	KM361441
	R: TCTTTGTGTATGTGTCCACTCC						
NLae04	F: AGGTCCCGACAAACAAAAGC	$(CAGA)_{9}$	M-NL2	VIC	142–165	5	KM361442
	R: TCCCATAGGGAAGTCAAGCC						
NLae05	F: ATACCTTACCGACCCACGTC	$(GA)_{11}$	M-NL1	NED	254–258	4	KM361443
	R: GAGCCGGAGTGAAATCCAAC						
NLae06	F: ATAACCAGGACGAGGAACTG	$(GGA)_{9}$	M-NL1	PET	89–129	8	KM361444
	R: TCCGTCCTACACCTGCAAAG						
NLae07	F: ACATGAAAAGCACCTGCCAC	$(TG)_{11}$	M-NL2	VIC	240–284	7	KM361445
	R: CTTTGCGCTCTCGGTAAGTC						
NLae08	F: GGGCTTAGGAAAAGTGTCCG	$(CTTG)_{15}$	M-NL1	6-FAM	207–240	3	KM361446
	R: AGTGCTGCTATATTATCACCTTTGG						
NLae09	F: AGATAACGTCGAGGTGGTGG	$(CAT)_{12}$	M-NL2	6-FAM	126–168	7	KM361447
	R: AGCAGTTTGGTCACGTTCTC						
NLae10	F: TCAGTCTTCCATTACCGGGG	$(TC)_{11}$	M-NL1	6-FAM	137–155	6	KM361448
	R: GCCTGAGTAGAAGGCTGGAC						
NLae11	F: TCGTCCAAGACACGAAAGGG	$(GGAT)_{7}$	M-NL2	PET	199–229	4	KM361449
	R: GACCTGCAATATGCGACCTG						
NLae12	F: AGCTGGTCATGTTATCCCC	$(CTT)_{9}$	M-NL1	VIC	205–212	3	KM361450
	R: GGAATCAGTGCAGTTGCTGG						
NPar01	F: CCGAGAGTACCCACTGAAGG	$(CA)_{11}$	M-NP2	VIC	207–221	3	KM361451
	R: TGAGATTGTGGAGGTGGCTC						
NPar02	F: CTAGACTCCGAGGAGACGC	$(TGG)_{8}$	M-NP1	PET	234–241	3	KM361452
	R: GCATGGAGCTGAACCACATC						
NPar03	F: GGCGCAACGAGGAGAATATG	$(AC)_{13}$	M-NP2	6-FAM	230–242	4	KM361453
	R: GTCAACAGGTCACTGCATCG						
NPar04	F: GGTACTTAGGGCCTTTAAATGACTG	$(TGA)_{9}$	M-NP1	NED	170–176	3	KM361454
	R: CGTCACTGAAGAAGAATCAGACG						
NPar05	F: TTCCCTAACGCAAATGCTCG	$(GA)_{13}$	M-NP1	VIC	244–246	2	KM361455
	R: CGCGGAAGTCACCATTCATC						
NPar06	F: AGCCATGCAGATTTCACACG	$(TGG)_{8}$	M-NP2	NED	194–224	4	KM361456
	R: CGATACCTGATCGCAGGAGG						
NPar07	F: TGTTTGCTTCCTGACGTGTG	$(GT)_{11}$	M-NP1	NED	233–244	3	KM361457
	R: TCGATAGGGCTCCTGTTTC						
NPar08	F: GCCTTTCAGGGGTTGGATTG	$(CAT)_{9}$	M-NP1	6-FAM	192–215	4	KM361458
	R: GGTACCATGGCTTGCATACG						
NPar09	F: ACCGGGGGTTTTTAATTGGTG	$(GT)_{11}$	M-NP2	NED	109–116	3	KM361459
	R: CAAAGCATTGCCTGCTACCC						
NPar10	F: CTTCGGTGGACTTTCGTTCG	$(CGT)_{7}$	M-NP2	PET	113–116	2	KM361460
	R: TCCCTTCATCTGCATACCCC						
NPar11	F: TTATGTGTGCCCGACTGGAG	$(GA)_{11}$	M-NP1	6-FAM	115–125	2	KM361461
	R: GTTTTCTTCCGAGTTCCCGC						
NPar12	F: AGCGATTTTGTGGGTCGTTG	$(GGT)_{8}$	M-NP2	VIC	145–151	3	KM361462
	R: GTTTCTCCGAAAGCGCTCC						
NPar13	F: TCCTACCCGACACAATTCCC	$(CA)_{13}$	M-NP1	NED	102–108	4	KM361463
	R: GTATGCCGGGCTGGATAAAC						
NPar14	F: GTAAACGCAATCCCTGGACG	$(GAG)_{7}$	M-NP2	PET	147–172	3	KM361464
	R: TACGTCAAACGCACAGTCAG						

Note: *A* = number of alleles.

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

biotin-labeled CT and GT repeat oligonucleotides. The genomic library was enriched for simple sequence repeats (SSRs) prior to 454 sequencing. The SSR content is species dependent, but usually less than 2% of the sequence read pool contains a microsatellite insert without enrichment (Ecogenics GmbH). The SSR-enriched library was analyzed on a Roche 454 platform using GS FLX Titanium reagents (454 Life Sciences, a Roche Company, Branford, Connecticut, USA). Based on fragment length, repeat motif, and no poly N present in each amplicon, the most promising markers were chosen for screening in *N. laevigatum* and *N. parile* using a Primer3 core code (available from http://primer3.sourceforge.net/releases.php; Rozen and Skaletsky, 2000). The remaining sequences were not suitable for primer design because flanking regions were absent or too short. In the end, the most promising markers were proved (1) to amplify, and (2) to be repeatable and polymorphic (i.e., bearing at least two alleles per population); the remaining were monomorphic, nonamplifying in some individuals, or too unspecific. For *N. laevigatum*, a total of 19,431 reads had an average length of 404 base pairs. Of these, 630 contained a microsatellite insert with a tetra- or a trinucleotide of at least six repeat units or a dinucleotide of at least 10 repeat units. Suitable primer design was possible in 265 reads (i.e., read sequence is not starting or ending with the repeat motif). In the case of *N. parile*, a total of 21,136 reads had an average length of 396 base pairs. Of these, 1386 contained a microsatellite insert with a tetra- or a trinucleotide of at least six repeat units or a dinucleotide of at least 10 repeat units. Suitable primer design was possible in 488 reads. Primers

TABLE 2. Results of microsatellite screening in four populations of *Nephroma laevigatum*.[a,b]

	Total			Dulsie (n = 19)			Torboll (n = 14)			Glen Nant (n = 17)			Ardery (n = 16)		
Locus	n	A	H_e	n	A	H_e	n	A	H_e	n	A	H_e	n	A	H_e
NLae02	66	10	0.75	19	4	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; H_e = Nei's unbiased gene diversity; n = total number of samples analyzed.

[a] See Appendix 1 for populations used in this study.

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

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 Ecogenics 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 that were polymorphic across samples are described in Table 1.

Of the set of 26 polymorphic primers obtained from the polymorphism test, a larger screen of marker variation was completed using four populations for each species (Tables 2, 3), totaling more than 60 samples per species. All PCR products obtained were multiplexed (Table 1). PCR reactions were performed in a total volume of 10 μL containing 1 μL of genomic DNA, 2 μL of water, 5 μL of HotStarTaq (QIAGEN), and 2 μL of primer mix (1 μM each of forward and reverse primer concentration). Cycling conditions were: 15 min at 95°C, 35× (30 s at 94°C, 90 s at 56°C, 60 s at 72°C), and a final extension of 30 min at 72°C. For the fragment analyses, 1 μL of 1:20 diluted multiplex products was used. PCR products were sized on an ABI3730 Genetic Analyzer (Applied Biosystems), and genotypes were assigned with a GeneScan 500 LIZ Size Standard (Life Technologies, Carlsbad, California, USA). Of the initial 26 microsatellite primer pairs, four were not included in the results of this screen due to their low amplification success on the evaluated samples (>8% nonamplifications; NLae01, NLae06, NPar04, NPar14). The number of alleles and unbiased genetic diversity were generated using GenAlEx 6.501 (Peakall and Smouse, 2006; Tables 2, 3). For the sample of 66 specimens for *N. laevigatum*, all 10 loci evaluated were polymorphic with between three and 13 alleles per locus, a total of 68 alleles across loci, and altogether comprising 46 multilocus genotypes detected over all populations. From the sample of 61 specimens of *N. parile*, all 12 loci evaluated were polymorphic with two to six alleles per locus, a total number of 48 alleles across loci, and 16 multilocus genotypes detected over all populations. Average gene diversities 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 3. Results of microsatellite screening in four populations of *Nephroma parile*.[a,b]

	Total			Dundonell (n = 16)			Glen Nant (n = 16)			Dreggie (n = 14)			Dulsie (n = 15)		
Locus	n	A	H_e	n	A	H_e	n	A	H_e	n	A	H_e	n	A	H_e
NPar01	59	3	0.42	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		2.75	0.50		1.83	0.28
Private alleles					1			6			6			4	

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

[a] See Appendix 1 for populations used in this study.

[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.

LITERATURE CITED

Hartl, D. L., and A. G. Clark. 1997. Principles of population genetics. Sinauer, Sunderland, Massachusetts, USA.

Lohtander, K., I. Oksanen, and J. Rikkinen. 2002. A phylogenetic study of *Nephroma* (lichen-forming *Ascomycota*). *Mycological Research* 106: 777–787.

Lowe, A., S. Harris, and P. Ashton. 2004. Gene flow and mating system. *In* A. Lowe, S. Harris, and P. Ashton [eds.], Ecological genetics: Design, analysis, and application, 106–149. Blackwell Publishing, Oxford, United Kingdom.

Peakall, R., and P. E. Smouse. 2006. GenAlEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.

Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. *In* S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

Scheidegger, C., and S. Werth. 2009. Conservation strategies for lichens: Insights from population biology. *Fungal Biology Reviews* 23: 55–66.

Schuelke, M. 2000. An economic method for the fluorescent labelling of PCR fragments. *Nature Biotechnology* 18: 233–234.

Seaward, M. R. D. 2008. Environmental role of lichens. *In* T. H. Nash III [ed.], Lichen biology, 274–298. Cambridge University Press, Cambridge, United Kingdom.

Sérusiaux, E., J. C. Villareal, T. Wheeler, and B. Goffinet. 2011. Recent origin, active speciation and dispersal for the lichen genus *Nephroma* (Peltigerales) in Macronesia. *Journal of Biogeography* 38: 1138–1151.

Smith, C. W., A. Aptroot, B. J. Coppins, A. Fletcher, O. L. Gilbert, P. W. James, and P. A. Wolseley. 2009. The lichens of Great Britain and Ireland. British Lichen Society, United Kingdom.

Werth, S. 2010. Population genetics of lichen-forming fungi: A review. *Lichenologist (London, England)* 42: 499–519.

Werth, S., S. Cheenacharoen, and C. Scheidegger. 2014. Propagule size is not a good predictor for regional population subdivision or fine-scale spatial structure in lichenized fungi. *Fungal Biology* 118: 126–138.

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.[a]

Species	Voucher[b]	Locality[c]	Latitude	Longitude	*n*	Collection date
Nephroma laevigatum	RB1	Ard Trilleachan SSSI, 31 m a.s.l.	56°33′09.8100″N	–005°05′08.1874″W	2[d]	15 Oct. 2012
Nephroma laevigatum	RB2	Glen Nant SSSI, 82 m a.s.l.	56°23′46.8924″N	–005°12′38.0419″W	2[d], 17[f]	20 Oct. 2012
Nephroma laevigatum	RB3	Birks of Abberfeldy SSSI, 241 m a.s.l.	56°36′25.4736″N	–003°52′18.4706″W	2[d]	10 Jan. 2013
Nephroma laevigatum	RB4	Glen Tilt SSSI, 221 m a.s.l.	56°48′22.2876″N	–003°50′09.0647″W	2[d]	15 Jan. 2013
Nephroma laevigatum	RB5	Ardura SSSI, 48 m a.s.l.	56°23′56.9184″N	–005°45′26.3063″W	4[e]	25 Oct. 2012
Nephroma laevigatum	RB6	Kyles Wood, 45 m a.s.l.	56°48′08.9964″N	–005°48′51.8998″W	4[e]	1 Nov. 2012
Nephroma laevigatum	RB7	Dulsie Bridge, 182 m a.s.l.	57°26′44.6460″N	–003°47′10.0759″W	4[e], 19[f]	18 Jan. 2013
Nephroma laevigatum	RB8	Dreggie Aspen Wood, 262 m a.s.l.	57°20′01.7592″N	–003°37′28.3181″W	3[e]	23 Jan. 2013
Nephroma laevigatum	RB9	Torboll Woods SSSI, 72 m a.s.l.	57°57′18.0036″N	–004°07′07.2178″W	14[f]	15 Apr. 2013
Nephroma laevigatum	RB10	Ardery, 36 m a.s.l.	56°41′38.2884″N	–005°40′09.1535″W	16[f]	5 Oct. 2012
Nephroma parile	RB11	Ard Trilleachan SSSI, 31 m a.s.l.	56°33′09.8100″N	–005°05′08.1874″W	2[d]	15 Oct. 2012
Nephroma parile	RB12	Glen Nant SSSI, 82 m a.s.l.	56°23′46.8924″N	–005°12′38.0419″W	2[d],16[f]	20 Oct. 2012
Nephroma parile	RB13	Kindrogan Field Centre, 280 m a.s.l.	56°44′50.1936″N	–003°32′41.6249″W	2[d]	15 Nov. 2013
Nephroma parile	RB14	Glen Tilt SSSI, 221 m a.s.l.	56°48′22.2876″N	–003°50′09.0647″W	2[d]	15 Jan. 2013
Nephroma parile	RB15	Ardura SSSI, 48 m a.s.l.	56°23′56.9184″N	–005°45′26.3063″W	4[e]	25 Oct. 2012
Nephroma parile	RB16	Kyles Wood, 45 m a.s.l.	56°48′08.9964″N	–005°48′51.8998″W	3[e]	1 Nov. 2012
Nephroma parile	RB17	Dulsie Bridge, 182 m a.s.l.	57°26′44.6460″N	–003°47′10.0759″W	4[e], 15[f]	18 Jan. 2013
Nephroma parile	RB18	Dreggie Aspen Wood, 262 m a.s.l.	57°20′01.7592″N	–003°37′28.3181″W	4[e], 14[f]	23 Jan. 2013
Nephroma parile	RB19	Dundonnell Woods SSSI, 26 m a.s.l.	57°50′18.0378″N	–005°11′16.818″W	16[f]	1 May 2013

Note: *n* = number of samples analyzed; SSSI = Site of Special Scientific Interest.
[a]All specimens were collected by R.B.
[b]Vouchers deposited at the Herbarium of the Royal Botanic Garden Edinburgh (E).
[c]Locality in Scotland.
[d]Specimens used for shotgun sequencing.
[e]Specimens used for polymorphism testing.
[f]Specimens used for microsatellite screening.