

Nephroma tangeriense new to Oceania, from Hawaii

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ABSTRACT. – *Nephroma tangeriense* was previously known from Macaronesia, the Mediterranean Basin, and western Europe north to Norway, and is here reported from the island of Kauai, Hawaii. The ITS sequence obtained from the specimen differs from the main European/Macaronesian genotype in only a single nucleotide substitution. A haplotype network of the *N. laevigatum* group is presented.

KEYWORDS. – Biogeography, haplotype network, lichenized fungi, *Nephroma washingtoniense*, Nephromataceae, Peltigerales.

INTRODUCTION

During a study of the *Nephroma helveticum* Ach./*N. tropicum* (Müll. Arg.) Zahlbr. species complex in Europe (Timdal et al. 2021), we examined additional material from other regions stored under those names in the herbarium of the University of Oslo (O) using thin-layer chromatography (TLC). One specimen from Hawaii contained a series of anthraquinones, similar to those occurring in *N. laevigatum* Ach. and *N. tangeriense* (Maheu & A. Gillet) Zahlbr. The specimen resembled the latter species morphologically, and an ITS sequence obtained later confirmed that identification. *Nephroma tangeriense* was previously known from Macaronesia (the Canary Islands, Madeira, and the Azores), the Mediterranean Basin, and western Europe north to Norway (Klepsland 2013, Sérusiaux et al. 2011). Here it is reported as new to Oceania.

MATERIALS AND METHODS

The specimen in O is a duplicate of the collection distributed as *Nephroma tropicum* by William A. Weber as *Lichenes Exsiccati COLO* No. 649. It was examined morphologically under a dissecting microscope at 6–50× magnification. Thin-layer chromatography (TLC) was performed in accordance with the methods of Culberson (1972) and Menlove (1974), using solvent C and aluminum plates. The abbreviations of the names of the terpenes follow James and White (1987; i.e., T3 = hopane-6 α ,22-diol [= zeorin], T4 = hopane-7 β ,22-diol, T5 = hopane-15 α , 22-diol, and T6 = hopane-6 α ,7 β ,22-triol).

ITS sequences were generated for Weber's specimen, one specimen of *Nephroma tangeriense* from Norway, and three specimens of *N. laevigatum* from Norway. Sequencing was performed at the Canadian Centre for DNA Barcoding (CCDB; <http://www.ccdb.ca>), using the primer pair ITS1-F/ITS4 (Gardes & Bruns 1993, White et al. 1990). All ITS sequences in GenBank (74) of the *N. laevigatum* group as delimited by Sérusiaux et al. (2011; i.e., *N. foliolatum* P. James & F.J. White, *N. laevigatum*, *N. tangeriense*, and *N. venosum* Degel.) and *N. washingtoniense* Gyeln., which is currently regarded as a synonym of *N. laevigatum* (Esslinger 2021), were downloaded on 2 June 2022. With the addition of the five sequences newly produced here, the data set consists of 79 sequences (Appendix I: Table 1).

Sequences were aligned in BioEdit (Hall 1999) with its bundled software ClustalW (Thompson et al. 1994). The alignment was manually inspected and slightly adjusted, then trimmed at the ends. There were no ambiguously aligned regions. A phylogenetic reconstruction under the maximum likelihood criterion was inferred by SATÉ-II ver. 2.2.7 (Liu et al. 2012), using MAFFT (Katoh & Toh 2008, Katoh et

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Figure 1. *Nephroma tangeriense*. U.S.A., Hawaii (W.A. Weber & D. Randolph s.n., Lich. Exs. COLO, No. 649, O L-131522). Scale bar = 2 mm.

al. 2005) as the aligner, MUSCLE (Edgar 2004) as the merger, FastTree (Price et al. 2010) as the tree evaluator, and with the default settings in the GUI except for that the number of iterations after the last improvement in the maximum likelihood score was set to 10. A haplotype network was constructed by PopART ver. 1.7 (Leigh & Bryant 2015) using the SATé alignment, the Median-joining algorithm (Bandelt et al. 1999), and geographical region as the displayed trait.

RESULTS

The Hawaiian specimen resembled European and Macaronesian specimens in its small, dark brown, fragile thallus with a yellow, K+ red medulla and numerous phyllidia along the margin and some on the lamina (Figure 1). One small apothecium was present. The thallus contained terpenes T6 (major), T3 (trace), and a series of anthraquinones similar to those of other analyzed specimens of *Nephroma tangeriense* from the Canary Islands and Norway.

The trimmed alignment was 507 bp long and contained 49 variable sites, including 28 parsimony informative sites. The aligned sequence from the Hawaiian specimens was 502 bp long (i.e., missing 5 bp at the end). Among the 20 sequences of *Nephroma tangeriense*, 14 were identical. The Hawaiian sequence (#58 in Table 1) differed from those in a single transversion (A→C). A Norwegian sequence (#57 in Table 1) shared that transversion, and contained an additional, neighboring transversion (also A→C). Three sequences (#52, #54, #55 in Table 1; all from Norway) contained five different, but among them identical, substitutions compared to the 14 identical sequences. The final sequence of *N. tangeriense* (#61 in Table 1; Tenerife) differed from the 14 identical sequences in containing a single nucleotide insertion.

The phylogenetic reconstruction (Appendix II: Supplementary Figure S1) was largely congruent with the previously published reconstructions and placed the Hawaiian specimen within a well-supported (Shimodaira-Hasegawa test support value: 0.94) clade with all but the three mentioned Norwegian specimens (#52, #54, #55 in Table 1) of *Nephroma tangeriense*. In the haplotype network (Figure 2), the Hawaiian specimen was placed between the 14 identical sequences of *N. tangeriense* and sequence #57.

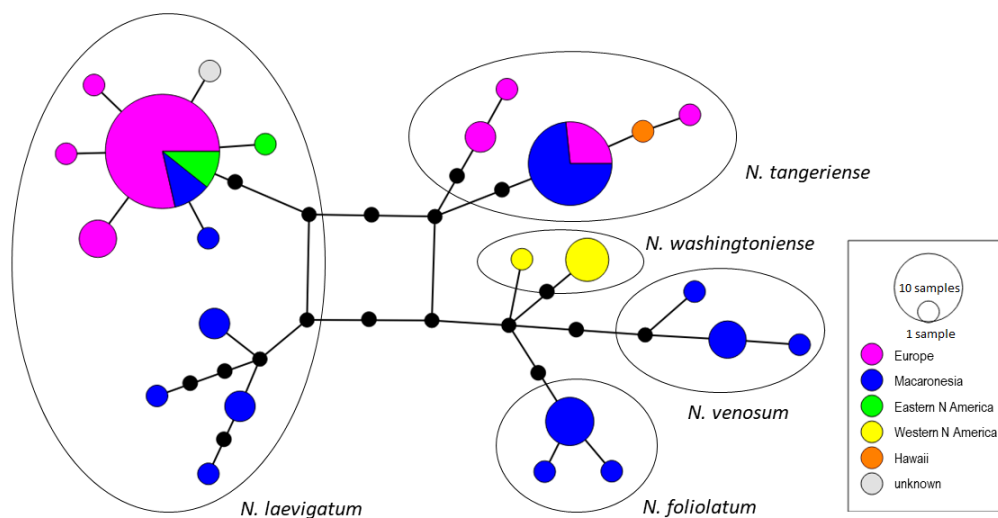


Figure 2. Median-joining haplotype network of the *Nephroma laevigatum* group based on the ITS marker. Genotypes are represented by circles whose sizes are proportional to the number of individuals. Different colors represent geographical region. Missing genotypes are indicated by small black circles.

DISCUSSION

Phylogenetic reconstructions of the *Nephroma laevigatum* complex have been published by Sérusiaux et al. (2011) and Fedrowitz et al. (2012). The current reconstruction is largely congruent with those and is here shown in Supplementary Figure S1 (Appendix II). Since it places the Hawaiian specimen within the major *N. tangeriense* clade, and the difference between that specimen and the majority of the *N. tangeriense* sequences is only a single nucleotide transversion, there seems to be no doubt about the taxonomic identity of the specimen. The sequences #52, #54, and #55 (all from Norway) are more distant in the haplotype network, differing in 5–7 substitutions, but the three specimens fit the current concept of *N. tangeriense* in morphology and chemistry.

The chemistry of the Hawaiian specimen is congruent with European and Macaronesian material of the species. The only *Nephroma* species known to contain anthraquinones are *N. laevigatum*, *N. tangeriense*, and *N. venosum* (James & White 1987). Two species of *Nephroma* are listed in the current checklist from Hawaii: *N. helveticum* (including *N. tropicum*) and *N. rufum* (C. Bab.) P. James (Elix & McCarthy 1998, 2008). They both belong in the *N. helveticum* complex and differ from *N. tangeriense* in, for example, having a white, K– medulla (White & James 1988). *Nephroma helveticum* contains terpene T4 as the major compound (James & White 1987, Timdal et al. 2021), and *N. rufum* contains T5 (White & James 1988).

Also of note, the sequences of *Nephroma laevigatum* from western North America differ markedly from the other sequences of the species. The name *N. washingtoniense* is available for what is apparently a morphologically cryptic species, as already pointed out by Sérusiaux et al. (2011) and Fedrowitz et al. (2012). Sequences from eastern North America, however, are similar to the major genotype in Europe (Figure 2, Appendix II: Supplementary Fig. S1). The molecular and geographical distinctions between the east and west coast populations of *N. laevigatum* in North America are strong, and it seems justified to treat the two populations as distinct species.

Specimen examined. – **U.S.A. HAWAII.** Island of Kauai, Kalua Puihi Trail, Kokee State Park, 3500 ft., on bark of saplings in deep shade, 24.iii.1985, W.A. Weber & D. Randolph s.n. = *Lich. Exs. COLO No. 649* (O L-131522).

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LITERATURE CITED

- Bandelt, H., P. Forster and A. Röhl. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16: 37–48.
- Culberson, C.F. 1972. Improved conditions and new data for the identification of lichen products by a standardized thin-layer chromatographic method. *Journal of Chromatography* 72: 113–125.
- Edgar, R.C. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5: 113.
- Elix, J.A. and P.M. McCarthy. 1998. Catalogue of the lichens of the smaller Pacific Islands. *Bibliotheca Lichenologica* 70: 1–361.
- Elix, J.A. and P.M. McCarthy. 2008. Checklist of Pacific Island lichens. Australian Biological Resources Study, Canberra. Version 21 August 2008. Published at https://www.anbg.gov.au/abrs/lichenlist/PACIFIC_introduction.html [accessed 2022-06-14].
- Esslinger, T.L. 2021. A cumulative checklist for the lichen-forming, lichenicolous and allied fungi of the continental United States and Canada, Version 24. *Opuscula Philolichenum* 20: 100–394.
- Fedrowitz, K., U. Kaasalainen and J. Rikkinen. 2012. Geographic mosaic of symbiont selectivity in a genus of epiphytic cyanolichens. *Ecology and Evolution* 2: 2291–2303.
- Gardes, M. and T.D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2: 113–118.
- Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.
- James, P.W. and F.J. White. 1987. Studies on the genus *Nephroma* I. The European and Macaronesian species. *Lichenologist* 19: 215–268.
- Katoh, K., K.-I. Kuma, H. Toh and T. Miyata. 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research* 33: 511–518.
- Katoh, K. and H. Toh. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics* 9: 286–298.
- Klepšland, J.T. 2013. *Nephroma helveticum* and *N. tangeriense* new to Norway. *Graphis Scripta* 25: 33–38.
- Leigh, J.W. and D. Bryant. 2015. PopART: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution* 6: 1110–1116.
- Liu, K., T.J. Warnow, M.T. Holder, S.M. Nelesen, J. Yu, A.P. Stamatakis and C.R. Linder. 2012. SATé-II: very fast and accurate simultaneous estimation of multiple sequence alignments and phylogenetic trees. *Systematic Biology* 61: 90–106.
- Lohtander, K., I. Oksanen and J. Rikkinen. 2002. A phylogenetic study of *Nephroma* (lichen-forming Ascomycota). *Mycological Research* 106: 777–787.
- Marthinsen, G., S. Rui and E. Timdal. 2019. OLICH: A reference library of DNA barcodes for Nordic lichens. *Biodiversity Data Journal* 7: e36252.
- Menlove, J.E. 1974. Thin-Layer Chromatography for the Identification of Lichen Substances. *British Lichen Society Bulletin* 34: 3–5.
- Price, M.N., P.S. Dehal and A.P. Arkin. 2010. FastTree 2 – approximately maximum-likelihood trees for large alignments. *PloS One* 5: e9490.
- Sérusiaux, E., A.J.C. Villarreal, T. Wheeler and B. Goffinet. 2011. Recent origin, active speciation and dispersal for the lichen genus *Nephroma* (Peltigerales) in Macaronesia. *Journal of Biogeography* 38: 1138–1151.
- Thompson, J.D., D.G. Higgins and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673–4680.
- Timdal, E., T.H. Hofton, M. Westberg and M. Bendiksby. 2021. The *Nephroma helveticum* complex (Peltigerales, lichenized Ascomycota) in the Nordic countries. *Graphis Scripta* 33: 86–110.
- White, F.J. and P.W. James. 1988. Studies on the genus *Nephroma* II. The southern temperate species. *Lichenologist* 20: 103–166.
- White, T.J., T. Bruns, S. Lee and J.W. Taylor. 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., D.H. Gelfand, J.J. Sninsky and T.J. White (eds.): *PCR Protocols: A Guide to Methods and Applications*. Academic Press Inc., New York. Pp 315–322.

APPENDIX I – METADATA FOR SEQUENCE DATA USED IN THIS STUDY

Table 1 (part 1 of 3). Specimens used for constructing the haplotype network, with GenBank ID, current species identification, voucher information, geographical origin, and original publication. GenBank IDs in bold mark sequences produced for this study.

#	GenBank ID	Species	Voucher	Geography	Published
1	HQ455060	<i>N. foliolatum</i>	LG 199	Madeira	Sérusiaux et al. (2011)
2	HQ455061	<i>N. foliolatum</i>	LG 27	Madeira	Sérusiaux et al. (2011)
3	HQ455062	<i>N. foliolatum</i>	LG 36	Madeira	Sérusiaux et al. (2011)
4	HQ455063	<i>N. foliolatum</i>	LG 38	Madeira	Sérusiaux et al. (2011)
5	HQ455064	<i>N. foliolatum</i>	LG 39	Madeira	Sérusiaux et al. (2011)
6	HQ455065	<i>N. foliolatum</i>	LG 201	Madeira	Sérusiaux et al. (2011)
7	HQ455066	<i>N. foliolatum</i>	LG 296	Madeira	Sérusiaux et al. (2011)
8	HQ455093	<i>N. laevigatum</i>	CONN 9200	USA, Maine	Sérusiaux et al. (2011)
9	HQ455094	<i>N. laevigatum</i>	42940 (NY)	Canada, Nova Scotia	Sérusiaux et al. (2011)
10	HQ455095	<i>N. laevigatum</i>	10270 (NY)	Canada, Newfoundland	Sérusiaux et al. (2011)
11	HQ455096	<i>N. laevigatum</i>	52538 (NY)	Canada, Newfoundland	Sérusiaux et al. (2011)
12	AY124141	<i>N. laevigatum</i>	Mayrhofer & Prugger 11583	Corsica	Lohtander et al. (2002)
13	AY124142	<i>N. laevigatum</i>	Skytén 6243	Norway	Lohtander et al. (2002)
14	AY124143	<i>N. laevigatum</i>	Kuusinen & Lommi 3745	Finland	Lohtander et al. (2002)
15	HQ455078	<i>N. laevigatum</i>	LG 190	UK	Sérusiaux et al. (2011)
16	HQ455079	<i>N. laevigatum</i>	LG 588	Corsica	Sérusiaux et al. (2011)
17	HQ455080	<i>N. laevigatum</i>	38581 LG 406	Spain	Sérusiaux et al. (2011)
18	HQ455086	<i>N. laevigatum</i>	35567 LG 408	Portugal	Sérusiaux et al. (2011)
19	HQ455087	<i>N. laevigatum</i>	35024 LG 407	Portugal	Sérusiaux et al. (2011)
20	HQ455088	<i>N. laevigatum</i>	34907 LG 409	Portugal	Sérusiaux et al. (2011)
21	HQ455089	<i>N. laevigatum</i>	34936 LG 410	Portugal	Sérusiaux et al. (2011)
22	HQ455090	<i>N. laevigatum</i>	35440 LG 411	Portugal	Sérusiaux et al. (2011)
23	HQ455091	<i>N. laevigatum</i>	35487 LG 412	Portugal	Sérusiaux et al. (2011)
24	HQ455092	<i>N. laevigatum</i>	35315 LG 413	Portugal	Sérusiaux et al. (2011)
25	JN857249	<i>N. laevigatum</i>	KUS30	UK	Fedrowitz et al. (2012)
26	JN857250	<i>N. laevigatum</i>	KUS37	UK	Fedrowitz et al. (2012)
27	JN857251	<i>N. laevigatum</i>	KUS39	UK	Fedrowitz et al. (2012)
28	JN857252	<i>N. laevigatum</i>	U370	Norway	Fedrowitz et al. (2012)
29	MK811868	<i>N. laevigatum</i>	L-48759 (TROM)	Norway	Marthinsen et al. (2019)
30	MK811969	<i>N. laevigatum</i>	L-196055 (O)	Norway	Marthinsen et al. (2019)
31	MK812002	<i>N. laevigatum</i>	L-204997 (O)	Norway	Marthinsen et al. (2019)

Table 1 (continued: part 2 of 3). Specimens used for constructing the haplotype network, with GenBank ID, current species identification, voucher information, geographical origin, and original publication. GenBank IDs in bold mark sequences produced for this study.

#	GenBank ID	Species	Voucher	Geography	Published
32	MK812108	<i>N. laevigatum</i>	L-175024 (O)	Norway	Marthinsen et al. (2019)
33	MK812261	<i>N. laevigatum</i>	L-201288 (O)	Norway	Marthinsen et al. (2019)
34	MK812687	<i>N. laevigatum</i>	L-168088 (O)	Norway	Marthinsen et al. (2019)
35	MZ159568	<i>N. laevigatum</i>	K M 201543	UK	Gaya et al. ined.
36	OP586781	<i>N. laevigatum</i>	L-37085 (TRH)	Norway	here
37	OP586779	<i>N. laevigatum</i>	L-225618 (O)	Norway	here
38	OP586780	<i>N. laevigatum</i>	L-167039 (O)	Norway	here
39	AY124139	<i>N. laevigatum</i>	Skult 27 March 1995	Madeira	Lohtander et al. (2002)
40	AY124140	<i>N. laevigatum</i>	Feurerer & Marth 19 July 1997	Canary Islands	Lohtander et al. (2002)
41	HQ455077	<i>N. laevigatum</i>	BR 10816 LG 244	Canary Islands	Sérusiaux et al. (2011)
42	HQ455081	<i>N. laevigatum</i>	LG 28	Canary Islands	Sérusiaux et al. (2011)
43	HQ455082	<i>N. laevigatum</i>	LG 262	Azores	Sérusiaux et al. (2011)
44	HQ455083	<i>N. laevigatum</i>	LG 271	Azores	Sérusiaux et al. (2011)
45	HQ455084	<i>N. laevigatum</i>	LG 292	Azores	Sérusiaux et al. (2011)
46	HQ455085	<i>N. laevigatum</i>	LG 293	Azores	Sérusiaux et al. (2011)
47	HQ455097	<i>N. laevigatum</i>	LG 35	Madeira	Sérusiaux et al. (2011)
48	HQ455098	<i>N. laevigatum</i>	BR 10818 LG 245	Canary Islands	Sérusiaux et al. (2011)
49	MN959975	<i>N. laevigatum</i>	98GB04	unknown	Slimani & Rambold ined.
50	AY124153	<i>N. tangeriense</i>	Burgaz 22 July 1995	Portugal	Lohtander et al. (2002)
51	AY124154	<i>N. tangeriense</i>	Aragon & Castillo 3 June 1993	Spain	Lohtander et al. (2002)
52	MK811986	<i>N. tangeriense</i>	L-186150 (O)	Norway	Marthinsen et al. (2019)
53	MK812084	<i>N. tangeriense</i>	L-181602 (O)	Norway	Marthinsen et al. (2019)
54	MK812530	<i>N. tangeriense</i>	L-183599 (O)	Norway	Marthinsen et al. (2019)
55	MK812579	<i>N. tangeriense</i>	L-197824 (O)	Norway	Marthinsen et al. (2019)
56	MK812593	<i>N. tangeriense</i>	L-197826 (O)	Norway	Marthinsen et al. (2019)
57	OP586778	<i>N. tangeriense</i>	L-222478 (O)	Norway	here
58	OP586777	<i>N. tangeriense</i>	L-131522 (O)	Hawaii	here
59	AY124152	<i>N. tangeriense</i>	Ceni & Vezda 14 Mar 1994	Canary Islands	Lohtander et al. (2002)
60	HQ455106	<i>N. tangeriense</i>	BR 10793 BR 246	Canary Islands	Sérusiaux et al. (2011)
61	HQ455107	<i>N. tangeriense</i>	LG 352	Canary Islands	Sérusiaux et al. (2011)
62	JN857298	<i>N. tangeriense</i>	KU544	Canary Islands	Fedrowitz et al. (2012)
63	JN857299	<i>N. tangeriense</i>	KU540	Canary Islands	Fedrowitz et al. (2012)
64	JN857300	<i>N. tangeriense</i>	KU541	Canary Islands	Fedrowitz et al. (2012)
65	JN857301	<i>N. tangeriense</i>	KU542	Canary Islands	Fedrowitz et al. (2012)

Table 1 (continued: part 3 of 3). Specimens used for constructing the haplotype network, with GenBank ID, current species identification, voucher information, geographical origin, and original publication. GenBank IDs in bold mark sequences produced for this study.

#	GenBank ID	Species	Voucher	Geography	Published
66	JN857302	<i>N. tangeriense</i>	KU545	Canary Islands	Fedrowitz et al. (2012)
67	JN857303	<i>N. tangeriense</i>	KU546	Canary Islands	Fedrowitz et al. (2012)
68	JN857304	<i>N. tangeriense</i>	KU547	Canary Islands	Fedrowitz et al. (2012)
69	JN857305	<i>N. tangeriense</i>	KU543a	Canary Islands	Fedrowitz et al. (2012)
70	HQ455108	<i>N. venosum</i>	LG 263	Azores	Sérusiaux et al. (2011)
71	HQ455109	<i>N. venosum</i>	LG 270	Azores	Sérusiaux et al. (2011)
72	HQ455110	<i>N. venosum</i>	LG 275	Azores	Sérusiaux et al. (2011)
73	HQ455111	<i>N. venosum</i>	LG 281	Azores	Sérusiaux et al. (2011)
74	HQ455112	<i>N. venosum</i>	LG 297	Azores	Sérusiaux et al. (2011)
75	AY124144	<i>N. washingtoniense</i>	McCune 4 Feb 2001B	USA, NW	Lohtander et al. (2002)
76	HQ455076	<i>N. washingtoniense</i>	759 LG 217	USA, Oregon	Sérusiaux et al. (2011)
77	JN857306	<i>N. washingtoniense</i>	U187	USA, California	Fedrowitz et al. (2012)
78	JN857307	<i>N. washingtoniense</i>	U143	USA, Oregon	Fedrowitz et al. (2012)
79	JN857308	<i>N. washingtoniense</i>	U146	USA, Oregon	Fedrowitz et al. (2012)

APPENDIX II – SUPPLEMENTAL FIGURE S1: PHYLOGENETIC RECONSTRUCTION

Supplementary Figure S1. Phylogenetic reconstruction of the *N. laevigatum* complex under the maximum likelihood criterion, inferred by SATé-II. Braces indicates current species hypotheses. Labels include sequence numbers as in Table 1, GenBank IDs, and geographical origin; those in bold are sequences newly produced here. Numbers above branches are Shimodaira-Hasegawa test support values. Branches with support value less than 0.6 are collapsed.

