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The Phylogenetic Position of *Normandina simodensis* (Verrucariaceae, Lichenized Ascomycota)

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Abstract The phylogenetic position of *Normandina simodensis* is demonstrated by Bayesian and Maximum Likelihood analyses of concatenated mtSSU, nucSSU, nucLSU and RPB1 sequence data. *Normandina simodensis* is placed basal in a well-supported clade with *N. pulchella* and *N. acroglypta*, thus confirming *Normandina* as a monophyletic genus within Verrucariaceae. *Normandina* species agree in general ascoma morphology but differ in thallus structure and the mode of vegetative reproduction: crustose and sorediate in *N. acroglypta*; squamulose and sorediate in *N. pulchella*; and squamulose and esorediate in *N. simodensis*.

Key words: Bayesian, growth form, Japan, maximum likelihood, pyrenocarpous lichens, taxonomy.

Normandina Nyl. is a small genus comprising only three species at the world level (Aptroot, 1991; Muggia *et al.*, 2010). *Normandina pulchella* (Borrer) Nyl. is almost cosmopolitan, lacking only in Antarctica, while the other two species are of more limited distribution. *Normandina acroglypta* (Norman) Aptroot is known from Europe (Aptroot, 1991; Orange and Aptroot, 2009), while *N. simodensis* (Asahina) Aptroot occurs in Japan (Asahina, 1933) and was reported once from Papua New Guinea (Aptroot, 1991). *Normandina* species usually grow over bryophytes or a thin layer of soil on rocks and trees in (sub-)oceanic to tropical climates, but are occasionally found growing directly on bark and rock.

Normandina species agree in general ascoma morphology (Aptroot, 1991; Orange and Aptroot, 2009) but the thallus structure is heterogeneous: crustose with goniocysts in *N. acroglypta*; bluish- to greenish-grey, rounded shell- to kidney-shaped squamules with upturned margins in *N. pulchella*; and greyish brown elongated, ± convex squamules with flat to downturned margins in *N. simodensis*. While the first two species usually bear maculate soralia and are often found in sterile condition, *N. simodensis* lacks soralia and is usually fertile. The latter species differs further by its thick paraplectenchymatic upper cortex and a ± well-developed medulla derived from the photobiont layer.

Normandina was recently confirmed in Verrucariaceae by Muggia *et al.* (2010) including *N. pulchella* and *N. acroglypta* in their phylogenetic analysis of Verrucariaceae. This study ended long debates on the phylogenetic position of *N. pulchella* which had been placed in, e.g., Basidiomycetes (Henssen and Jahns, 1974), Fungi incertae sedis (Henssen, 1976) and Verrucariaceae (Aptroot, 1991; Zschacke, 1934 [as Dermatocarpaceae]) by previous authors. These conflicting hypotheses regarding the placement of *Normandina* depended on whether or not the perithecia of *N. pulchella* were interpreted as ascomata or as a lichenicolous fungus. *N. simodensis* (Asahina) Aptroot was not included in the phylogeny

of Muggia *et al.* (2010) and its placement in *Normandina* needed confirmation given the differences in thallus structure to *N. pulchella* and *N. acroglypta*. *N. simodensis* is a rare species in Japan and known only from a restricted area of central Honshu southwest of Tokyo. The species is most often collected from exposed coastal rocks, where it usually grows over a thin layer of bryophytes or soil. The report of *N. simodensis* from Lake Aunde (elev. 3927 m) in the Mt. Wilhelm area of Papua New Guinea (Aptroot, 1991), however, indicates a much wider distribution and ecology for this species.

Materials and Methods

In the course of floristic field work in Japan, the type locality of *Normandina simodensis* was visited and material for sequencing and voucher preparation was collected. Additional recent collections of *N. pulchella* from Japan were also sequenced. All collections are stored in the herbarium of the National Museum of Nature and Science (TNS) in Tsukuba. Sequences of selected species in the phylogeny of Verrucariaceae in Muggia *et al.* (2010) were obtained from GenBank and used for comparison.

DNA extraction followed a modified CTAB protocol (Hosaka, 2009). For DNA amplification, 10 μl of PCR mix contained 1 μl genomic DNA extraction, 0.25 μl of each primer (10 pmol/μl) and 5 μl EmeraldAmp PCR Master Mix (TaKaRa Bio Inc., Japan). The following primers were used for PCR amplification: mtSSU1 (Zoller *et al.*, 1999) and MSU7 (Zhou and Stanosz, 2001) for mtSSU, LIC24R (Miadlikowska and Lutzoni, 2000) and LR5 (Vilgalys and Hester, 1990) for nucLSU, nSSU131 (Kauff and Lutzoni, 2002) and NS24 (Gargas and Taylor, 1992) for nucSSU, and RPB1-AF, RPB1-DF2asc, RPB1-6R1asc (Hofstetter *et al.*, 2007) and RPB1-G2R (Stiller and Hall, 1997) for RPB1. PCR cycling conditions were 94℃ (3 min), followed by 11 cycles of 95℃ (30 sec), 62℃ to 52℃ (30 sec) with annealing temperatures lowered by 1℃ between cycles, and 72℃ (1 min), followed by 30 cycles at 52℃ annealing temperature and a final extension at 72℃ (7 min). Sequencing was done on an ABI Prism 3130x genetic analyzer (Applied Biosystems) using the BigDye Terminator ver. 3.1 Cycle Sequencing Kit according to the manufacturer's instructions.

Sequences were aligned in MAFFT as implemented in the MEGA 5 (Tamura *et al.*, 2011) and manually corrected. Confidence scores were calculated for all single-gene alignments using the GUIDANCE web-server (Penn *et al.*, 2010) and columns with confidence values <0.95 were removed from further analysis. The alignments were further checked for obvious aligning errors and all remaining phylogeny uninformative insertions removed. The final alignment (5379 nucleotide positions) contained the concatenated mtSSU (523), nucLSU (1245), nucSSU (1548) and RPB1 (2063) gene loci. A partitioned dataset was used for the phylogenetic analyses to enable independent parameter estimation for the four genes. The RPB1 data set was further partitioned according to codon positions to allow for the higher evolutionary rates of the 3rd codon position.

Bayesian analysis was performed with MrBayes 3.2.1 (Ronquist and Huelsenbeck, 2003) implemented in the CIPRES Science Gateway (Miller *et al.*, 2010). A GTR-I-Γ model of sequence evolution was applied to the partitioned dataset, and the model parameters were estimated during the run for each gene partition separately starting from a default flat Dirichlet distribution. The analysis was run for 5,000,000 generations in 8 chains and every 100th generation was sampled. The first 50% of trees were discarded as burn-in and a 50% majority rule consensus tree calculated with the sumt command implemented in MrBayes 3.2.1.

Maximum likelihood was performed on the partitioned dataset with the RAxML-HPC black box implemented in the CIPRES Science Gateway (Miller *et al.*, 2010) using rapid bootstrapping and full ML analysis under a GTR + GAMMA approximation allowing for a proportion of invariable sites (I). The analysis was

Table 1. Sample numbers (from Muggia *et al.*, 2010 and own collection numbers) and GenBank accession numbers

Species	Sample	nLSU	nSSU	mtSSU	RPB1
Agonimia allobata	L467	FJ455771	—	GU121589	—
Agonimia tristicula	L469	FJ455772	—	GU121590	—
Bagliettoa parmigera	AFTOL 2271	EF643805	EF689825	—	EF689746
Catapyrenium cinereum	AFTOL 2230	EF643747	EF689829	FJ225671	EF689747
Flakea papillata	L442	FJ455773	FJ455776	GU121592	—
Heteroplacidium imbricatum	AFTOL 2281	EF643756	EF689839	FJ225679	EF689758
Hydropunctaria adriatica	AFTOL 2251	EF643783	EF689862	FJ225680	EF689786
Hydropunctaria maura	AFTOL 2263	EF643801	EF689876	FJ225681	EF689803
Normandina acroglypta	L538	GU121555	—	GU121595	—
Normandina acroglypta	L621	GU121557	—	GU121597	—
Normandina acroglypta	L619	GU121556	GU121576	GU121596	—
Normandina pulchella	L613	GU121561	—	GU121603	—
Normandina pulchella	L614	GU121562	—	GU121604	—
Normandina pulchella	L615	GU121563	—	GU121605	—
Normandina pulchella	L616	GU121568	—	GU121612	GU121619
Normandina pulchella	L617	GU121564	—	GU121606	—
Normandina pulchella	**A. Frisch 12/Jp145 (TNS)**	**KF972456**	**KF972464**	**KF972460**	**KF972465**
Normandina pulchella	**Y. Ohmura 7853 (TNS)**	**KF972457**	**KF972463**	**KF972459**	**KF972467**
Normandina simodensis	**Y. Ohmura 8475 (TNS)**	**KF972458**	**KF972462**	**KF972461**	**—**
Parabagliettoa dufourii	AFTOL 2254	EF643792	EF689868	FJ225684	EF689793
Placidiopsis cartilaginea	AFTOL 2283	EF643758	EF689841	FJ225685	EF689760
Placidium lacinulatum	AFTOL 2287	EF469158	EF689847	FJ225688	EF689765
Placopyrenium bucekii	AFTOL 2238	EF643768	EF689852	FJ225693	EF689772
Staurothele frustulenta	AFTOL 697	DQ823098	DQ823105	FJ225702	DQ840553
Thelidium incavatum	AFTOL 2248	EF643780	EF689860	—	EF689783
Thelidium papulare	AFTOL 2249	EF643781	EF689861	—	EF689784
Verrucula arnoldaria	AFTOL 2302	EF643816	EF689886	FJ225713	EF689816
Verrucula inconnexaria	AFTOL 2307	EF643821	EF689892	FJ225718	EF689821
Verruculopsis lecideoides	AFTOL 2295	EF643798	—	—	EF689800
Wahlenbergiella mucosa	AFTOL 2264	EF643802	EF689877	FJ225720	EF689804
Wahlenbergiella striatula	AFTOL 2267	EF643810	EF689882	FJ225721	EF689810

New sequences obtained in this study are in bold.

stopped automatically after 1000 bootstrap replicates using the bootstopping option implemented in RAxML 3.2.7 (Pattengale *et al.*, 2009). The analysis was repeated thrice to test for consistency of the results and no significant differences in support values were observed.

Results and Discussion

New mtSSU, nucSSU, nucLSU, and RPB1 sequences were generated for one specimen of *Normandina simodensis* and two specimens of *N. pulchella* collected in Japan, except that RPB1 could not be amplified from *N. simodensis* (Table 1). Comparison of the sequences available for *N. acroglypta* and *N. pulchella* in GenBank prior to the analysis showed for several specimens serious conflict between loci. Such specimens are not included in the present study.

The Bayesian and Maximum Likelihood analyses confirm the genus *Normandina* as being monophyletic and show *N. simodensis* as a basal lineage within the genus (Fig. 1). With the present selection of specimens, *N. acroglypta* and *N. pulchella* are supported as distinct species and are more closely related to each other than to *N. simodensis*. *Normandina* is included as sister to *Wahlenbergiella* in our phylogeny, which is supported by Bayesian posterior probabilities, but the branch support value is low in the ML bootstrap analysis. In contrast, *Agonimia* is shown as the closest relative of *Normandina* in Muggia *et al.* (2010).

With our reduced set of taxa and *Endocapon pusillum* as the outgroup, the backbone of the phylogenetic tree is well supported in the Bayes-

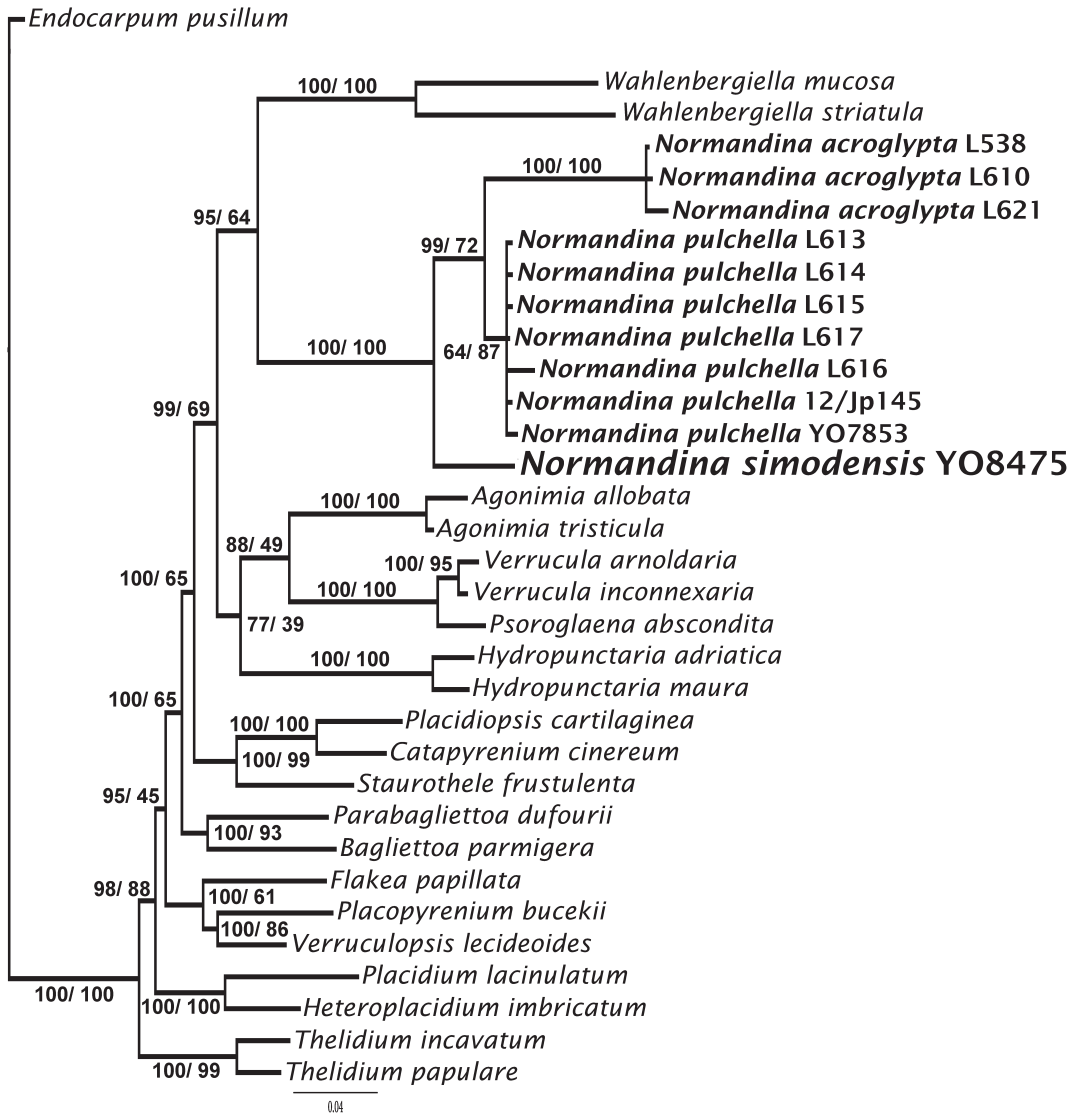

Fig. 1. Bayesian 50% majority rule consensus tree showing the phylogenetic position of the genus *Normandina* and *N. simodensis* in Verrucariaceae. Bayesian posterior probabilities are given first followed by ML support values.

ian analysis but only partly supported in the ML analysis (Fig. 1).

Normandina was first recognized as a natural group by Aptroot (1991) who, in addition to *N. pulchella*, accepted *Thelidium erichsenii* Keissler (a younger name for *Thelidium acroglyptum* Norman; = *Lauderlindsaya acroglypta* (Norman) R. Sant.) and *Heterocarpon simodense* Asahina for the genus. This classification was based on the observation that the perithecia which occasionally form on the thallus of *N. pulchella* represent the ascomata of the lichen and not a parasitic fungus, and the concordant ascoma morphology of the three species. *Normandina pulchella*, *N. acroglypta* and *N. simodensis* share semi-immersed globular to slightly conical perithecia with a moderate orange-brown pigmentation in the wall of textura angularis, the absence

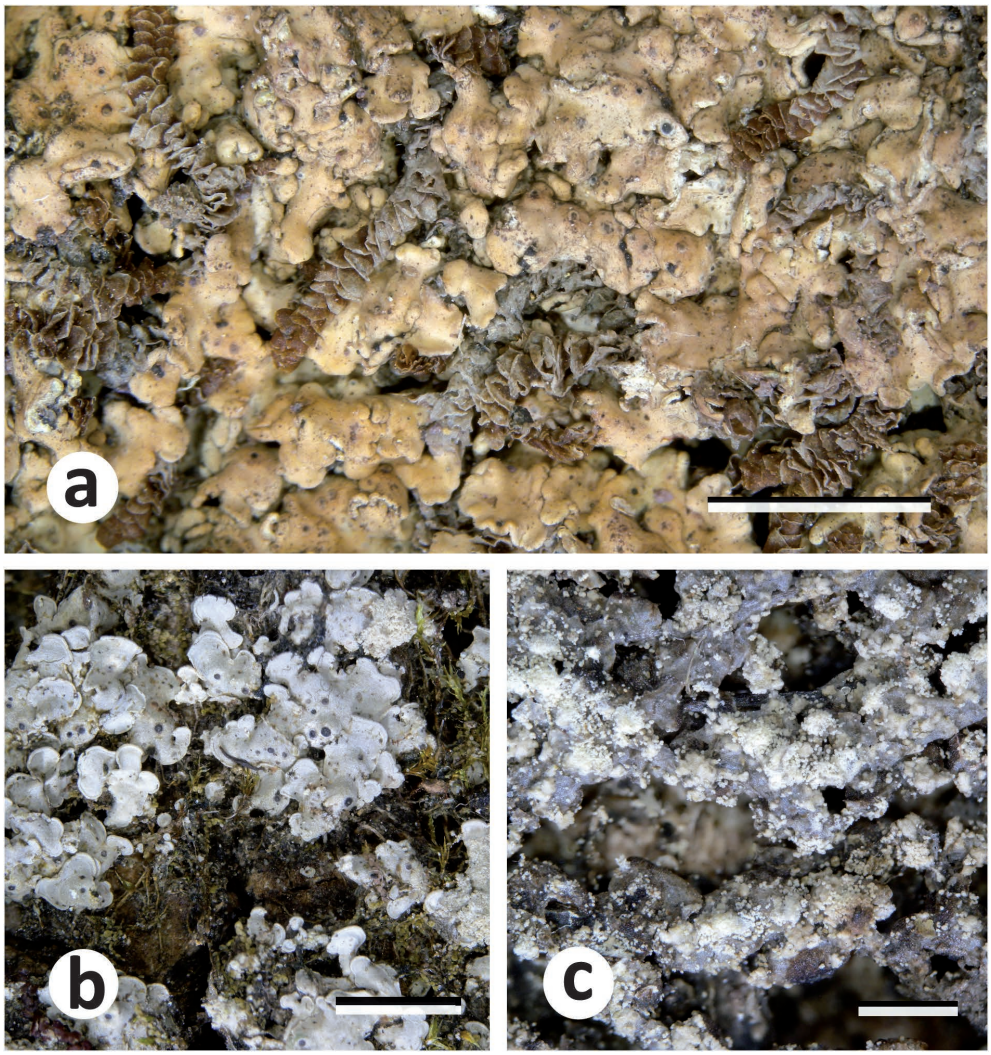

Fig. 2. Thallus morphology in the genus *Normandina*. a) *N. simodensis*, fertile thallus lobes (Kurokawa 58634, TNS); b) *N. pulchella*, fertile thallus lobes with soralia (Kashiwadani 47377, TNS); c) *N. acroglypta*, sterile thallus with soralia (W. Obermayer: Lichenotheca Graecensis 295, TNS); scales: a) = 5 mm, b) = 2 mm, c) = 1 mm.

of an involucrelum, abundant periphyses, absence of paraphyses and hymenial algae, I+ red/ KI+ blue hymenial gel, clavate asci with poorly defined apical chamber, and the hyaline transversely (5–)7-septate ascospores which get pale brownish at late maturity (Aptroot, 1991; Orange and Aptroot, 2009).

Different thallus morphologies as crustose, squamulose and foliose have traditionally been used as one of the main characters for distinguishing genera in Verrucariaceae, beside spore septation, presence or absence of hymenial algae, and involucrellum development (e.g., Zahlbruckner, 1905; Zschacke, 1933–1934; Servít, 1953). Recent phylogenetic studies (Gueidan *et al.*, 2007, 2009; Muggia *et al.*, 2010) have shown these characters as symplesiomorphic or homoplastic in Verrucariaceae, though individual genera as, e.g., *Baglietoa*, *Dermatocarpon* or *Placidium* may be homogeneous with respect to

them (Gueidan *et al.*, 2007, 2009). *Normandina* was probably the first genus in Verrucariaceae accepted to include both squamulose and crustose species. This concept of the genus was previously verified using molecular data by Muggia *et al.* (2010), but is here validated for the first time by including all three accepted species. *Normandina simodensis*, previously not included in molecular phylogenies, is a squamulose species like *N. pulchella*, from which it can be separated by the esorediate elongated squamules with downturned margins and the well-developed cortical and medullary layers.

In our phylogeny, the crustose *N. acroglypta* takes a position distal from the squamulose *N. pulchella* and *N. simodensis*, which is supported both by the Bayesian and ML analyses. Two additional examples of genera in Verrucariaceae comprising both squamulose and crustose species include *Endocarpon* and *Heteroplacidium* (Gueidan *et al.*, 2007, 2009; Muggia *et al.*, 2010). It is interesting to note that the position of the crustose species in relation to squamulose taxa differs in these genera. While the crustose *Endocarpon diffractellum* takes a statistically supported proximal position in *Endocarpon* in all phylogenetic studies, *Heteroplacidium fusculum* is proximal of the squamlose taxa in Muggia *et al.* (2010) but distal in Gueidan *et al.* (2007, 2009). Statistical support for this in the latter two studies, however, is weak or absent.

The spore size in *N. simodensis* has been given as 7-septate, 45–55 × 9–12 μm by Aptroot (1991), but we observed spores in all investigated specimens from Japan including the type collections predominantly (4–)5(–6)-septate and 17–29 × 5–7 μm in size. This agrees with the protologue (Asahina, 1933) that gives the spores as 5-septate and 20–24 × 4–6 μm.

Normandina simodensis (Asahina) Aptroot

Selected specimens examined (TNS). JAPAN. Honshu. Prov. Izu (Pref. Shizuoka): Shimoda, 4 January 1933, Y. Asahina s.n. (holotype and isotypes); Tumekizaki, Suzaki, Shimoda-city, Y. Ohmura 8475; Matsuzaki, H. Kashiwadani 15011; Tsumeki Cape, S. Kurokawa 701001; Cape Irohzaki, S. Kurokawa 58561. Prov. Mikawa (Pref. Aichi): Kawai, Horai-machi, Minami-Shidara-gun, Y. Asahina, S. Kurokawa & M. Nuno s.n. (TNS-L-22197).

Exsiccate specimens examined (TNS). Y. Asahina, Lichenes Japoniae Exsiccati 233; A. Zahlbruckner, Lichenes Rariores Exsiccati 341.

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