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Is *Nephroma silvae-veteris* the cyanomorph of *Lobaria oregana*? Insights from molecular, chemical and morphological characters

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Abstract- The question has recently been raised whether *Nephroma silvae-veteris* Goward et Goffinet, an endemic species of the Pacific Northwest, might not appropriately be interpreted as the cyanomorph of *L. oregana*. This view is supported by comparison of fungal nucleotide sequences of the internal transcribed spacer and the 5.8S gene of the nuclear ribosomal DNA repeat from *N. silvae-veteris* with those of *N. arcticum* and *L. oregana*. The fact, however, that *N. silvae-veteris* and *L. oregana* differ in several morphological and anatomical characters, as well as in the nature of their associated chlorobiont, suggests they are not entirely genetically identical. *Nephroma silvae-veteris* appears to be morphologically intermediate between *L. oregana* and *N. arcticum*, and may perhaps be interpreted as a species that arose through hybridization between *L. oregana* and *N. arcticum*. It is concluded that although *N. silvae-veteris* may appropriately be accomodated in *Lobaria*, it should for the time being be considered distinct from *L. oregana*. Consequently the combination *L. silvae-veteris* (Goward & Goffinet) Goward & Goffinet is made.

Introduction

The epiphytic lichen *Nephroma silvae-veteris* was recently described from old-growth forests of the Pacific Northwest of North America by Goward and Goffinet (1993). This may be described as a small cyanophilous foliose lichen, in which chlorophilous secondary lobules are often borne laminally. Although *N. silvae-veteris* is not known to produce apothecia, its placement in *Nephroma* was assumed to be justified by its close morphological resemblance to the cyanomorph of

N. arcticum (L.) Torss, from which, however, it clearly differs on chemical grounds. Goward and Goffinet (1993) also called attention to the morphological similarity of *N. silvae-veteris* to *Lobaria oregana* (Tuck.) Müll.Arg., with which it also shares an identical chemical profile. More recently, this latter similarity led McCune and Geiser (1997) to interpret *N. silvae-veteris* as the cyanomorph of *L. oregana*.

Photomorph pairs have been reported primarily from the Peltigerineae (*sensu* Tehler 1996), in which the chloromorphs are invariably foliose in habit whereas the cyanomorphs may be foliose or fruticose. In *Nephroma*, for example, all cyanomorphs reported to date are foliose (Tønsberg and Holtan-Hartwig 1983; White and James 1987, 1988). By contrast, secondary cyanomorphs in *Lobaria* appear to be invariably fruticose (Jordan 1972; Purvis 1992). Chloromorphs of *Nephroma* and *Lobaria* primarily differ in characters of their apothecia, and the morphology of their lower surface. These species further differ in the nature of their symbiotic partner. Chloromorphic *Lobariae* are lichenized with either *Myrmecia* (i.e., *M. biatorellae* [Tsch.-Woess & Plessl] Petersen; Tschermak-Woess 1981) or *Dictyochloropsis* (i.e., *D. reticulata* [Tsch.-Woess] Tsch.-Woess; Tschermak-Woess 1978 as *Myrmecia reticulata*), whereas the chlorobiont of *N. arcticum* is assumed to belong to *Coccomyxa* (Wetmore 1960; White 1992). *Nephroma silvae-veteris* likewise appears to be lichenized with a species of *Coccomyxa* (Goward and Goffinet 1993).

Consensus has yet to be reached on the appropriate taxonomic disposition of alternative photomorphs. The controversy is centered primarily on two points: first, the genetic nature of the mycobiont in either symbiotic relationship is in most cases not known; and second, physical independence, as well as ecological, geographic and sometimes also chemical differentiation could be seen as supporting the recognition of two distinct taxa (Galloway 1988; White and James 1988; see Laundon 1995 and Goffinet and Bayer 1997 for review). Armaleo and Clerc (1991) were the first to test the "one-fungus-photomorphs" hypothesis using molecular techniques. Their results suggest that the mycobionts of a photomorph pair are genetically very similar if not identical. Based on actual nucleotide sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal repeat sequences, Goffinet and Bayer (1997) recently reached a similar conclusion for joined photomorphs in *Peltigera* and *Nephroma*, suggesting that the mycobionts of both photomorphs are conspecific.

The present study attempts to answer three questions: 1) Do the chloromorphic and the cyanomorphic lobes of *N. silvae-veteris* share the same mycobiont, as defined by ITS sequence data? 2) Can molecular data provide insights regarding the systematic affinities of *N. silvae-veteris* to either *N. arcticum* or *L. oregana*? and 3) Do comparisons of vegetative

characters among the three species yield results congruent with the molecular data?

MATERIALS AND METHODS

DNA extraction, PCR amplification, sequencing, and sequence alignment: Thallus fragments (green lobules in the case of the chloromorph of *N. silvae-veteris*) were removed from single herbarium collections of *N. arcticum*, *N. silvae-veteris* and *L. oregana* (Table 1), then cleaned in distilled water and lyophilized. DNA extraction followed a modification of Doyle and Doyle's (1987) method (protocol available from senior author). The ITS region was amplified as outlined in Goffinet and Bayer (1997) using the universal primer ITS1 (White et al. 1990) and the ascomycete-specific primer NL6A (Egger 1995). The PCR protocol followed the one outlined in Goffinet and Bayer (1997). The double-stranded templates were sequenced using the Dye Terminator Cycle Sequencing Kit (Perkin Elmer), and the resulting fragments were separated on polyacrylamide gels (Long Ranger Singel ™; FMC BioProducts) using a ABI Prism ™ 377 DNA Sequencer (Perkin Elmer). Sequences obtained were edited using Sequencher 3.0 (Gene Codes Corporation), entered in PAUP 3.1 (Swofford 1993) and aligned to an available sequence of *N. arcticum* (Goffinet and Bayer 1997; U92881). The beginning of the first spacer (ITS1) was determined by comparisons with available sequences of Sclerotiniaceae (Carbone and Kohn 1993); whereas the length of the 5.8S gene and the end of the second spacer were inferred from comparisons with *Saccharomyces cerevisiae* (J01355 and K01048; see Gutell et al. 1993).

Table 1: Voucher specimens and associated GenBank accession number for sequences of the ITS region ([1] private herbarium of B. Goffinet; [2] UBC)

Taxon	GenBank accession number	Voucher
Nephroma arcticum	AF014109	Canada, Goffinet 1310[1]
N. silvae-veteris (joined photomorphs)	AF014110	Canada, Goward 92-336[2]
Lobaria oregana	AF014111	Canada, Goffinet 3141[1]

Morphological study: Several specimens of *N. silvae-veteris* (Goward 81-1965, 91-1149a, 91-1400, 92-336), *N. arcticum* (Goffinet 1310, Goward 92-354, 91-988), and *L. oregana* (Goffinet 3141, Goward 81-1878, 83-137) were selected from different parts of temperate western North America for examination of vegetative characters. Hand-cut sections were mounted in water. All specimens are deposited at UBC, OSU or in the herbarium of B. Goffinet.

Chemical study: Selected specimens of *N. silvae-veteris* (Goward 95-603-cyanomorph, 92-336 and 91-1400 - chloromorph), *N. arcticum* (Goward 91-988-chloromorph, 92-354 both photomorphs), and *L. oregana* (Goward 81-1878, 83-137) were examined for their chemical constituents. Secondary substances were extracted in acetone or toluene, and each extract loaded on precoated Merck Silica gel 60 F_{254} plates, and chromatographed in solvent A, B, or C following Culberson et al. (1981).

RESULTS

ITS sequences: Nucleotide (nt) sequences of the ITS region generated using ITS-1 and ITS-4 as sequencing primers vary between 468 nts (*N. silvae-veteris*, *L. oregana*) and 507 nts (*N. arcticum*; Table 2). Both photomorphs of *N. silvae-veteris* share identical coding (5.8S) and non-coding (ITS1 and ITS2) sequences (absolute distance=0). The 5.8S sequence of *N. silvae-veteris* is identical to that of *L. oregana* but differs from the homologous sequence in *N. arcticum* by eight point mutations and one insertion or deletion (indel). The sequences of both spacers of *N. silvae-veteris* also completely overlap with those in *L. oregana*. ITS sequences of *N. arcticum* are nearly identical to those obtained previously by Goffinet and Bayer (1997). Except for one point mutation, all differences are accounted for by additional G (5nt) or C (2) in the *N. arcticum* sequence obtained here. These may reflect the increased sensitivity and resolution of the automated sequencing method rather than a true genetic divergence, as was observed between different populations of *Peltigera leucophlebia*, for example (Goffinet and Bayer 1997). Aligning the ITS sequences of *L. oregana* and *N. arcticum* required incorporation of numerous gaps and was not pursued here.

Table 2: Length in nucleotides (nt) of the spacers (ITS1&2) and 5.8S gene in *Nephroma arcticum*, *N. silvae-veteris*, and *Lobaria oregana*.

	ITS1	5.8S	ITS2	total
N. arcticum	201	159	147	507
N. silvae-veteris - chloromorph	162	158	148	468
N. silvae-veteris - cyanomorph	162	158	148	468
L. oregana	162	158	148	468

Morphological characters (Table 3): The dominant cyanomorph of *N. silvae-veteris* often produces numerous green "stress lobules" that are about 4-5 mm in diameter. The lobules may occasionally be produced in great numbers, e.g. to about 20 lobes per cm^2. Their early development is signalled by the occurrence of brown, pycnidiiform initials over the upper surface of the cyanomorph. These lobules are morphologically nearly identical to "homologous" chloromorphic lobules

Table 3: Comparison of molecular, morphological and chemical characters for *Nephroma arcticum*, *N. silvae-veteris*, and *Lobaria oregana*.

	N. arcticum	*N. silvae-veteris*	*L. oregana*
Chloromorph			
margin of lobules	**downturned**	**downturned**	upturned
cortex of lobules	**subparaplectenchymatous**	**subparaplectenchymatous**	paraplectenchymatous
upper surface	**dull,** **not maculate**	**dull,** **not maculate**	shiny, (weakly) maculate
Chlorobiont	***Coccomyxa***	***Coccomyxa***	*Myrmecia* or *Dictyochloropsis*
Cyanomorph			
growth-from	**foliose**	**foliose**	? (fruticose in all *Lobariae*)
Tomentum of lower surface	**continuous** (both photomorphs)	**continuous** (cyanomorph)	reticulate (chloromorph)
ITS sequences	very different from those of *N.sv.*	- -	**identical to those of *N.sv***
depsidones	all absent	**constictic, cryptostictic, norstictic, stictic acids**	**constictic, cryptostictic, norstictic, stictic acids**
others secondary metabolites	nephroarctin, phenarctin, zeorin, ± methyl gyrophorate	**all absent**	**all absent**

of *N. arcticum*, that is young green lobules that arise from the surface of the cyanomorph of *N. arcticum*. In both species, the lobes may be characterized by their distinctly downturned tips, and dull to occasionally weakly scabrid upper surface that also lacks maculae and is not at all ridged. By contrast, the tips of young lobes of *L. oregana* are upturned, and the upper cortex is smooth and somewhat shiny, bearing weakly effigurate maculae, and is usually weakly to strongly ridged. The ridging in this species is very pronounced, and is readily observed in lobes as small as 5 mm in diameter. The lower surface of the cyanomorphic lobes of *N. silvae-veteris* is covered by a continuous tomentum as in *N. arcticum*, and not by a reticulate tomentum as in *L. oregana*.

Morphogical similarities between the chloromorphs of *N. silvae-veteris* and *N. arcticum* are also seen at the anatomical level. In both species the upper cortex is rather delicate, and may be described as subparaplectenchymatous compared to the distinctly paraplectenchymatous cortex of *L. oregana*. Furthermore, *N. silvae-veteris* and *N. arcticum* share a similar photobiont (*Coccomyxa* sp.?) that measures 3-5(-7) µm in length, whereas the photobiont of *L. oregana* , whose cells are 10-12 µm in diameter, appears to belong either to *Myrmecia* (*M. biatorellae*) or to *Dictyochloropsis* (*D. reticulata*; see Tschermark-Woess 1984 for distinguishing characters). The cyanobacteria in all three species appear to belong to the genus *Nostoc*.

Chemical profile (Table 3): Thin layer chromatography of extracts of either photomorph of *N. silvae-veteris* confirmed the presence of constictic, cryptostictic, stictic, norstictic and usnic acids. The presence of phenarctin in the chloromorph of this species (Goward et Goffinet 1993) could not be confirmed, nor were trace amounts of PCr4 detected. *Lobaria oregana* shared an identical array of secondary substances. *Nephroma arcticum* lacks all the above depsidones, and instead produces nephroarctin, phenarctin, usnic acid and zeorin, as well as methyl gyrophorate which is however absent from the cyanomorph.

DISCUSSION

The 5.8S gene is a small gene characterized by a low evolutionary rate, which yields characters generally phylogenetically informative at the level of deep divergences dating back to the Paleozoic (Hillis and Dixon 1991). Although evolutionary rates for a given locus may vary among organisms (see Britten 1986), the lack of sequence variation between taxa most likely reflects the lack of genetic divergence as opposed to homoplastic changes leading to identical sequences shared by "distantly" related organism. The complete identity in nucleotide sequences of the 5.8S gene between *N. silvae-veteris* and *L. oregana* therefore suggests that these species are more closely related to each other than either species

is to *N. arcticum*, whose 5.8S gene sequence differs by eight point mutations and one indel.

The ITS sequences of both photomorphs of *N. silvae-veteris* are completely identical, suggesting that these photomorphs share a single mycobiont, as do other pairs of attached photomorphs (Goffinet and Bayer 1997). Furthermore, this mycobiont shares with *L. oregana* identical nucleotide sequences for the transcribed spacers (ITS1 and ITS2). Along a taxonomic gradient, minimal or lack of divergence of ITS sequences is generally found at the species level. Although homologous sequences are not available for other species of *Lobaria*, ITS sequences in *Lobaria*, as in other Peltigerineae and other fungi or plants (see Baldwin et al. 1995; Goffinet and Bayer 1997, and references therein) are likely to differ "significantly" and consistently among species. The complete identity in nucleotide sequence of the ITSs between *N. silvae-veteris* and *L. oregana* therefore suggets that their mycobionts are very closely related if not conspecific. This hypothesis is further supported by the identical chemical profile of both species. A complete identity of ITS sequences does not, however, exclude genotypic differentiation between *N. silvae-veteris* and *L. oregana*, even if they merely represent alternative photomorphs of a single species (see Goffinet and Bayer 1997).

Chloromorphs of *L. oregana* and *N. silvae-veteris* differ in the nature of their chlorobiont. *Lobaria oregana* is lichenized with a species seemingly belonging to *Myrmecia*, whereas the green lobules of *N. silvae-veteris* include a much smaller photobiont, similar to that found in *N. arcticum* (i.e., a species of *Coccomyxa*). The genetic basis for the choice of the photobiont is not understood, but varying degrees of selectivity have been demonstrated between eukaryotic and prokaryotic photobionts (Stocker-Wörgötter and Türk 1994; Yoshimura et al. 1994) and between various taxa of chloroccocalean algae (Ott 1988; see also Honegger 1996 for review). The differences in the chlorobiont between *N. silvae-veteris* and *L. oregana* could be interpreted in two ways: 1) although closely related (see above), the mycobionts may be genetically distinct, at least with regard to the gene(s) controlling selection of the symbiotic chlorobiont; or 2) the two mycobionts are identical but may produce only small, juvenile lobes when occurring with *Coccomyxa*, as opposed to larger thalli when occurring with *Myrmecia*.

Although a single fungus species is apparently capable of establishing a symbiotic relationship with more than one algal species (e.g., *Parmelia saxatilis* Ach., Friedl and Büdel 1996; Honegger 1996), the chlorobionts involved are typically congeneric, and may thus entail minimal changes in the actual symbiotic relationship. By contrast, *Coccomyxa* and *Myrmecia* differ significantly in their cell wall composition (e.g., a resistant biopolymer is present in *Coccomyxa*, but is absent in *Myrmecia*; Brunner and Honegger 1985), and are on this account

unlikely to provide suitable photobionts for a single mycobiont. It may be significant that the sporopollininlike polymer in the wall of *Coccomyxa* prevents the formation of haustoria (Honegger and Brunner 1981). Intraparietal haustoria formation was not studied in the symbiosis between *L. linita* and *M. biatorellae* (Tschermark-Woess 1981), but other *Lobariae* (i.e., those lichenized with *D. reticulata*, which shares cell wall charateristics with *M. biatorellae*; Brunner and Honegger 1985) do form haustoria on the cell wall of the photobiont (Tschermak-Woess 1978). Cell-wall characteristics of the photobiont may thus determine the type of cytological interactions between the symbionts. It appears likely that such cytological differences have a genetic basis, and do not merely reflect the phenotypic plasticity of the fungus. Consequently, the presence of distinct photobionts in the chloromorphs of *N. silvae-veteris* and *L. oregana* may be seen as evidence of genetic differentiation; and the resulting taxa may be interpreted as sibling species (Culberson 1986).

As mentioned above, an alternative explanation for the observed morphological and anatomical differences between the chloromorphs of *N. silvae-veteris* and *L. oregana* may be provided by thallus ontogeny. From this perspective, the occurrence of *Coccomyxa* in the chloromorph of *N. silvae-veteris* may represent an initiating or "stop-gap" measure in lobes that will eventually mature into typical *Myrmecia*-containing lobes of *L. oregana*. We tentatively reject this hypothesis on the following grounds. First, careful searching has failed to turn up any material in which the chloromorph of *N. silvae-veteris* is accompanied by *Myrmecia*. Second, no instances of joined thalli between these species have yet been detected. Third, *Myrmecia*/*Dictyochloropsis* are the only algae genera reported to date for chloromorphic *Lobariae*; *Coccomyxa*, by contrast, appears to be the sole photobiont genus present in chloromorphic *Nephromae*. And fourth, in all chlorophilous *Lobariae* for which cyanomorphs are known (e.g., *L. amplissima* [Purvis 1992], and *L. ravenelii* [Jordan 1972]), the cyanomorphs are fruticose, not foliose.

In our opinion, the above observations support the hypothesis that *N. silvae-veteris* may be genetically distinct from *L. oregana* and may thus appropriately be considered a distinct albeit closely related species. The strict identity of the ITS sequences is not in conflict with this interpretation, though it does strongly suggest that both species have diverged only recently. The mode of speciation is not clear, and a hybrid origin involving participation of *N. arcticum* cannot be excluded. This rather startling claim seems to be supported by four characteristics of *N. silvae-veteris*. First, the lower surface of its cyanomorph is covered by a continuous tomentum, as in both photomorphs of *N. arcticum* (Tønsberg and Holtan-Hartwig 1983), rather than by a reticulate tomentum as in *L. oregana* (Jordan 1973). Second, the green lobules of *N. silvae-veteris* and *N. arcticum* share a thin upper cortex as well as downturned margins, whereas "homologous" lobules in *L. oregana* have a thicker cortex and

upturned margins. Third, the chloromorphs of *N. silvae-veteris* and *N. arcticum* both contain *Coccomyxa* as their chlorobiont. And fourth, the foliose growth form of the cyanomorphic thalli is more consistent with *Nephroma* than it is with *Lobaria* (see above). A hybrid origin may also perhaps be consistent with the extraordinarily narrow ecology and distribution of *N. silvae-veteris* (see Goward and Goffinet 1993).

A final taxonomic disposition for *N. silvae-veteris* must await further study, particularly regarding its ontogeny and genetic structure. Molecular and chemical evidence suggest that *N. silvae-veteris* is most appropriately placed in *Lobaria*, and may actually represent the cyanmorph of *L. oregana*. Given, however, the existence of various morphological, anatomical, and especially symbiological points of distinction between these two taxa, we prefer for now to recognize *N. silvae-veteris* as a distinct species. Doing so not only accords with our belief that this is an independent taxon, it also considerably improves the long term prospects for one of western North America's most endangered epiphytic macrolichens (Goward 1996).

Lobaria silvae-veteris* (Goward & Goffinet) Goward & Goffinet *comb. nov.

Nephroma silvae-veteris Goward & Goffinet. Bryologist 96: 242. 1992. TYPE: Canada: British Columbia. Skeena River Basin, Date Creek, 10 km WNW Kispiox, 55°22'N, 127°50' W, elev. 760 m, shrubby semi-forested seeps with old-growth *Tsuga heterophylla* and *Abies amabilis*, on branches of *Tsuga*, 15 July 1992, *Goward 92-336* with *Allen Banner* (holotype, UBC!; isotypes BM, CANL, H).

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