Molecular support for the recognition of the Mycoblastus fucatus group as the new genus Violella (Tephromelataceae, Lecanorales)

Toby SPribille, Bernard Goffinet, Barbara Klug, Lucia Muggia, Walter Obermayer and Helmut Mayrhofer

Abstract: The crustose lichen genus Mycoblastus in the Northern Hemisphere includes eight recognized species sharing large, simple ascospores produced 1–2 per ascus in strongly pigmented biatorine apothecia. The monophyly of Mycoblastus and the relationship of its various species to Tephromelataceae have never been studied in detail. Data from ITS rDNA and the genes coding for translation elongation factor 1-α and DNA replication licensing factor mini-chromosome maintenance complex 7 support the distinctness of Mycoblastus s. str. from the core of the Tephromelataceae, but recover M. fucatus and an undescribed Asian species as strongly supported within the latter group. We propose accommodating these two species in a new genus, Violella, which is characterized by its brownish inner ascospore walls, Fucatus-violet hymenial pigment granules and secondary chemistry, and discuss the position of Violella relative to Calvitimela and Tephromela. We describe the new species Violella wangii T. Sprib. & Goffinet to accommodate a new species with roccellic acid from Bhutan, China, India and the Russian Far East. We also exclude Mycoblastus indicus Awasthi & Agarwal from the genus Mycoblastus and propose for it the new combination Malmidea indica (Awasthi & Agarwal) Hafellner & T. Sprib.

Key words: ascus types, Asia, Calvitimela, EF1-α gene, fatty acid, lichens, Malmidea, Mcm7 gene, phylogeny, pigment, taxonomy

Introduction

The genus Mycoblastus is a widely distributed group of mainly epiphytic species found in cool temperate to arctic regions of both hemispheres. Its type species, M. sanguinarius (L.) Norman, is one of the common and familiar crustose lichens of boreal conifer forests, and is circumboreal. Despite being easily recognized and often collected, the genus has never been subjected to a complete global revision. Northern Hemisphere species concepts in Mycoblastus developed gradually through the description of forms and varieties of M. sanguinarius that were later raised to species rank. More species were added to the genus as regions of the Southern Hemisphere became better explored and species previously described under Lecidea were combined into Mycoblastus (e.g., Müller-Argoviensis 1894; Zahlbruckner 1926). Recent European taxonomic concepts and nomenclature were outlined by Schauer (1964), who recognized two species, and were expanded by James (1971), who provided a key. Recently Kantvilas (2009) revised cool temperate Southern Hemisphere material, recognizing eight species, which he considered to belong to two different species groups, the ‘M. sanguinarius group’ which always contains atranorin, and the ‘M. dissimulans group’, the members of which always contain perlatolic acid. Mycoblastus in the Northern Hemisphere is currently considered to include eight species,
namely *M. affinis*, *M. alpinus*, *M. glabrescens* (Kantvilas 2009), *M. sanguinarius*, *M. sanguinioides* (Spribille et al. 2011), *M. japonicus* (Müller-Argoviensis 1891), *M. fucatus* (James 1971) and *M. caesius* (Tønsberg 1992). A dichotomy between atranorin- and perlatolic acid-containing species is present in the Northern Hemisphere as well, with *M. caesius* containing perlatolic acid and all other taxa containing atranorin and other substances. The atranorin-containing *Mycoblastus* species of the Northern Hemisphere have been accorded renewed attention recently with a detailed study of the *M. sanguinarius* group by Spribille et al. (2011). Specifically, these authors inferred the phylogenetic relationships with an emphasis on testing monophyly of *M. sanguinarius* in a phylogeny in which all known atranorin-containing Northern Hemisphere species were represented. *Mycoblastus fucatus* was represented by a single specimen, and was resolved to be only distantly related to the core group of *Mycoblastus*.

*Mycoblastus fucatus* is enigmatic among the Northern Hemisphere atranorin-containing species, for at least two reasons. First, its brilliant violet hymenial pigment, termed ‘Fucatus-violet’ by Kantvilas (2009), sets it apart from other *Mycoblastus* species, which contain the dull greenish to green-blue pigment ‘Cinereorufa-green’. Second, it is the common and sole host of a lichenicolous fungus, *Tremella lichenicola*, which does not invade any other *Mycoblastus* species (Coppins & James 1979; Diederich 1986, 1996). Apart from James (1971), little attention has been paid to the ascocarps of *M. fucatus*, in part because they are so rare; in Norway, apothecia were observed in only three of 103 specimens studied by Tønsberg (1992). Sterile forms were described in Britain as a separate species, *M. sterilis* (Coppins & James 1979) until it was later realized that they were sterile forms of *M. fucatus* (Tønsberg 1992).

The recovery of *Mycoblastus fucatus* outside of the core of *Mycoblastus* by Spribille et al. (2011) motivated us to expand our sampling in line with our previous phylogenetic work on *Tephromela* s. lat. (Muggia et al. 2008), a lineage which has repeatedly been found to be related to *Mycoblastus* (Miadlikowska et al. 2006; Arup et al. 2007; Ekman et al. 2008). We also wanted to explore the possible relationship of *M. fucatus* with the saxicolous genus *Calvitimela* and some of the species groups discussed by Kantvilas (2009). Sequence motifs in *M. fucatus* indeed suggested affinities to *Tephromela* or *Calvitimela* rather than to *Mycoblastus*. At the same time, another taxon clearly related to *M. fucatus* was collected by the first two authors of this paper in Russia and China, providing more fresh material and further solidifying the concept of this as a recognizable species group with distinct morphological characters. Here, we present the results of molecular phylogenetic and morphological investigations on the *M. fucatus* group and propose for it the new genus *Violella*.

**Materials and Methods**

**Taxon sampling and hypothesis testing**

We designed our taxon sampling to include the core groups of *Mycoblastus* for which we could obtain fresh material, as well as representatives of major groups in the *Tephromelataceae* identified by Hertel & Rambold (1985), including *Tephromela*, *Calvitimela* and the “Lecidea” *aglaea* group, which has been treated as belonging to both *Tephromela* and *Calvitimela* in the past. We also generated sequences for several taxa of *Parmelaceae*, which is a group often retrieved in BLAST searches of *Mycoblastus* sequences in GenBank. We included one specimen of *Japanula* (*Lecanoraceae*), hypothesized as being close to *Mycoblastus* by Kantvilas (2009), and spent some sequencing effort examining the possibility of relationships to *Megalaria*, also proposed as a relative of *Mycoblastus* by Kantvilas (2009), and *Psorinia*, suggested as a possible relative to *Calvitimela* by Hafellner & Türk (2001). We ultimately excluded *Megalaria* and *Psorinia* from our sampling because 1) morphological evidence, especially the strongly gelatinized proper exiple of *Megalaria*, argues against close relationships with that genus, and 2) DNA sequence data we obtained for single loci for both *Megalaria* and *Psorinia* were so different from the other taxa in our dataset as to be easily ruled out as close relatives. *Heppssora indica*, a species and genus described from Tamil Nadu state, India (Awasthi & Singh 1977; Singh & Sinha 2010: photograph), exhibits clear morphological affinities to *Tephromelataceae* (Poelt & Grube 1993). Unfortunately we did not have access to any fresh material; a specimen distributed under this name in a recent exsiccate

Laboratory methods

Material for DNA extraction was taken from apothecia if present, otherwise from parasite-free thallus fragments inspected in water droplets on a microscope slide under ×20 magnification. Prepared material was transferred into reaction tubes, dried and pulverized using a TissueLyser II (Retsch). DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) extraction kit using the manufacturer’s instructions. For Tephromela specimens already studied by Muggia et al. (2008), existing extractions were used. Dilutions (mostly $5 \times 10^{-2}$) of the genomic DNA extractions were used as a template for the PCR reactions. After screening potential markers (Spribille et al. 2011), we settled on using three loci: two protein-coding genes, namely translation elongation factor 1-α (EF1-α) and the DNA replication licensing factor mini-chromosome maintenance complex 7 (Mcm7), and the nuclear ribosomal internal transcribed spacer region (ITS). For amplification of EF1-α from Mycoblastus japonicus, we employed a Mycoblastus-specific primer pair which will be described in detail elsewhere.

PCR reactions were performed with Illustra Ready-To-Go RT-PCR Beads (GE Healthcare) in a thermocycler (Alphamatrix) using conditions detailed by Spribille et al. (2011). Two μl aliquots of PCR products were viewed on 1% agarose gels stained with GelRed™ (Biotium, VWR); whole products were subsequently viewed on 1% agarose gels stained with GelRed™ and the nuclear ribosomal internal transcribed spacer region (ITS). For amplification of EF1-α from Mycoblastus japonicus, we employed a Mycoblastus-specific primer pair which will be described in detail elsewhere.

Phylogenetic analyses

Alignments were performed using ClustalW (Thompson et al. 1994) and subsequently optimized by hand in BioEdit (Hall 1999). Non-conserved regions and positions with missing data in >50% of sequences were removed using Gblocks (Talavera & Castresana 2007). Candidate nucleotide substitution models were identified for each partition using the likelihood ratio test implemented in jModelTest (Posada 2008); likelihood scores were then compared based on the Akaike Information Criterion (AIC). Individual gene alignments were analyzed using a maximum likelihood (ML) and Bayesian Markov Chain Monte Carlo (B/MCMC) approach. We tested for conflict between partitions by examining frequencies of bipartitions for the same taxon sets across all three partitions using a set of B/MCMC gene trees; a conflict was interpreted as significant if two well supported different relationships were detected for the same taxon set (Kauff & Lutzoni 2002); we used the threshold of $\geq 95\%$. Maximum likelihood analyses were performed using the program PhyML 3.0 (Guindon et al. 2010). Bootstrapping was carried out on 500 tree replicates. B/MCMC analyses were performed using the program MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001) using substitution models approximated by jModeltest (see above). For each analysis, two runs with ten million generations each starting with a random tree and running four simultaneous chains was employed. Every 1000th tree was sampled and saved to a file. The first 5 000 000 generations (5000 sampled trees) were discarded as chain ‘burn-in’. Of the remaining 5001 trees a majority consensus tree with averaged branch lengths and annotated with posterior probability values at every node was calculated using the sumtree command in MrBayes. The program TRACER v. 1.5 [http://tree.bio.ed.ac.uk/software/tracer] was used to assess whether likelihood values had reached stationarity within the allocated burn-in window by plotting log likelihood against the number of generations. In addition, we examined the distributions of split frequencies using the online program AWTY (Nylander et al. 2007) to test whether runs had converged. Only clades that received bootstrap values $\geq 70\%$ in ML and posterior probabilities $\geq 0.95$ were considered significantly robust. Phylogenetic trees were visualized in TreeView (Page 1996).

Morphological and chemical analyses

To test whether our phylogenetic results could be matched by morphological traits, we sorted specimens under a Leica Wild M3Z dissecting microscope and examined anatomical sections on material mounted in water with a Zeiss Axioskop light microscope fitted with Nomarski differential interference contrast and outfitted with a Zeiss AxioCam MRc5 digital camera. Some images were digitally optimized through ‘stacking’ using CombineZM open source image processing software [www.hadleyweb.pwp.blueyonder.co.uk/CZM]. Ascospore, areole, soredia and apothecia measurements are given as (smallest absolute measurement–)smallest average – largest average(–largest absolute measurement) asci if present, otherwise from parasite-free thallus fragments. Mycoblastus fucatus would be included as Mycoblastus fucatus. 2011 Molecular support for Violella gen. nov. 447.

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Table 1. DNA vouchers and GenBank Accession Numbers of the species used in this study; bold species names and accession numbers indicate new accessions

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<td>Italy, Campania, Napoli, Capri Island, Muggia (TSB 37119)</td>
<td>JN009698 EU558648 JN009754</td>
</tr>
</tbody>
</table>
**Table 1. Continued**

<table>
<thead>
<tr>
<th>Species</th>
<th>Ref. number</th>
<th>Voucher</th>
<th>GenBank Accession Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T. atra</strong></td>
<td>L228</td>
<td>Italy, Campania, Napoli, Capri Island, <em>Muggia</em> (TSB 37124)</td>
<td>JN009699 EU558650 JN009755</td>
</tr>
<tr>
<td><strong>T. atra</strong></td>
<td>L248</td>
<td>Italy, Campania, Napoli, Capri Island, <em>Muggia</em> (TSB 37133)</td>
<td>– EU558656 JN009756</td>
</tr>
<tr>
<td><strong>T. atra calcarea</strong></td>
<td>L284</td>
<td>Italy, Sardinia, Nuoro, Mt. Albo, <em>Muggia</em> (TSB 37465)</td>
<td>JN009700 JN009729 JN009758</td>
</tr>
<tr>
<td><strong>T. atra calcarea</strong></td>
<td>L403</td>
<td>Greece, Epirus, Tzoumerka, <em>Spribille</em> 15951 (GZU)</td>
<td>– EU558681 JN009759</td>
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<tr>
<td><strong>T. atra calcarea</strong></td>
<td>L280</td>
<td>Italy, Sardinia, Nuoro, Mt. Albo, <em>Muggia</em> (TSB 37461)</td>
<td>– EU558660 JN009760</td>
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<tr>
<td><strong>T. cf. pertusarioides</strong></td>
<td>L280</td>
<td>Russia, Khabarovskiy Krai, Bureinskiy Zapovednik, near Staraya Medvezhka, <em>Spribille</em> 31797 &amp; Yakovchenko (GZU)</td>
<td>JN009701 JN009730 JN009761</td>
</tr>
<tr>
<td><em>Tephromela</em> sp. Björk 18057†</td>
<td>629</td>
<td>Canada, British Columbia, Fraser Canyon, Björk 18057 (UBC)</td>
<td>JF744875 JF744986 JF744821</td>
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<tr>
<td><strong>Usnea intermedia</strong></td>
<td>609</td>
<td>Austria, Styria, Gurktaler Alpen, <em>Obermayer</em> 11839 (GZU)</td>
<td>JN009702 JN009731 JN009762</td>
</tr>
<tr>
<td><strong>Violella fucata</strong></td>
<td>844</td>
<td>Germany, Bavaria, Bayerischer Wald, Dreisselwels, <em>Spribille</em> 32112 (GZU)</td>
<td>– JN009732 –</td>
</tr>
<tr>
<td><strong>V. fucata</strong></td>
<td>600</td>
<td>USA, Massachusetts, Mt. Greylock, <em>Spribille</em> 32161 (GZU)</td>
<td>JN009703 JF744968 JF744818</td>
</tr>
<tr>
<td><strong>V. fucata</strong></td>
<td>835</td>
<td>Slovenia, Snežnik area, <em>Spribille</em> 30276 &amp; Mayrhofer (GZU)</td>
<td>– JN009733 JN009763</td>
</tr>
<tr>
<td><strong>V. wangii</strong></td>
<td>796</td>
<td>China, Yunnan, Laojunshan, <em>Goffinet</em> 10029 (KUN)</td>
<td>JN009704 JN009734 JN009764</td>
</tr>
<tr>
<td><strong>V. wangii</strong></td>
<td>840</td>
<td>China, Yunnan, Laojunshan, <em>Goffinet</em> 10033 (UPS)</td>
<td>JN009705 JN009736 JN009765</td>
</tr>
</tbody>
</table>

*first confirmed record for North America (TLC: α-collatolic and alectoronic acids)
†reported as *M. affinis* by Spribille et al. (2011), this specimen actually corresponds to the *alpinus* morphotype
‡first modern record for Japan
§first record for Russia
¶previously published as *T. atra* s.lat. by Spribille et al. (2011), but probably an undescribed taxon
Pigments were examined under the light microscope and named according to Meyer & Printzen (2000), except for Fucatus-violet, which was not treated by those authors. Fucatus-violet would key in Meyer & Printzen’s key under lead 2 as N+ violaceous as it goes from its natural violet colour in H2O to a deep raspberry red. It has the following standard reactions: K+ peacock-blue, N+ raspberry-red, HCl− (slowly fading but maintaining hue), C+ grey, eventually bleaching altogether; after pretreatment with N: K greenish yellow ↔ HCl completely clear. The pigment was mentioned already by Stirton (1879) as an ‘intense violaceous colour’ and has also been previously referred to as ‘gentian violet’ (James 1971; James & Watson 2009). We adopt the name proposed by Kantvilas (2009).


Mycoblastus sanguinarius (L.) Norm.: USA: Alaska: Kenai Peninsula, Russian River, 2008, Sprüille 27359 & Wright (GZU).


**Results of phylogenetic analysis**

We obtained 91 new DNA sequences from 43 individuals, including 30 of EF1-α, 31 of ITS and 30 of Mcm7. Following exclusion of positions with missing or ambiguous data, the sequences consisted of 852, 478 and 564 characters, respectively, for a combined total of 1894 characters. Tests of nucleotide substitution models returned TIM3ef+I+G for EF1-α, GTR+I+G for ITS, and HKY+I+G for Mcm7. We ran individual B/MCMC analyses for each locus but detected no significant conflict between the loci, and thus combined them. Our partitioned B/MCMC analysis employed six, six and two substitution rate categories, respectively, for the three partitions; four rate categories, predicted in the TIM3ef model, are not possible to implement in current software. Overall rate heterogeneity was modelled using a gamma density function. ML and B/MCMC returned congruent phylogenies for the concatenated data set. Analysis of B/MCMC log likelihood outputs in Tracer indicated that convergence was reached well before our burn-in threshold; plotting of split frequencies between runs in AWTY also showed stationarity had been reached. The average standard deviation across runs for splits with a frequency of at least 0·1 was 0·003493.

We recovered two strongly supported core groups (Fig. 1), one of which includes Tephromela, Calvitimela s.lat. and the Mycoblastus fucatus group (which we call here ‘core Tephromelataceae’), and another including Mycoblastus s.str. Both of these clades were separated from the five taxa of Parmeliaceae at the base of the tree and Japewia subsaurifera, which was recovered close to Miriquidica intrata (Lecanoraceae). The combined Mycoblastus clade consists of a strongly supported monophyletic M. sanguinarius, M. sanguinarioides and M. globrescens. Mycoblastus alpinus was recovered within a strongly supported M. affinis clade and the single individual of M. japonicus, which for the first time is represented by two markers in a molecular phylogeny, is recovered as strongly supported sister to M. affinis.

The ‘core Tephromelataceae’ clade consists of four distinct, well supported groups; the relationships to each other are, however, not supported. These groups correspond to Tephromela (T. atra, T. cf. pertusarioides and the undescribed Tephromela sp. Björk 18057), Calvitimela s.str. (C. armeniaca and C. melaleuca), the Mycoblastus fucatus group, interpreted here as the new genus Violella (see below), and “Lecidea” aglaea on its own long branch separate from the rest of Calvitimela.

**Discussion**

Hertel & Rambold (1985) provided an overview of species groups in what they considered Tephromela, and later Kantvilas (2009) proposed a range of potential relatives for Mycoblastus. Our results shed new light on potential relationships and invite a reassessment of meaningful morphological characters (Table 2). In his study of Lecanoralean
Fig. 1. Majority rule B/MCMC consensus tree of the concatenated EF1-α, ITS and Mcm7 data set. Posterior probabilities ≥ 95% are shown as thick branches; bootstrap support results of maximum likelihood analysis are shown where ≥ 70%. Reference numbers refer to Table 1.
ascus types, Hafellner (1984) implied deep differences between _Tephromela_ and _Mycoblastus_, sufficient for him to recognize them as belonging to different families, _Tephromelataceae_ and _Mycoblastaceae_. Indeed, our results strongly support the distinctness of _Mycoblastus s. str._ from a ‘core _Tephromelataceae_’ (Fig. 1). This does not necessarily translate to different families, however. We did not structure the taxon sampling of our phylogenetic analysis to test family-level relationships within a broader Lecanoralean context, and cannot predict the outcome of such a study. Morphologically, however, the distinction of two families would appear to be untenable. _Mycoblastus_ shares a similar ascus apical apparatus with members of _Tephromelataceae_, similar development of a peculiar thalline cushion below the apothecia (see below), similar pycnidal development, conidiophores, shared ascocarp pigments and widely overlapping thallus secondary chemistry. Morphologically, the only difference we have found may relate to the basic type of hymenial matrix formed by the paraphyses. In ‘core _Tephromelataceae_’, paraphyses can be branched and anastomosing, but more often than not they form long, straight, multicellular ‘beams’ that separate easily in K and are substantially thicker than the cross-bridges (Fig. 2F). In _M. sanguinarius_, by contrast, paraphyses almost never form straight segments even within a single paraphysis cell, the anastomosing network is intricate, with bridges often nearly as thick as the main beams (Fig. 2E), and the entire network enmeshes the asci; even in K, squashing of the hymenium results in breakage of the hymenium rather than separation of asci and paraphyses. We never found the extreme degree of branching and anastomosing without straight beams depicted by James (1971: fig. 7) for _M. fucatus_ but instead always found the paraphysis beams to be much thicker than the bridges and easily separable in K, and thus similar to other core _Tephromelataceae_.

Another enigmatic structure linking _Tephromelataceae_ and _Mycoblastaceae_ is the so-called thalline exciple, especially evident in _Tephromela_. Hertel & Rambold (1985) and Kantvilas (2009) have interpreted the ‘thalline exciple’ of _Tephromela_ to be homologous, or at least worthy of providing in the same table category, to the proper exciple in other genera. We have, however, found apparently homologous thalline tissue, in addition to the presence of a rudimentary proper exciple, in all genera of _Tephromelataceae_ and _Mycoblastus_. We hesitate to refer to this as an amphitheciun or thalline exciple because it lacks an algal layer and consists of differentiated, dense, prosoplectenchymatous tissue not normally found in the thallus. Instead we will refer to it as a ‘thalline cushion’. The thalline cushion occasionally emerges to outer view as a thin or thick white line in _M. sanguinariusoides_ (T. Spribille, unpublished data), is visible in section in the _M. fucatus_ group (Fig. 3C & 3F), and in _Tephromela_ it forms a ‘thalline rim’. However, it is even present in _Calvitimela_, where it forms a dense layer between the subhymenium and the thallus medulla.

Our phylogenetic results re-open a discussion on the generic boundaries in _Tephromelataceae_, begun by Hertel & Rambold (1985) and continued by Hafellner & Türk (2001), with the description of _Calvitimela_. _Tephromela_ possesses _Biatrica_-type asci with a sometimes bulbous masse axiale (Fig. 2B). Hafellner & Türk (2001) separated out _Calvitimela_ in part based on its _Lecanora_-type ascus, though even in describing their new genus they already anticipated that the “_Lecidea_” _aglaea_ group, with its _Biatrica_-type asci (Fig. 2C), might not be closely related to the type species _C. armeniaca_ (Fig. 2A). Even so, they transferred it to _Calvitimela_. Our results confirm that the two are not closely related and we thus maintain this taxon in the genus _Lecidea_ in the broad sense until its generic disposition can be resolved. To this medley can now be added the _M. fucatus_ group with its _Biatrica_-type asci (Fig. 2D). _Mycoblastus fucatus_ has long been recognized for its unusual hymenial pigmentation, a character absent from _Mycoblastus s. str._ Furthermore, _M. fucatus_, and in particular material from Asia that will be described here as a new taxon, possesses a character not known from any of the other associated genera studied here, namely the tendency of the
internal ascospore wall to turn brown. This character was already noted by Leighton (1879, see also below). These characters also do not reconcile with those of *Tephromela* and *Calvitimela*, which differ in hymenium pigmentation, ascus type and, in part, secondary chemistry (Table 2). We accordingly propose recognizing *M. fucatus* and this new taxon as constituting the new genus *Violella*. The alternative generic solution would require all

Fig. 2. Selected asci and paraphyses. A–D, ascus variation in the *Tephromelataceae*, showing asci with immature ascospores; A, *Calvitimela armeniaca* (Hafellner 71304); B, *Tephromela atra* (Spribille 16260); C, “Lecidea” aglaea (Hafellner 72944); D, *Violella wangii* (holotype). E & F, paraphyses; E, *Mycoblastus sanguinarius* (Spribille 27359); F, *Violella wangii* (holotype). A–D in *I*<sub> Lugol </sub>, after pretreatment with *K*, E & F in *K*. Scales: A–F = 10 μm.
Table 2. Characters of genera and major groups in the *Tephromelaceae* and *Mycoblastus*

<table>
<thead>
<tr>
<th></th>
<th>Violella</th>
<th>Calvitimela</th>
<th>&quot;Lecidea&quot; aglaea group</th>
<th>Heppsora*</th>
<th>Tephromela</th>
<th>Mycoblastus</th>
<th>M. dissimulans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascospore walls</td>
<td>yes, in endospore</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Ascospore walls</td>
<td>double†</td>
<td>apparently single weakly amyloid, internal content clearly visible</td>
<td>apparently single weakly amyloid, internal content clearly visible</td>
<td>apparently single weakly amyloid, internal content clearly visible</td>
<td>apparently single weakly amyloid, internal content clearly visible</td>
<td>double strongly amyloid, internal content concealed except when iodine dissipates</td>
<td>double strongly amyloid, internal content concealed except when iodine dissipates</td>
</tr>
<tr>
<td>Ascus wall in I⊙'s</td>
<td>double†</td>
<td>apparently single weakly amyloid, internal content clearly visible</td>
<td>apparently single weakly amyloid, internal content clearly visible</td>
<td>apparently single weakly amyloid, internal content clearly visible</td>
<td>apparently single weakly amyloid, internal content clearly visible</td>
<td>strongly amyloid, internal content concealed except when iodine dissipates</td>
<td>strongly amyloid, internal content concealed except when iodine dissipates</td>
</tr>
<tr>
<td>Ascus apical</td>
<td>Biatora-type</td>
<td>Lecanora-type</td>
<td>Biatora-to Bacidia-type</td>
<td>±Lecanora-type</td>
<td>±Biatora-type</td>
<td>Biatora-to Bacidia-type</td>
<td>±Bacidia-type</td>
</tr>
<tr>
<td>apparatus</td>
<td>c. 1/4 to 1/5 of ascus length</td>
<td>c. 1/5 of ascus length</td>
<td>c. 1/5 of ascus length</td>
<td>not studied</td>
<td>c. 1/5 of ascus length</td>
<td>c. 1/3–1/4 of ascus length</td>
<td>c. 1/3–1/4 of ascus length</td>
</tr>
<tr>
<td>Ascus ocular chamber</td>
<td>c. 1/4 to 1/5 of ascus length</td>
<td>c. 1/5 of ascus length</td>
<td>c. 1/5 of ascus length</td>
<td>not studied</td>
<td>c. 1/5 of ascus length</td>
<td>c. 1/3–1/4 of ascus length</td>
<td>c. 1/3–1/4 of ascus length, ascus often becoming pyriform</td>
</tr>
<tr>
<td>Number of ascospores</td>
<td>mostly 2 (1–3)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>1–2</td>
<td>2</td>
</tr>
<tr>
<td>Paraphyses</td>
<td>stout with thin cross-bridges</td>
<td>stout with thin cross-bridges</td>
<td>stout with thin cross-bridges</td>
<td>not studied</td>
<td>stout with thin cross-bridges</td>
<td>netted, cross-bridges of similar thickness to main beams</td>
<td>netted, cross-bridges of similar thickness to main beams</td>
</tr>
<tr>
<td></td>
<td>Violella</td>
<td>Calvitimela</td>
<td>“Lecidea” aglaea group</td>
<td>Heppsora*</td>
<td>Tephromela</td>
<td>Mycoblastus</td>
<td>M. dissimulans</td>
</tr>
<tr>
<td>----------------------</td>
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<td>------------------------</td>
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<td>------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>Hymenial pigmentation</strong></td>
<td>Fucatus-violet, secondary Cinereorufa-green</td>
<td>Cinereorufa-green</td>
<td>Cinereorufa-green</td>
<td>Atra-red</td>
<td>Atra-red</td>
<td>Cinereorufa-green</td>
<td>Cinereorufa-green</td>
</tr>
<tr>
<td><strong>Proper exciple</strong></td>
<td>reduced, hyphae similar to paraphyses rudimentary, thin layer below proper exciple</td>
<td>reduced, hyphae similar to paraphyses rudimentary, thin layer below proper exciple</td>
<td>reduced, hyphae similar to paraphyses rudimentary, thin layer below proper exciple</td>
<td>reduced, hyphae similar to paraphyses highly reduced or appearing absent</td>
<td>reduced, hyphae similar to paraphyses well developed and forming ‘thalline margin’§</td>
<td>reduced, hyphae similar to paraphyses rudimentary to well developed and forming ring around apothecia</td>
<td>reduced, hyphae similar to paraphyses rudimentary, thin layer below proper exciple</td>
</tr>
<tr>
<td><strong>‘Thalline cushion’</strong></td>
<td>rudimentary to well developed and forming ring around apothecia</td>
<td>rudimentary to well developed and forming ring around apothecia</td>
<td>rudimentary to well developed and forming ring around apothecia</td>
<td>rudimentary to well developed and forming ring around apothecia</td>
<td>rudimentary to well developed and forming ring around apothecia</td>
<td>rudimentary to well developed and forming ring around apothecia</td>
<td>rudimentary to well developed and forming ring around apothecia</td>
</tr>
<tr>
<td><strong>Conidia</strong></td>
<td>bacilliform</td>
<td>bacilliform*</td>
<td>ellipsoid to bacilliform*</td>
<td>bacilliform</td>
<td>filiform*</td>
<td>crustose to fruticose</td>
<td>bacilliform</td>
</tr>
<tr>
<td><strong>Thallus morphology</strong></td>
<td>crustose</td>
<td>crustose</td>
<td>crustose</td>
<td>crustose</td>
<td>crustose</td>
<td>crustose</td>
<td>crustose</td>
</tr>
<tr>
<td><strong>Thallus secondary chemistry</strong></td>
<td>atranorin, fumarprotocetraric acid + fatty acid</td>
<td>ateloric acid, psoromic acid, stictic acid + fatty acids</td>
<td>atranorin, usnic acid + fatty acids</td>
<td>atranorin, alectoric acid, α-collatolic acid</td>
<td>atranorin, alectoric acid, Usnic acid, fumarprotocetraric acid + fatty acids</td>
<td>atranorin, plauanic, fumarprotocetraric acid + fatty acids</td>
<td>perlatolic acid + fatty acids</td>
</tr>
</tbody>
</table>

*description based on Awasthi & Singh (1977) and Poelt & Grube (1993);
†outer wall considered an epispore by Stirton (1879), but not dissolving in C;
‡Fucatus-violet not seen in Chilean material but reported from Tasmania by Kantvilas (2009);
§‘exciple’ of Kantvilas (2009, p. 158: table);
¶illustrated by Hertel & Rambold (1985);
**in T. siphuloides (Poelt & Grube 1993).
taxa from *Tephromela* s. str. through *Calvitimela*, *Violella* and the “*Lecidea*” *aglaea* group to be referred to *Tephromela* s. ampl. but in our opinion this would defeat the purpose of genera to circumscribe like species groups, and make *Tephromela*, which even in its narrow definition has more than 20 described species, unnecessarily large.
and unwieldy. We expect “Lecidea” aglaea will eventually be placed in its own genus, possibly together with C. perlata (Haugan & Timdal) R. Sant., which has Bacidia-type asci, and some of the various entities currently treated as chemotypes of “Lecidea” aglaea (Haugan & Timdal 1994). Already Andreev (2004) has postulated that these two taxa are closely related, though he retained them in Calvitimela. We leave these problems unresolved until a better sampling of Calvitimela s. lat. has been achieved, perhaps including Southern Hemisphere taxa (Fryday 2011) and Heppsora, a south Asian genus (Awasthi & Singh 1977), for which DNA could not be obtained for the current study.

Taxonomy

Violella T. Sprib. gen. nov.

MycoBank No: MB 519831

Genus novum ad Tephromelataceae pertinet. Generi Calvitimela simile sed differt pigmentis hymenialibus violaceis (haud viridibus), ascis ut in Biatora constructis (haud ut in Lecanora), ascosporis primum hyalinis, demum strato interno fuscascenti (haud persistenter hyalinis) et substanciis chimicis alis (atranorinum vice acidi alectorialici).

Typus: Violella fucata (Stirt.) T. Sprib.

Thallus crustose, areolate to rimose; photobiont chlorococcoid algae. Thallus chemistry includes the depside atranorin, a depsidone and a fatty acid.

Apothecia apparently biatorine, macroscopically black, formed on a rudimentary thalline cushion, this prospectenchymatous, tawny with brown streaks; proper exciple reduced; epihymenium not differentiated as a distinct layer, epipsamma lacking; hymenium inspersed with violet granules (“Fucatus-violet”) that react N+ raspberry red, K+ peacock green; paraphyses straight or slightly curved with thinner cross-bridges; asci Biatora-type; ascospores simple, in the known species two per ascus (reported as occasionally 1 or 3: Stirton 1879; Leighton 1879; James 1971), initially with a single wall, eventually a differentiated internal wall turning brown.

Pycnidia apparently rare, colourless or with light brown pigment around ostiole, sunken in thallus areoles; conidiophores Parmelia-type; conidia bacilliform.

Etymology. Diminutive of Viola, a reference to the characteristic pigment in the hymenium of both known species (Fig. 3).

Comments. Species of Violella are distinguished from related genera first and foremost by their abundant Fucatus-violet pigment and the tendency of the inner ascospore walls to become brown. The latter character appears to have been recorded only once previously in the literature, by Leighton (1879: 545), who noted the tendency of the ‘protoplasm’ of the ascospores of V. fucata to turn a ‘nigro-fulvaceous colour’ in K. However, this colour, apparently produced in the internal ascospore wall, is present even without treatment with K and seems to occur in all mature ascospores in both species of the genus (Figs. 2D, 3C & F, 4A, B & F). It appears that many ascospores with a brown inner wall are collapsing internally and are possibly abortive (Fig. 4A & B). However, healthy ascospores with brown internal walls were also observed (Fig. 4F) and no mature ascospores were observed in either species that had not turned brown internally.

Kantvilas (2009) speculated that the perlatolic acid-containing species of Mycoblastus, the so-called M. dissimulans group, may constitute a distinct genus. We were not able to sample that group for our phylogeny, but we note that Violella differs from M. dissimulans in 1) its paraphyses linked over small bridges to other paraphyses, as opposed to the dense network paraphyses similar to those in Mycoblastus s. str. formed in M. dissimulans; 2) its ascospores, in which the internal ascospore wall frequently becomes brown or olivaceous (remaining hyaline in M. dissimulans); and 3) its secondary thallus chemistry. We suspect that M. dissimulans will ultimately be found to cluster more closely with Mycoblastus s. str., which also has brittle, anastomosing paraphysis networks. We are not aware of any existing generic
Fig. 4. Microscopic characteristics of Violella apothecia and pycnidia. A, V. fucata ascospore in water (Tønsberg 19004). B–I, V. wangii internal anatomy; B, mature and immature ascospore, in water; C–F, asci at different stages of development, ending in nearly mature ascospore, using differential interference contrast following pretreatment with C; G, section of pycnidium in water (holotype); H, conidiophores (in water); I, conidia (in water). B–F from Goffinet 10033, G–I from holotype. All scales = 10 μm.
name in this group that would need to be considered before describing Violella.

Violella fucata (Stirt.) T. Sprib. comb. nov.

MycoBank No.: MB 519832
Basionym: Lecidea fucata Stirton, Scottish Naturalist 5: 16 (1879); type: Great Britain, Scotland, Mid Perth, Tyndrum, on wood, July 1878, Stirton s.n. (BM—holotype).—Megalospora fucata (Stirt.) H. Olivier, Bull. de Géogr. Bot. 21: 187 (1911; on p. 207 Olivier incorrectly attributes the combination to Leighton 1879).—Mycoblastus fucatus (Stirt.) Zahlbruckner, Cat. Lich. Univ. 4: 3 (1926).

(Figs 3A–C, 4A)

The first species of the genus to be described was Violella fucata (Stirton 1879, as Lecidea fucata), but this taxon rarely produces apothecia. A detailed description is provided by James (1971). Violella fucata is widely reported from western Europe (e.g., Tønsberg 1992; James & Watson 2009), the Pacific Coast of North America (British Columbia and Washington: Tønsberg 1993; Alaska: Spribille et al. 2010) and eastern North America (Massachusetts: Spribille et al. 2011 and below; Newfoundland: Tønsberg 1993; New York: Schmull et al. 2002; Harris 2004). A distribution map of its obligate parasite Tremella lichenicola (Diederich 1996: 102) includes many European and some western North American records.


Violella wangii T. Sprib. & Goffinet sp. nov.

MycoBank No.: MB 519833
A Violella fucata areolis maioribus bullatisque, apothecis maioribus et substanciis chimicis alis (atranorinum et acidum roccellicum/angardianum vice atranorini et acidi fumarprotocetrarici) differt. Habitat in montibus altis Asiae extratropicace.

Typos: China, Yunnan, Lijianga Prefecture, Lijiang Co. 8 of Lijiang, Jinhue village, Laojunshan Mountain, at the border with Jianchuan Co., 26°38′53″–37°936′N, 99°43′509″–45°992″E, 3510–3900 m, montane forest dominated by Abies and further up by Rhododendron, along trail from parking lot to peak, epiphytic, 16 July 2010, B. Goffinet 10029, with L. Wang, S.L. Guo and S.Y. Huang (KUN—holotypus; CONN, GZU—isotypi); same locality, same date, B. Goffinet 10033, with L. Wang, S.L. Guo and S.Y. Huang (TNS, UPS).

(Figs 3D–F, 4B–I)

Thallus crustose, covering patches as much as 8 cm diam., consisting of discrete areoles (0.15–)0.2–0.6 (–1.5) mm diam., these sometimes confluent forming a rimose thallus; colour white to ashen grey, surficial thallus granules corticate, corticate surface finely pruinose; cortex in esorediate thalli prosoplectenchymatous, 30–55 μm thick; algal layer c. 50 μm thick, grading into medulla that is variably thin to as much as 200 μm thick, to 300 μm thick under apothecia; soredia when present borne in soralia at tips of areoles, rarely areoles dissolving into soredia, internal and external soredia white; soredia roundish, (40–)64–88 (–110) μm diam., sometimes forming consoredia; hypophyllum not observed; photobiont chlorococcoid, cells rounded to irregularly angular, (7–)8.4–11.1 (–17) μm diam.

Apothecia always present, rounded, single or clustered in groups of 2–3 and becoming confluent, (0.7–)1.3–2.6 (–4.1) mm diam., base broadly adnate, disc ± flat to weakly convex, jet black and shiny; margin indistinct, visible from above only in the youngest apothecia, concolorous with the disc; ‘thalline cushion’ present, rarely visible from above and forming a thin white line, in section prosoplectenchymatous, variable in thickness, 25–230 μm thick, typically tawny brown with streaks of darker brown pigment, clearly differentiated from subhymenium above and
medulla below; proper exciple similar in structure to the hymenium, hyphae radiate, similar to paraphyses, when well developed in young apothecia to 170 μm wide laterally, filled with Fucatus-violet granules and often suffused with Cinereorufa-green pigment; differentiated hypothecium absent; subhymenium consisting of a thin layer of ascogenous hyphae, c. 20–50 μm tall, filled like the hymenium with Fucatus-violet granules but sometimes also infused with Cinereorufa-green; hymenium highly variable in thickness even within one and the same apothecium, (80–) 100–300(–350) μm tall, strongly infused with Fucatus-violet granules and collectively forming a deep violet impression in section, but hymenial gel itself hyaline in thin section; epithecium not differentiated, epipsamma lacking; paraphyses mostly simple, arranged vertically and linked to each other in their lower halves by thin bridges, the main beams stouter than the bridges and not readily breaking when squashed in K; paraphysis tips not or scarcely expanded, 4–6 μm wide including gel sheaths, lumina to 1·5 μm wide, paraphyses completely coated on the outside by Fucatus-violet granules; ascii clavate, 85–110 × 25–33 μm when mature, inner and outer walls staining blue, tholus strongly I_{Lugol}+ blue, pierced by a broad, conical non-amyloid structure, thus similar to the Biatora-type; ascospores 2 per ascus, beginning colourless and apparently with a single wall, eventually developing a secondary inner wall, which quickly turns brown while still in the ascus; outer wall thick, to 5 μm in some cases, the inner brown wall thin, often collapsing (spore aborting?), live, healthy ascospores also with brown endospore, (35–)41·7–54·2 (–65) × (15–)20·8–30·8(–35) μm in water.

Pycnidia apparently rare, barely visible externally, in small colourless bumps on the thallus, to 60 μm diam.; wall 10–20 μm thick, pigmented a pale rufous brown or hyaline; conidiophores of Parmelia-type (type VI of Vobis 1980), with zig-zag cells sprouting conidia in upper part of each cell; conidia bacilliform, c. 4–5 × 1 μm.

Chemistry. Atranorin and roccellic/angardianic acid detected by TLC.

Etymology. The species is named in honour of Dr. Wang Li-Song, for his ongoing efforts to describe the lichen diversity in western China.

Habitat and distribution. Found on bark of Rhododendron sp. in China (Hengduan Shan, Yunnan) and on wood of Pinus pumila in the Russian Far East (Bureinskiy Khrebet, north-western Khabarovskiy Krai). Substratum was not recorded for the Indian and Bhutanese material. Collections came from elevations of 3500 to 4000 m in the southern area and c. 1000 m in the northernmost collection. In two of the collections it was associated with Mycoblastus affinis; one of these specimens is included in our phylogeny.

Comments. Violella wangii is a distinct species that seems to be widespread, if rarely collected, in the mountains of high Asia. It occurs in two intergrading morphs, one esorediate with granular, corticate areoles that can become heaped and almost phyllocladioid, and another in which these areoles remain small and erupt in apical soralia, in one specimen even disintegrating completely into soredia in parts of the thallus. The two morphs exhibit no other consistent differences however and several specimens are intermediate. The apparently fluid gradient between esorediate and sorediate morphs recalls the case of Mycoblastus sanguinarius (Tønsberg 1992), in which fully leprous morphs have not been found to be genetically distinct from esorediate morphs (T. Spribille, unpublished data).

Violella wangii differs from the only other species in the genus, V. fucata, in possessing much larger thalli (frequently covering patches 4–8 cm in diam. (rarely >3 cm diam. in V. fucata), robust areoles 0·2–0·6 mm across (to 0·3 mm in V. fucata), external soredia, if present, which remain white (often turning bluish grey in V. fucata), and chemistry (roccellic/angardianic instead of fumarprotocetraric acid). Ascospores average larger in V. wangii than in V. fucata; though based on a limited number of apothecia available and paucity of ascospores, our measurements in V. fucata (38·5 ± 6·7 ×
18.5 ± 3.3 μm, n = 24) fall exactly within the ranges given by Stirton (1879) and James & Watson (2009). The apothecia of V. wangii are larger than anything we have measured in V. fucata but this may not be a reliable character given that apothecia are rare and often poorly developed in V. fucata, a primarily sterile species.

Specimens examined (V. wangii). Bhutan: Tongsa District: Black Mountains NW of Nubji, 27°12′N, 90°22′E, 4040 m elev., Rhododendron thicket with Abies densa at treeline on ridges, on Rhododendron, 2000, G. & S. Miehe 00-13-07/06 (GZU).—India: Darjeeling: Phalut-Dentam, 11 v 1960, Togashi et al. s.n. (TNS). Sikkim: Jongri, elev. 4000 m, 20 v 1960, Togashi et al. s.n. (TNS).—Russia: Khabarovskiy Krai: Chegdomyn-Sofisky road, high pass, watershed between Niman and Umal’ta Rivers, c. 7·1 km S of the bridge over the Niman River, 26 km (air line) SW of Sofisky, 52°05·866′N, 133°42·433′E, Pinus pumila-Rhododendron xerewn woodland under Larix gmelinii, on hard wood of P. pumila, 1016 m, 2009, T. Spriggle 31621 & L. Yakovenken (H).

Vězda (1993) issued an excisate of a specimen from China under the name Mycoblastus fucatus, but as Kantvilas (2009) has pointed out, it is distinct from this taxon. It was collected near the type locality of V. wangii but is distinct from that species in its chemistry (fumarprotocetraric instead of roccelic/angardianic acid, in this respect recalling V. fucata) and thallus morphology (larger, flatter areoles). It is also distinct from the chemically concordant V. fucata in, amongst other characters, developing larger thalli, large, flattened areoles and large apothecia, and apparently lacking soredia. We regard this as probably another species distinct from V. wangii and V. fucata based on thallus chemistry and morphology. However, we were unable to obtain fresh material of this species and hesitate to describe it without getting a better overview of its variability. We have seen three specimens conforming to this morphology and chemistry, all from China.


**Status of Mycoblastus indicus**

A candidate name for our new taxon that required examination was Mycoblastus indicus (Awasthi & Agarwal 1968, as “indicum”), described from Darjeeling district, India, near to where Violella wangii has also been collected. We did not receive a response to repeated requests for type material from Lucknow (LWU), but we did find a specimen of M. indicus at UPS, collected and identified by Awasthi and Agarwal only days before they collected the type specimen. The specimen fits the description provided by Awasthi & Agarwal (1968) and in habit resembles the photograph of the holotype provided by Singh & Sinha (2010), though the latter appears to have more mature apothecia. Mycoblastus indicus is clearly not a member of Mycoblastus or Tephromelataceae. Instead, detailed study of the UPS specimen (Fig. 5) revealed brown ephymenial and hypothecial pigments, a strongly developed proper exciple, mostly simple, loose paraphyses, and asci with a dark apical amyloid cylinder. We obtained an unknown phenolic substance from the thallus, with Rf values similar to confluentic acid in TLC. We concur with Awasthi & Agarwal’s original statement that the species appears similar to the group of tropical species around Lecidea granifera Vain., for which the genus Malmidea has been erected by Kalb et al. (2011). We accordingly combine the species into that genus, where it appears similar to M. coralliformis Kalb. We note that it has larger ascospores than any of the members of the genus discussed by Kalb et al. (2011).

**Malmidea indica** (Awasthi & Agarwal)

**Hafellner & T. Sprib. comb. nov.**


Fig. 5. Malmidea indica (Awasthi & Agarwal 67.224, UPS). A, habit; B, section of apothecium; C, section through hymenium showing paraphyses; D, ascus, squash preparation stained in I$_{L}$, after pretreatment with K; E, ascospore, in I$_{L}$-stained. Scales: A = 2 mm; B = 200 μm; C–E = 10 μm.
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References


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