Gremmeniella balsamea sp. nov.
on balsam fir in Canada

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**Abstract:** Gremmeniella balsamea sp. nov. is described from specimens collected on balsam fir (*Abies balsamea*) in eastern Canada. This is a fungal pathogen specific to balsam fir. Until now, it was considered to be *G. abietina* var. *balsamea*, this variety including also a *Gremmeniella* fungus on spruces. Consequently, this new species causes changes in the taxonomy within the genus *Gremmeniella*.

**Key words:** *Gremmeniella*, *Abies balsamea*, cankers.
Introduction: The genus *Gremmeniella* was proposed by Morelet (1969) and is now widely accepted. The type species, *Gremmeniella abietina* (Lagerb.) Morelet, is found mainly on stems and shoots of pines. A taxonomic re-evaluation of *Gremmeniella* by Petrini et al. (1986) recognized *G. abietina var. balsamea* on balsam fir (*Abies balsamea* (L.) Mill.) and spruces (*Picea* spp.) in the North American boreal forest as being distinct from var. *abietina*. However, Petrini et al. (1989) decided not to create new species for these fungi on balsam fir and spruces because they lacked well-defined morphological differences.

Since that reappraisal, it is now known that *Gremmeniella* found on balsam fir is a pathogen specific to that host (Laflamme et al. 1996) and this has been confirmed by completing Koch's postulates (Smerlis and Laflamme, 2011). This fungus causes cankers on stems and branches of seedlings and saplings. It is relatively common in the boreal forest and was first collected in the Réserve faunique des Laurentides, north of Quebec City, Canada, by the second author in 1956.

Our objectives are to underline morphological differences between *Gremmeniella* species found on balsam fir, spruces and pines, and to give a detail description of the proposed new *Gremmeniella* species on balsam fir. We are providing the GenBank accession numbers of two sequences of this new proposed species that we obtained from a molecular study in progress in our laboratory.

Materials and Methods: For morphological measurements, thirty four fungal specimens representing *G. abietina* from pines, and *Gremmeniella* sp. from spruce were obtained from the René Pomerleau Herbarium (QFB) of the Laurentian Forestry Centre, the Forest Research Station, Wageningen, The Netherlands, the ETH, Zurich, Switzerland, and the Norwegian Forest Research Institute, Aas, Norway. All the specimens from the René Pomerleau Herbarium (QFB) are listed here in numerical order; these numbers are followed by the host species, the locality, the county (Co), the date of collection and the collector. Samples from other herbarium are at the end of the list:

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Measurements of fungal specimens were performed with an image analysis system (Software from Bioquant Image Analysis Corporation, Nashville, TN, USA) connected to a research microscope (Polyvar, Reichert-Jung, Wien, Austria) at 400 x for ascospores and conidia. Length and width of thirty ascospores and/or conidia were measured for each specimen. Minimum and maximum lengths and widths for each element of all specimens were used to characterize these fungi.

For comparison of G. abietina var. balsamea on balsam fir in cultures with two other Gremmeniella species, we used the isolates CF-79-0620 on balsam fir, CF-85-0168 of G. abietina from Pinus resinosa Ait., and CF-82-419 of Gremmeniella sp from Picea mariana (Mill.) B.S.P. Isolates of G. abietina var. balsamea from balsam fir used for sequence analysis are CF-79-0620, CF-87-0061 and CF-87-0063. These cultures are deposited in the collection and stored in liquid nitrogen at the Laurentian Forestry Centre.

The DNA extraction protocol used was according to Zolan and Pukkila (1986), Gardes and Bruns (1993), and Sokolski et al. (2006) with some modifications. A sample of approximately 0.5-1 cm² plug of mycelium of each fungal culture was transferred into a sterile 1.5 mL Eppendorf tube and stored at -20°C until processing. The tissue was disrupted by homogenization with a micropestle in 600 µL of extraction buffer as described by Gardes and Bruns (1993) (2% CTAB, 100 mM Tris [pH 8], 20 mM EDTA, 1.4 M NaCl, and 0.2% beta-mercaptoethanol) and incubated for 1 h at 65°C. To extract the DNA, 600 µL of phenol-chloroform-isoamyl alcohol (25:24:1) was added and the tube was mixed by inversion for 2 min. The emulsion was centrifuged at room temperature at 13,000 rpm (18327 g) for 10 min. The upper aqueous phase was transferred to a clean tube and the genomic DNA was precipitated with 600 µL of ice cold isopropanol. The tubes were incubated at -20°C for 30 min and centrifuged at 10,000 rpm (10844 g) for 10 min at 4°C. The pellet was rinsed twice with 300 µL of ice cold 70% ethanol followed each time by a centrifugation at 10,000 rpm for 10 min, dried, dissolved in 50 µL of storage buffer TE [pH 8.0], and stored at -20°C.

The ITS region of rDNA was amplified using the universal primer ITS4 (White et al. 1990) and the universal fungal primer ITS1F (Gardes and Bruns 1993). The amplification was done according to Sokolski et al. (2006) with minor modifications. Briefly, 1 µL of DNA diluted 100-fold was added to the PCR mixture containing 1 X PCR buffer.
supplied with the enzyme (20 mM Tris-HCl [pH 8.4], 50 mM KCl, and 3 mM MgCl₂), 0.4 mM each of dATP, dCTP, dGTP and dTTP, 0.125 µM of each PCR primer, 1 U of recombinant Taq DNA Polymerase (Invitrogen Life Technologies, Grand Island, NY, USA), and molecular biology-grade water to a final volume of 25 µL. The amplification was performed on a thermal cycler (PTC 200, MJ Research, Waltham, MA, USA) programmed with an initial denaturation at 94°C for 3 min followed by 40 cycles at 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min and 30 s. The amplification was terminated by strand completion at 72°C for 10 min. The PCR products were analyzed on a 1.5% agarose gel in 1X TAE buffer (Invitrogen, 24710030). The gels were stained 10 min in an aqueous solution of 1% ethidium bromide and photographed under UV light using the GeneSnap Gel/Blot Imaging Software from Syngene, Frederick, MD, USA. Sequencing of the PCR fragments was done by the Sequencing and Genotyping Platform of the CHUL (CHUQ) Research Centre, Québec City, Canada, where the samples were analyzed by capillary electrophoresis using the ABI 3730/XL sequencing apparatus (Applied Biosystems). The sequence analyses were performed with BioEdit version 7.0.5.2, designed by Hall (1999). The sequences were aligned with Clustal W (Thompson et al. 1994) and compared with those in GenBank (NCBI) by BLAST.

**Results:** The ascospores of *G. balsamea* measured 15.4 – 40.6 x 3.5 – 4.9 µm and conidia 24.5 – 56.0 x 2.1 – 3.5 µm (Table 1). In specimens of *G. abietina* collected on pine in Québec, the ascospores measurements are 10.5 – 27.3 x 3.5 – 6.3 µm and conidia measures 11.2 – 49.0 x 2.8 – 3.5 µm (Table 1). In specimens of *Gremmeniella* sp. collected on spruce in Québec, ascospores measures 14 – 26.1 x 3 – 5 µm and conidia 24 – 48 x 1.8 – 4 µm (Table 1). Illustrations of ascus, ascospores, secondary spores and conidia are presented (Figure 1-4) as well as apothecia (Figure 5). *G. balsamea* is well distributed in the boreal forest (Figure 6).

Cultures of all three species, *G. balsamea, G. abietina* and *Gremmeniella* sp. on spruces, are slow growing, reaching 22 to 28 mm on 3% malt agar at 15°C in 4 weeks and the underside is dark brown or black. Culture color of *G. balsamea* is yellow, yellowish green or yellowish brown (Figure 7), while culture color of *G. abietina* isolates from pine are dark green and of *Gremmeniella* sp. isolates from spruce are bright yellow green.

ITS sequences were obtained from two isolates (CF-87-0063 and CF-79-0620) of *G. balsamea* and were deposited in GenBank to be accessible to all (Table 2). These sequences are 100% identical to those already present of *Gremmeniella* fungi on *A. balsamea* in GenBank and obtained from one isolate (CF-87-0061) and four herbarium specimens. The specimens from the René Pomerleau Herbarium (QFB) at the Laurentian Forestry Centre are QFB 15131, QFB 17317, QFB 17333 and QFB 19956 (Table 2).

All the specimens listed under the descriptions of the new fungal species are deposited in the René Pomerleau Herbarium (QFB) at the Laurentian Forestry Centre, Québec City, Canada.


*In caulibus et ramis Abies balsamea. Apothecia erumpentia, solitaria vel subcaespitosa, brevistipitata, cupulata, fusco-brunnea, coriacea, margine involuto, glabra vel tenuiter squamosa, rotunda, oblonga vel triangula, 300 – 1 500 µm dia., 350 – 1000 µm latit.; stipes 160 – 300 µm latit. et 160 – 280 µm crassis; excipulum 50 – 240 µm crassum ad basim et 25 – 120 µm ad apicem; stipes et excipulum medulosum textura angularis, e cellulis fusco-brunneis et crasse
Table 1: Comparison of ascospores and conidia (length and width in µm) measured by the authors and reported in literature for the following three fungal species: *Gremmeniella balsamea* on balsam fir, *Gremmeniella* sp. on spruces and *G. abietina* on pines.

<table>
<thead>
<tr>
<th>Fungal species and references</th>
<th>Ascospores</th>
<th>Conidia</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gremmeniella balsamea</em></td>
<td>15.4–40.6 x 3.5–4.9</td>
<td>24.5–56 x 2.1–3.5</td>
</tr>
<tr>
<td><em>Gremmeniella</em> sp. on spruces</td>
<td>14–26.1 x 3–5</td>
<td>24–48 x 1.8–4</td>
</tr>
<tr>
<td>Laflamme 1988</td>
<td>11.2–24.3 x 2–4.8</td>
<td>15–56 x 1.8–3.7</td>
</tr>
<tr>
<td><em>Gremmeniella abietina</em></td>
<td>10.5–27.3 x 3.5–6.3</td>
<td>11.2–49.0 x 2.8–3.5</td>
</tr>
<tr>
<td>Morelet 1980*</td>
<td>12–24 x 3–6</td>
<td>19–55 x 2.5–4</td>
</tr>
<tr>
<td>Ettlinger 1945</td>
<td>10.5–25.8 x 3.6–6.5</td>
<td>12.3–63.3 x 2.4–4.0</td>
</tr>
<tr>
<td>Van Vlotten and Gremmen 1953</td>
<td>15.2–16.1 x 3.8–4.7</td>
<td>26.0–47.7 x –</td>
</tr>
<tr>
<td>Roll-Hansen 1964</td>
<td>12–23 x 3.5–5.0</td>
<td>30–43 x 2.7–3.6</td>
</tr>
<tr>
<td>Schläpfer-Bernhard 1969</td>
<td>14–20 x 3.5–5.0</td>
<td>24–50 x 2.5–3</td>
</tr>
</tbody>
</table>

* The description of the type species *Gremmeniella abietina* by Morelet (1969) does not include any measurement of the fungus elements.

Table 2: Information including GenBank accession numbers obtained from *Gremmeniella balsamea* isolates, or directly from herbarium material (QFB) collected on *Abies balsamea*. Numbers between brackets are included as references only.

<table>
<thead>
<tr>
<th>Isolate Number</th>
<th>QFB Number</th>
<th>Locality</th>
<th>Collector</th>
<th>Date</th>
<th>GenBank Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF-79-0620</td>
<td>(17321)</td>
<td>Secteur Launière</td>
<td>A. Carpentier</td>
<td>31-07-1979</td>
<td>JN896311</td>
</tr>
<tr>
<td>CF-87-0063</td>
<td>(19957)</td>
<td>Secteur Jumeau</td>
<td>G. Laflamme et al.</td>
<td>30-06-1987</td>
<td>JN896310</td>
</tr>
<tr>
<td>(CF-87-0063)</td>
<td>19957</td>
<td>Secteur Jumeau</td>
<td>G. Laflamme et al.</td>
<td>30-06-1987</td>
<td>JN131825</td>
</tr>
<tr>
<td>-</td>
<td>17317</td>
<td>Secteur Jumeau</td>
<td>R. Paquet</td>
<td>04-07-1979</td>
<td>JN131823</td>
</tr>
<tr>
<td>-</td>
<td>17333</td>
<td>Lac Ball</td>
<td>A. Carpentier</td>
<td>12-06-1979</td>
<td>JN131824</td>
</tr>
<tr>
<td>-</td>
<td>15131</td>
<td>Secteur Jumeau</td>
<td>G. Laflamme et al.</td>
<td>30-06-1987</td>
<td>JN131822</td>
</tr>
<tr>
<td>CF-87-0061</td>
<td>(19956)</td>
<td>Secteur Launière</td>
<td>G. Laflamme et al.</td>
<td>30-06-1987</td>
<td>GAU72259</td>
</tr>
</tbody>
</table>

*tunicatis; excipulum ectalum e textura angularis ad basim et textura oblita ad apicem; discus concavus, flavidus; hypothecium 30 – 50 µm crassum, hyalinum et textura intricata, e cellulis elongatis et implicatis; asci octospori, cylindracei, brevi-stipitati, tenuiter tunicati, rotundatis ad apicem, poro in iodo non coerulescente, 75 – 140 x 7 – 9 µm; ascosporae biseriatae, hyalinae, non septatae vel 1-5-septatae, fusiformae, ellipsoideae vel fere cylindraceae, rectae, curvatae vel interdum ramosae, ad apicem leviter incrassatis, epithecium formantus.


On stems and shoots of Abies balsamea. apothecia innate-eruptent, single or cespitose, short-stipitate, cup-shaped, dark brown, coriaceous, inrolled and brittle when dry, smooth or somewhat scabrous, circular, ellipsoid or triangular, 300 – 1 500 µm in dia., 350 – 1 000 µm in height; stipe 160 – 300 µm long, 160 – 280 µm wide; excipulum 50 – 240 µm thick at the base, tapering to 15 – 30 µm in the middle part and flaring to 25 – 120 µm at the top; stipe and medullary excipulum of textura angularis, composed of thick-walled, isodiametric or somewhat elongated brownish cells 3 – 16 µm in dia.; lower part of the ectal excipulum consists of dark brown, parallel hyphae 2 – 5 µm in dia. which are gradually transformed into textura oblita consisting of dark brown, parallel hyphae 2 – 5 µm in dia. in the upper part; disc concave, pale yellowish; hypothecium 30 – 50 µm thick, of textura intricata, composed of hyaline, interwoven hyphae; asci 8-spored, thin-walled, cylindrical, short-stipitate, obtuse at the apex, the pore not staining blue in iodine, 75 – 140 x 7 – 9 µm; ascospores hyaline, aseptate to 5-septate, fusiform, narrowly ellipsoid or nearly cylindrical, straight, curved or sigmoid, smooth or slightly constricted at the septa, granular and pluriguttulated, irregularly biseriate, 15.4 – 40.6 x 3.5 – 4.9 µm; secondary spores, formed after the discharge of ascospores on minute phialides of each cell, hyaline, aseptate, ellipsoid, oblong or globular, 2.0 – 4.9 x 1.4 – 2.1 µm; paraphyses filiformes, septatae, simplices vel interdum ramosae, ad apicem leviter incrassatis, epithecium formantus.
simple or compound with 2 to 3 phialides, 10 – 30 x 2 – 3 µm, lining the base and sides of the cavity. The anamorph is a Branchorostia type.

Cultures of G. balsamea are slow growing, reaching 23 to 26 mm on 3% malt agar at 15°C in 4 weeks. Advancing zone white, margin even or irregular, raised. Mat white, tuft-like, 3 to 4 mm in height during the first week, becoming yellow, yellowish green or yellowish brown and felt-like, except for a raised centre in older cultures (Figure 7). Reverse dark brown or black. Aerial hyphae 1.4 to 4.2 µm in dia., relatively straight and infrequently branched, thin-walled, hyaline or golden yellow with hyaline or brown oil globules and frequently incrusted. Submerged mycelium extensive, hyphae 1.4 to 6.3 µm in diameter, thin-walled, hyaline or golden yellow and containing large oil globules. Medium and fine hyphae characterized by a) terminal or intercalary hyaline to golden brown swellings up to 25 µm in dia. and b) by terminal, lateral or intercalary dark segments smaller in diameter than the supporting or adjacent cells.

Other specimens examined, all on Abies balsamea located in eastern Canada (Figure 6), are listed here in the numerical order used in the René Pomerleau Herbarium (QFB) of the Laurentian Forestry Centre; these numbers are followed by the locality, the county (Co), the date of collection and the collector.

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Discussion: It is possible to differentiate the following three species, Gremmeniella balsamea, G. abietina and Gremmeniella sp. on spruces on the basis of their color in culture. Cultures of G. balsamea are yellowish green to yellowish brown (Figure 7), while cultures of G. abietina on pine and Gremmeniella sp. on spruce are respectively dark green and bright yellow green. G. balsamea has also longer ascospores than the two other species reaching 40 μm compared to 20 – 27 μm for the two other species measured by the authors, as well as five different authors (Table 1). On the Figure 4 in Petrini et al. (1989), the graph shows clearly longer ascospore measurements on balsam fir than the ones from pines and spruces. However there is some overlaps between the ascospore longer length of G. abietina and the ascospore shorter length of G. balsamea. The shapes of G. balsamea’s ascospores are slightly different than the ones of G. abietina. The ascospores of G. balsamea are generally fusiform, occasionally narrowly ellipsoid or nearly cylindrical as compared with the ascospores of G. abietina which are ellipsoid (Ettlinger 1945, van Vloten and Gremmen 1954) or ellipsoid to slightly clavate (Schläpfer-Bernhard 1969). However, the secondary spores formed by the ascospores of G. balsamea are indistinguishable from those of G. abietina from pines and Gremmeniella from spruces collected in Québec. They all are globose, oblong to nearly cylindrical, 2.1 – 4.2 x 2.1 – 2.8 μm in size. These secondary spores are formed after the discharge of ascospores, or, in older apothecia, after the release from ruptured asci. Secondary spore formation by G. abietina was reported earlier by Petrini et al. (1989).

The length of conidia cannot differentiate the three species. While Figure 5 in Petrini et al. (1989) shows slightly longer conidia for some specimens of G. balsamea, conidia measurements are indistinguishable in size from those of G. abietina and Gremmeniella sp. from spruce (Table 1). Nevertheless, there is a slight difference in conidial shape between G. balsamea and G. abietina. Typical conidia of G. balsamea are acicular or narrowly cylindrical and occasionally narrowly fusiform. The conidia of G.
abietina have been described as fusoid (Ettlinger 1945, van Vloten and Gremmen 1954, Schläpfer-Bernhard 1969).

Finally, G. balsamea is specific to its host, A. balsamea. This has been demonstrated by two pathogenicity tests performed in Canada, where the respective authors inoculated isolates from Picea spp., Pinus spp. and Abies, which confirmed both host preference (Laflamme et al. 1996) and host specificity (Smerlis and Laflamme 2011) for each sample of these three groups of isolates to their respective hosts.

The preliminary work on the ITS sequences provided in this paper strongly support our morphological and microscopic data. Further investigations are in progress with more Gremmeniella species.

In conclusion, in light of this information, the var. balsamea should no longer exist. Gremmeniella found on balsam fir is distinct from the type species G. abietina, and thus the new species Gremmeniella balsamea on A. balsamea is introduced. Another distinct Gremmeniella species on spruces in eastern Canada remains yet to be described, and a further revision of the genus Gremmeniella should be undertaken.

Acknowledgement: This description was done mainly by the second author before his retirement from the Canadian Forest Service in 1987. This information was brought to the attention of the first author after Smerlis’s death in 2003. The authors are grateful to J. Gremmen, Forest Research Station, Wageningen, the Netherlands, from L. Ettlinger, ETH, Zurich, Switzerland and the late F. Roll-Hansen, Norwegian Forest Research Institute, Aas, Norway, for lending herbarium specimens. Assistance of the late Marcel Saint-Laurent, Laurentian Forestry Centre, for numerous specimen collections. Thanks to Jacques Morissette from the Laurentian Forestry Centre for the preparation of the distribution map and to Gilles Bélanger for his technical assistance. We appreciated very much the collaboration of Dr. Pierre DesRochers, curator of the René Pomerleau Herbarium, for the numerous specimens borrowed from that collection and for his comments on the manuscript. Thanks to Dr. S.A. Redhead, Eastern Cereal and Oilseed Research Centre, Ottawa, Ontario for his advice on nomenclatural rules. Finally, special thanks to Chantal Côté, biologist at the Laurentian Forestry Centre, for all the molecular work, from the DNA extraction to the interpretation of sequences. Thanks to the anonymous linguist for the Latin description.

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Pathology 18:330-334. http://dx.doi.org/10.1080/07060669609500584


Figures 1-4: Gremmeniella balsamea. Fig. 1. Ascus and paraphyses. Fig. 2. Ascospores. Fig. 3. Ascospore with secondary spores. Fig. 4. Conidia.

Figure 5: Apothecia of Gremmeniella balsamea on a twig of Abies balsamea.
Figure 6: Known distribution of *Gremmeniella balsamea* in Canada (Map by J. Morissette).
Figure 7: Culture of Gremmeniella balsamea, isolate 79-620, on PDA medium after 6 weeks of growth.