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Taxonomy of *Calicium victorianum* (F. Wilson) Tibell (*Caliciaceae*, *Lecanorales*), a lichenized ascomycete new to Europe

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Abstract: The morphological features and chemical compounds found in the first European collection of *Calicium victorianum* are compared with type material from Australia of *C. piperatum* F. Wilson. The phylogenetic relationships of the species are discussed by comparing its nuclear rDNA ITS1-5.8S-ITS2 with that of other species of *Calicium*.

Introduction

The genus *Calicium* Pers. was one of the first two calicioid lichen genera (or 'pin lichens') to be described late in the Eighteenth century, some 30 years before it was placed by Fée in the family *Caliciaceae* (Tibell 2003). The family and other taxa of pin lichens (up to 28 genera, see Poelt 1974) were later included in the order *Caliciales*. Recent molecular phylogenies of these taxa (Wedin & Tibell 1997) strongly support morphological observations by Tibell (1984) that the group was most likely polyphyletic, and the *Caliciaceae* have been since grouped with the *Physciaceae* in the order *Lecanorales* (Wedin *et al*. 2000; Tibell 2006), although the delimitation of these families is still uncertain (Helms *et al*., 2003). Thus, various species that once were described as *Calicium* have now been transferred to other genera, with the total number of accepted species in the genus currently at around 40. *Calicium* was confined by Tibell (1984) to pin lichens with a verrucose to granular thallus (sometimes endosubstratal), associated with *Trebouxia*, stalked (rarely sessile) ascomata, well-developed mazaedia, and thick-walled, dark brown, one-septate, mostly ornamented ascospores.These characters are also shared by members of the genus *Cyphelium* as presently circumscribed, and since *Calicium* and *Cyphelium* are paraphyletic with respect to each other and *Tholurna* (Tibell 2003), new generic circumscriptions are needed. Typical *Cyphelium* species have immersed ascomata with yellowish thalli, and some of the species have submuriform ascospores. However, the differences between these genera are rarely clearly stated, and, indeed, keys to calicioid genera misleadingly, but almost universally, use 'sessile' as opposed to 'stipitate' ascomata as a fundamental dichotomy. In terms of chemistry, β-orcinol depsides and depsidones and pulvinates variably occur in the species of *Cyphelium* as the main secondary metabolites. *Calicium* species, on the other hand, accumulate xanthones and anthraquinones in addition to these substances (Tibell 1984; Tibell 2003).

Calicium victorianum was first described from Australia in the genus *Trachylia* by Wilson (1889) for a species with sessile to only shortly stipitate ascomata occurring on wood of *Eucalyptus*. Owing presumably to the virtually sessile habit of the ascomata, Wilson found it difficult to decide whether the taxon was a *Trachylia* (i.e. *Cyphelium* as currently circumscribed) or a *Calicium*. Two

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years later, Wilson (1891) described several new species of *Calicium* from the same area of Victoria, Australia, and provided a Latin diagnosis for *T. victoriana*. When Tibell (1987) re-examined the types of these species for a monograph on Australasian calicioid fungi, he concluded that *T. victoriana* should be transferred to the genus *Calicium* and that *Calicium piperatum* F. Wilson (Wilson 1891: 365) with virtually sessile ascomata, also described from Australia, was conspecific. The species had previously been reported from New Zealand (Tibell, in Galloway 1985 as *C. piperatum*), where it occurs on the hard wood of *Nothofagus* and is considered to be rare. It has been considered endemic to Australasia (Tibell 1987), and is here reported from the Northern Hemisphere for the first time. The results of chemical and DNA sequence analyses of this material are also presented in this paper.

Material and Methods

Material

Collections deposited in the mycological herbaria of the Royal Botanic Gardens, Kew (K) and the Natural History Museum, London (BM), were studied morphologically and chemically. DNA analysis was performed only on the recently collected material deposited in K.

Specimens studied in addition to type material of Calicium piperatum (*i.e.* C. victorianum). *Calicium glaucellum* Ach.: **British Isles:** *England*: **V.C.3**, South Devon, Bovey Valley, Foxworthy, on bare wood, 13 December 1975, *F. Rose* [K(M) 134523].

Calicium victorianum (F. Wilson) Tibell: **British Isles:** *England*: **V.C. 13**, West Sussex, Wisborough Green, Sparr Rough, on a fence post (probably *Quercus robur*), 18 April 1999, *B. M. Spooner* [K(M) 60853 as *Cyphelium sessile*]; *ibid.*, with *Lecanora conizaeoides* Nyl. ex. Cromb., 6 April 2004, *B. M. Spooner & M. B. Aguirre-Hudson* [K(M) 122465 as *Calicium* sp.]; *ibid.*, 25 July 2004, *B. M. Spooner & R. D. Roberts* [K(M) 124262 as *Calicium* sp.].—**Australia:** *Victoria*: Kilmore, on fence, 1890, *F. R. W. Wilson* 104 [BM 730862 as *Calicium piperatum*]. *Tasmania*: Ben Lomond National Park, 2·6 km S of Upper Blessington, along Ben Lomond Road, 41°30′S, 147°34′E, 750 m, on decorticated stump of *Eucalyptus* in open *Eucalyptus* forest, 1981, *L. Tibell* 11467 [L. Tibell, *Caliciales Exsiccate* no. 104 *Calicium piperatum* ssp. *piperatum*] (BM 730860).

Light microscopy

Morphological studies were carried out using a Leica DM LB2 and MZ 16 compound and stereomicroscopes, respectively, supplemented by a DFC digital camera. Measurements were taken using the same camera and associated computer software QWin; all ascospore measurements were made at × 100 magnification on slides stained with lactic acid plus Cotton blue.

HPLC-DAD-ESI-MS analyses

Analysis was performed using a Waters Alliance HPLC system (2695) connected to a Waters photodiode array detector (DAD, 2996) and an orthogonal quadrupole mass spectrometer (Micromass ZQ) in tandem, both controlled by MassLynx software (V 4.0, Micromass, Manchester, UK). An ESCi multiprobe was used but the atmospheric pressure chemical ionization mode was turned off during the analyses. An Agilent Technologies Zorbax SB-C_{18} column (3·0 × 150 mm i.d., *dp* 5 µm) with a guard column was used at 30°C. The elution was with a methanol-water gradient (containing 2 per cent acetic acid throughout) at 0·5 ml min^{-1}, programmed as follows: initial condition 50 per cent methanol, changing to 100 per cent in 20 min, and then holding for 15 min, before returning to the initial condition at 37 min. The UV detector was set to scan from 200 to 600 nm, at a resolution of 3·6 nm, every 1 sec. For ESI-MS detection the conditions were set as follows: vaporizer at 450°C; nitrogen flow rate (desolvation), 500 l hr^{-1}; source temperature, 120°C; capillary voltage, 3·0 kV; cone voltage, 25·0 V; Rf lens voltage, 0·2 V. The acquisition times were set to 0·4 sec for both positive and negative ionization modes scanning over the m/z ranges of 150–2000 (positive) and 120–2000 (negative), and the change-over time of 0·2 sec was allowed between the modes.

Samples. Four ascomata from each accession, excised from their substratum under a dissecting microscope, were used for the analysis. The excised ascomata were extracted with 20 µl of acetone within a microsyringe for 30 min at room temperature. The whole contents were injected altogether via an external Rheodyne valve (time 0) and the gradient elution commenced. The deposit on the guard column was removed after the end of each run, and the system washed and equilibrated thoroughly prior to the next injection.

Standards. The standard reference compound physodalic acid was isolated from *Hypogymnia physodes* (L.) Nyl. (*Parmeliaceae*) (deposited at herbarium K(M) 108739) and identified using spectroscopic methods (UV, mass spectrometry, and 1- and 2-dimensional nuclear magnetic resonance spectroscopy) and comparison of physical properties with published data (Huneck & Yoshimura 1996). Physodalic acid was dissolved in acetone and analysed in the same manner as described above for the lichen extracts. The maximum variation in the elution time between injections was 0·13 min.

DNA sequence and phylogenetic analysis

Isolation, PCR-amplification of the nuclear rDNA ITS1-5.8S-ITS2, sequencing and phylogenetic analyses were carried out in accordance with the methods indicated in Tibell (2003).

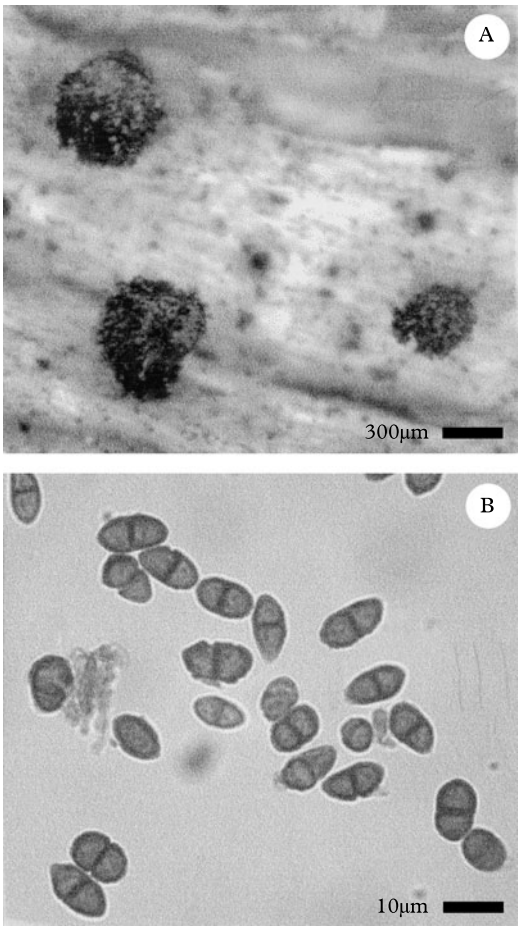

FIG. 1. *Calicium piperatum* (isolectotype BM 730861). A, general view of the ascomata; B, ascospores.

Results

Calicium victorianum (F. Wilson) Tibell

Symb. Bot. Upsal. **27**(1)**:** 59–65 (1987); *Trachylia victoriana* F. Wilson, *Victorian Naturalist* **6:** 67 (1889); *Cyphelium victorianum* (F. Wilson) Zahlbr. *Cat. Lichen. Univ.* **1:** 676 (1922).

Calicium piperatum F. Wilson, *J. Linn. Soc. Bot.* **28:** 365 (1891); type: Australia, Mt. Macedon, ad lignum *Eucalypti*, *F. R. M. Wilson* 45 (BM 730861—isolectotype!).

Calicium obconicum Müll. Arg., *Bull. de l'Herb. Boiss.* **4:** 87 (1896).

(Figs 1 & 2)

Thallus inconspicuous, endoxylic and rather thin, *c.* 200 µm thick; fungal hyphae associated with chlorococcoid algae, 7–10 µm diam.

Ascomata apothecial, short stalked, not more than 100 µm high, appearing sessile, bell-shaped, (195–)235–400(–500) µm diam. (see Fig. 2A & B); *exciple* carbonized, dark brown to black, 45–65 µm wide, consisting of isodiametric cells tightly compressed, 4–5 µm diam., with carbonized lumens and forming a prosoplectenchyma; with a distinct non-pruinose dark brown mazaedium, which sometimes appears greyish owing to the presence of colourless hyphae amongst the ascospores. *Hypothecium* dark brown, becoming carbonized and up to 120 µm high at its widest point. *Hymenium* consisting of very thin (5·5 µm in width), unitunicate and cylindrical 8-spored asci (Fig. 2C), that become evanescent as the ascospores mature. *Paraphyses* observed only in sections, sparse, colourless, simple, and not capitate, *c.* 1 µm in width. *Ascospores* uniseriately arranged in the ascus, broadly ellipsoidal, pale brown and aseptate within the ascus but becoming darker brown, one-septate (only slightly constricted at the septum) and ornamented with fine, irregular verrucae at maturity (see Fig. 2D), (9–)11–13 × 4–6 µm.

Chemistry. Physodalic acid was detected in *C. victorianum* K(M)124262 as the single major component as well as in the type of *C. piperatum* [*Wilson* 45 BM 730861], using HPLC, but not as a spot test with PD. It was also found in *C. glaucellum* [K(M)134523] and in *C. piperatum* [*Wilson* 104, BM730862]. Xanthones (arthothelin and thiophanic acid), however, were not detected in any of the specimens.

Total DNA was isolated from three ascomata (*Spooner & Aguirre-Hudson* [K(M) 122465]), and a sequence of the nuclear rDNA ITS1-5.8S-ITS2 was obtained (GenBank EU010389).

Ecology and distribution. In Britain *Calicium victorianum* is currently known from a single locality in West Sussex, the site being

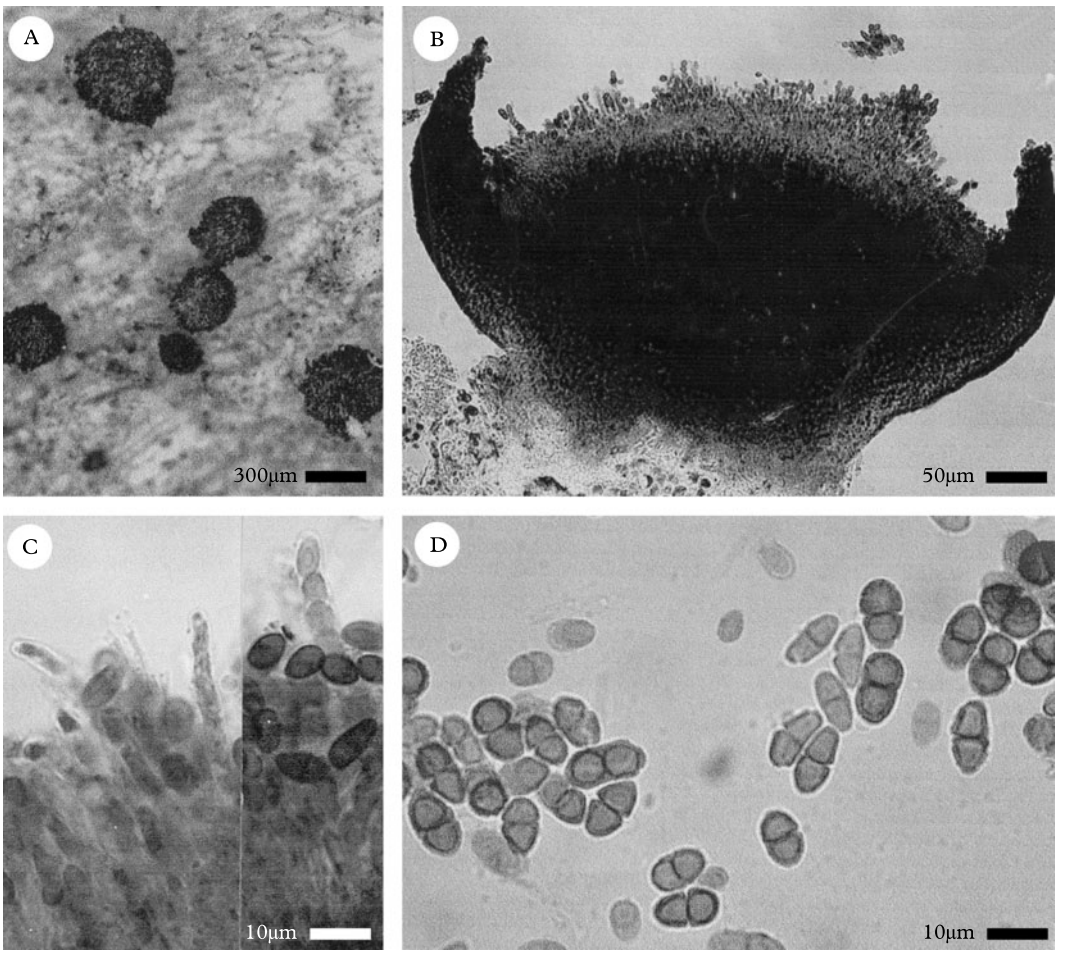

FIG. 2. *Calicium victorianum*. A, general view of the ascomata [K(M)124262]; B, vertical section through ascoma [K(M)122465]; C, immature asci and free ascospores in section [K(M)122465]; D, ascospores showing ornamentation in a squash mount [K(M)124262].

a privately owned ancient oak woodland. The fungus grows on the north face of vertical oak fence posts around a small compound in cleared woodland, positioned mostly in full sun. It is growing abundantly on three posts and occurs with *Chaenotheca ferruginea* (Turner ex Sm.) Mig., *Lecanora conizaeoides* and *Mollisia melaleuca* (Fr.) Sacc. Elsewhere, it is known from Australia on fence posts and stumps of *Eucalyptus* trees and from New Zealand on the hard wood of *Nothofagus*.

Discussion

Morphology

The subsessile to virtually sessile ascomata of *Calicium victorianum* distinguish the taxon from most other species of the genus. However, other sessile or subsessile taxa are known, notably *Calicium diploellum* Nyl. in the British Isles. This species, however, has smaller ascomata and ascospores than *C. victorianum*, and presents a yellow pruina when young. Ecologically *C. diploellum*

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appears to have an oceanic distribution as it is restricted to north-west Scotland and western Ireland, and is found on the bark of old *Ilex* trees (Purvis *et al.* 1992). Tibell (1991) suggested that it might be an ecological variant of *Calicium adspersum* Pers., also with yellow pruina, although with larger ascospores (for ascoma differences see also Tibell 1999). It was, however, accepted as occurring in Africa (Tibell 2001). Other sessile or subsessile taxa described in *Calicium* are little known and require further evaluation. These include the sessile *Calicium acaule* Eitner, *C. pusiolum* f. *sessile* Arnold, and *C. adspersum* f. *sessile* (Fr.) Nádv., and the short-stalked taxa *C. abietinum* var. *brevicaule* (Arnold) Zahlbr., *C. abietinum* var. *brachypodium* (de Lesd.) Zahlbr., *C. adspersum* var. *brevicaule* Szatala, *C. corynellum* var. *subsessile* (Vain.) Zahlbr., and *C. lenticulare* var. *brachypus* (Jatta) Zahlbr. These species have received no modern revision so their true position is unknown. The evident disjunct distribution of *C. victorianum* cannot be assessed until the positions of these species have been resolved.

Tibell (1987) recognized two subspecies in *Calicium victorianum*: *C. victorianum* subsp. *victorianum*, and *C. victorianum* subsp. *desidiosum* Tibell. These taxa were separated on the shape of the ascomata, with *C. victorianum* subsp. *victorianum* sometimes shortly stipitate, and on the size and ornamentation of the ascospores. The latter are larger (13·4–16·3 × 6·5–8·2 μm) in *C. victorianum* subsp. *desidiosum* and bear minute, mostly isolated verruculae. On the basis of these characters, the British material reported here belongs to *C. victorianum* subsp. *victorianum*.

Chemistry

Galloway (1985) noted that no secondary metabolites of *C. victorianum* (as *C. piperatum*) had been identified. However, the HPLC analysis of the acetone extract revealed the presence of one major compound in *C. victorianum* eluting at 11·5 min. The retention time, UV absorption spectrum, and the mass spectra both in positive and negative modes matched those of physodalic acid. The same compound was also detected in *C. glaucellum* K(M) 134523, collected in England, and in *C. piperatum* (*Wilson* 45 and 104) collected in Australia, with the aid of mass chromatograms and time-averaged UV scans. This compound is new to the family *Caliciaceae*. The production of β-orcinol depsidones (and the cortical depside atranorin) is considered to be rare in the *Caliciaceae*, but a more common feature in the *Physciaceae* (Wedin *et al.* 2000).

In addition, three further metabolites were found in *Calicium glaucellum* [K(M) 134523] by using mass chromatograms. Their apparent molecular weights (retention times) are 418 (16·8 min), 432 (15·2 min) and 344 (17·7 min), which correspond to two *m*-depsides sekikaic acid and 2-*O*-methylsekikaic acid, and a depsidone 4-*O*-methylhypoprotocetraric acid, respectively. All have also been reported to occur in Swedish and Japanese material of the species (Tibell 1998; Tibell & Thor 2003). These compounds were, however, notably absent in the British material of *C. victorianum* and the two accessions of *C. piperatum*.

The secondary chemistry of the two accessions of *C. piperatum*, however, were not identical, as *Wilson* 104 [BM 730862] contained, in addition to physodalic acid, an unidentified substance eluting at 11·2 min. Its UV spectrum suggested a β-orcinol depside or depsidone; however, the apparent molecular weight of 400 indicates this is yet another lichen substance new to the family.

The more characteristic lichen substances in the *Caliciaceae*, such as pulvinic acid derivatives and anthraquinones, could not be detected with certainty in this study. Despite this, the present finding is in agreement with the more frequent occurrence of depsidones in *Calicium* than any other genera of the family (Tibell 1984; Tibell & Kalb 1992; Tibell & Thor 2003). Outside *Calicium* only *Cyphelium brunneum* W. Weber, *C. lecideinum* (Nyl.) Trevis., *Thelomma mammosum* (Hepp in Hartung) A. Massal. and *T. californicum* (Tuck.) Tibell are known to produce β-orcinol depsidones variably (Tibell 1984).

DNA

A comparison of the ITS-sequence of *Calicium victorianum* with 65 additional sequences representing 29 species in *Caliciaceae*, plus *Diplotomma alboatrum* (Hoffm.) Flot. as outgroup was carried out but is not shown here. This is the same matrix as used in Tibell (2006), but with *C. victorianum* added and the matrix realigned and re-analyzed by maximum-parsimony. *Calicium victorianum* in this analysis belongs to 'Clade I' (Tibell 2006), and has *C. tricolor* F. Wilson as sister-species. The support for this placement is, however, weak. These two species in the analysis join *C. pyriforme* Tibell, a species described from the Himalayas. The sister-group to these is formed by *C. denigratum* (Vain.) Tibell, *C. montanum* Tibell, *C. pinastri* Tibell, *C. glaucellum* and *C. trabinellum* (Ach.) Ach., all these species forming a moderately well-supported subclade of Clade I. This differs from the analysis of Tibell (2006) only in so far that *C. pyriforme* there, is not part of this clade. Clade I does not include the type species of *Calicium* (*C. viride* Pers.), but it and some of its subclades, for example, the *Calicium glaucellum*-clade, are strongly supported in molecular analyses based on ITS and LSU nuclear rDNA sequences, and may be suitable for generic recognition.

Conclusion and additional notes

The ITS sequence obtained from *C. victorianum*, the presence of physodalic acid, and the apparent lack of xanthones support the distinctiveness of the species. *Calicium tricolor* has well-stalked ascomata with a thick, Pd+ orange pruina and a C+ thallus that contains the xanthones arthothelin and thiophanic acid. *Calicium pyriforme* has, in contrast to all the other species mentioned here, clavate asci, a K+ yellow to dirty red thallus and contains squamatic, baeomycesic, pseudoplacodiolic and placodiolic acids and alternariol. *Calicium denigratum*, *C. glaucellum*, *C. montanum*, *C. pinastri* and *C. trabinellum* all occur in Europe, with only *C. glaucellum* and *C. trabinellum* known from the British Isles (Coppins 2002), although the latter is represented only by a single 19th century collection (Church *et al.* 1997). *Calicium pinastri* is a very minute species with comparatively short stalks and unidentified secondary chemistry. *Calicium montanum* has quite short-stalked ascomata with a distinct, white pruina and a well-developed thallus containing divaricatic and 2-*O*-methyldivaricatic acids, whereas the other species are clearly stalked. *Calicium denigratum* has well-stalked, non-pruinose, shining ascomata and an immersed thallus, and no secondary substances have been detected. *Calicium trabinellum* also usually has an immersed thallus, but has a more short-stalked ascoma and a yellow pruina consisting of vulpinic acid at the edge and outside of the capitulum. It is, apart from the pruina and the shortness of the stalk, very similar to *C. glaucellum*. None of the above-mentioned compounds could be detected in the post-analysis of the HPLC-DAD-ESI-MS analytical runs on our materials.

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