Genetic confirmation for the presence of the rare moss *Atrichum angustatum* (Brid.) Bruch & Schimp. in Belgium

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Abstract. – Atrichum angustatum *(Polytrichaceae) had not been recorded in Belgium for more than a century, until it was rediscovered in 2014. In this article, we disclose the coordinates of that find, and add two new observations from the same area (Zedelgem-Oostkamp). We also report on a validation of the species' identity by means of DNA barcoding. This genetic test clearly confirmed the latest sample as* A. angustatum. *Given the extremely scarce observations, and taking into account the inherent risks associated with its dioecious life-history, the current status as a red-listed species remains justified.*

Samenvatting. – ***Genetische bevestiging voor de aanwezigheid van het zeldzame bladmos Atrichum angustatum (Brid.) Bruch & Schimp. in België.*** Atrichum angustatum *(Polytrichaceae) was sinds meer dan een eeuw niet waargenomen in België, tot het opnieuw werd ontdekt in 2014. In dit artikel onthullen we de coördinaten van die vondst, en voegen we twee nieuwe waarnemingen uit hetzelfde gebied toe (Zedelgem-Oostkamp). We rapporteren ook over een validatie van de soort door middel van DNA-barcoding. Deze genetische test bevestigde het recentste staal duidelijk als* A. angustatum. *Gelet op de uiterst schaarse waarnemingen, en rekening houdend met de inherente risico's die aan tweehuizigheid zijn verbonden, lijkt de status als rodelijstsoort gerechtvaardigd.*

Résumé. – ***Confirmation génétique de la présence de la mousse rare Atrichum angustatum (Brid.) Bruch & Schimp. en Belgique.*** Atrichum angustatum *(Polytrichaceae) n'avait pas été observé en Belgique depuis plus d'un siècle, jusqu'à sa redécouverte en 2014. Dans cet article, nous révélons les coordonnées de cette découverte et ajoutons deux nouvelles observations de la même localité (Zedelgem-Oostkamp). Nous rapportons également une validation de l'espèce par le barcoding moléculaire. Ce test génétique a clairement confirmé que le dernier échantillon correspond à* A. angustatum. *Compte tenu des observations extrêmement rares, ainsi que des risques inhérents à la dioécie, le statut d'espèce sur la liste rouge semble justifié.*

Illustrations:
Fig. 1: geopunt.be, modified by authors; Fig. 2: authors; Fig. 3: BopCo.

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Background

Atrichum angustatum (Brid.) Bruch & Schimp. is a dioicous, acrocarpous member of the moss family Polytrichaceae (Siebel & During 2006). The species is red-listed as endangered for the European Union, and as vulnerable for Europe (Hodgetts *et al.* 2019). In Belgium, the only records dated back to the 19th and early 20th century (provinces of Liège and Namur; Sotiaux & Vanderpoorten 2015), until the species was newly discovered in 2014. As described by Stieperaere (2014) and De Beer *et al.* (2014), *A. angustatum* was found growing at two localities within the mixed forest-heathland reserve Doeveren (municipalities of Zedelgem and Oostkamp, province West-Vlaanderen).

In this brief report, we disclose the coordinates of those first findings, add two new observations and, most importantly, report on a genetic validation of the species' identity. Indeed, Bell *et al.* (2013) recommended DNA barcoding as a suitable, easy-to-use technique for species confirmation in *A. angustatum*. As the species is poorly known to the Belgian bryological community, given its rarity and morphological similarities to its congeners (*A. tenellum* and *A. undulatum*), we considered it worthwhile to have its identity checked on genetic grounds.

Records of *A. angustatum*

Four clumps of *A. angustatum* have been recorded in the Doeveren reserve so far. These locations are very close together, all situated within IFBL grid cell c2-51-12 (Figure 1).

› April and May 2014: coordinates 51° 7' 19.86'' N, 3° 11' 18.57'' E and 51° 7' 17.54'' N, 3° 11' 14.50'' E. Details were provided by

Figure 1. Localities of *Atrichum angustatum* at nature reserve Doeveren, according to the year of observation (1: 2014; 2: 2016; 3: 2022). IFBL grid cell: c2-51-12.

De Beer *et al.* (2014). In summary, one clump was adjacent to a strongly humified trunk of a fallen *Betula* tree; another clump was situated on a humus-rich bump of sand. Accompanying species were *Dicranella heteromalla*, *Hypnum cupressiforme*, *Mnium hornum* and *A.* cf. *undulatum*.

› May 2016: 51° 7' 20.28'' N, 3° 11' 17.88'' E. Terrestrial clump of plants on sparsely vegetated, sandy soil, accompanied by *Hypochaeris radicata* L., *Carex pilulifera* L. and seedlings of *Betula*, but no other bryophytes.

› November 2022: 51° 7' 21.36'' N, 3° 11' 9.96'' E. One clump growing on a strongly humified tree stump, with only *Dicranella heteromalla* in its immediate vicinity (Figure 2).

Figure 2. *Atrichum angustatum* growing on a decayed tree stump. IFBL grid cell: c2-51-12. Pictures taken in December 2022.

In all four cases, the identification relied on the cell size at mid-leaf, which is distinctly smaller than in *A. undulatum* and *A. tenellum* (≤ 20 µm; Touw & Rubers 1989 in Smulders 2003, Bell *et al.* 2013). Some doubt nonetheless remained, in part because the width of the zone taken by the foliar lamellae was below the thresholds provided by Siebel & During (2006). This mismatch, described by De Beer *et al.* (2014) for the initial observations, also applied to the specimens from 2016 (BD, pers. obs.).

Genetic confirmation

In order to remove any ambiguity regarding the species' identification, we opted to sample the specimen from 2022 for genetic analysis. Two separate stems were subjected to DNA barcoding. DNA was extracted by means of the NucleoSpin Plant II kit (Macherey-Nagel), using the manufacturer's instructions. The marker regions used, with the highest species representation and success rate, were the trnL-trnF intergenic spacer region, the rps4 gene and the chloroplast intergenic spacer region trnH-psbA. The primer pairs used for PCR amplification and bi-directional Sanger sequencing were, respectively, trnL_15/trnF_39, rps4_TRNS/rps4_RPS5 and trnH05f/psbA3F.

The resulting sequences could subsequently be compared to available sequences from other (vouchered) bryophytes in the GenBank database, using the BLAST search algorithm (Altschul *et al.* 1990). The best scoring match, with the highest percentage sequence identity, was an *Atrichum angustatum* sequence for trnH-psbA (97.75%) and rps4 (93.85%).

We also constructed a Bayesian inference tree (GTR + G model), based on all available sequence data of the trnL-trnF intergenic spacer region for members of the genus *Atrichum*. The results are shown in Figure 3. Our sample clearly clustered among other samples of *A. angustatum*, with high bootstrap support. This thus provides proof for its identification. Note that all current *A. angustatum* reference sequences originate from North America, where the species is widespread and locally common (references below). This explains the relatively long branch of our European sample.

Discussion

Inspired by the work of Bell *et al.* (2013), we have genetically confirmed the current presence of this rare bryophyte in Belgium. We also confirm leaf cell size to be the most reliable morphological characteristic in identifying non-sporulating plants.

Atrichum angustatum is currently listed as a vulnerable species in the Region of Flanders (Van Landuyt & De Beer 2017). Leaving aside the historical observations, the species has not yet been

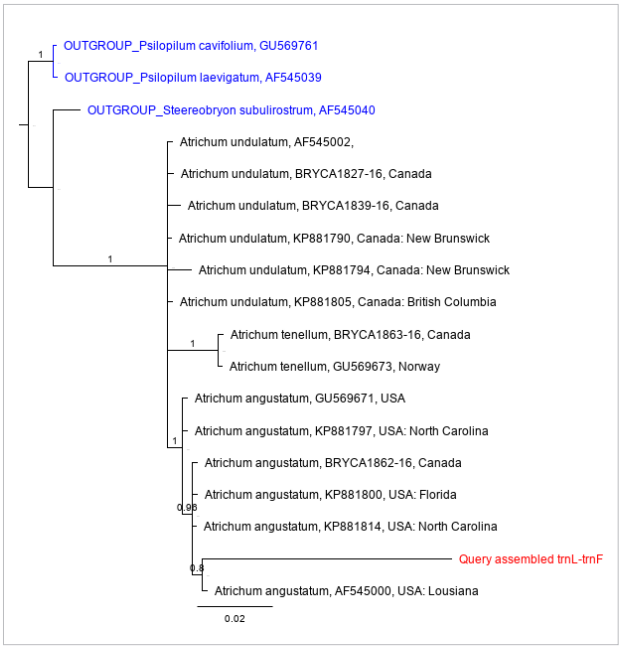

Figure 3. Phylogenetic tree with posterior probability at the branch of the trnL-trnF intergenic spacer data for samples of *Atrichum*. Our sample is denoted as 'Query'. Samples of *A. altecristatum*, *A. androgynum*, *A. crispum*, *A. crispulum*, *A. flavisetum*, *A. oerstedianum* and *A. selwynii* have been omitted from the figure; their topology did not affect the position of our sample.

recorded from any area other than the above. Its abundance and dynamics at this location are also not well understood. Its presence for almost a decade on the one hand suggests that a stable population is present. On the other hand, it is fair to assume that the species is also locally rare at least, given the fact that two bryological inventories in the nature reserve did not yield more observations (April 2014 and November 2022, see Stieperaere 2014 for a report).

There are also no recent records of the species from the neighbouring regions of Flanders, apart from those mentioned by Smulders (2003) for The Netherlands, and one French find of which we have been notified (Benoit Toussaint, pers. comm.). Since that latter find – the only recent one from the region of Hauts-de-France – has not been published elsewhere, we take the opportunity to include it here.

› September 2012: La Fausse Taille, municipality of Raismes, Nord department. On bare earth at the edge of a track in a humid forest lane. Found by Mr. Jean-Michel Lecron. Annotated sample conserved at the Conservatoire Botanique National de Bailleul.

Given this minimal number of localities, suitable habitat conditions in our region are difficult to generalize. Varied microhabitats are mentioned from forested and open environments in North America and Europe: tree-fall mounds, ditch banks, disused sand pits, sandy track edges, rabbit burrows, ant hills, or other openings in grassland swards (e.g. Bremer & Ott 1990, Stoneburner *et al.* 1992, Smulders 2003, Kimmerer 2005, Lansdown *et al.* 2016). They generally classify as disturbed sites, the mineral fraction of which is sandy or loamy, with varying levels of organic material (which may dominate over the mineral fraction). Given such a broad habitat range, the apparent contrasts in rarity, local abundance and trend among both continents is intriguing.

Peristome teeth of *Atrichum* capsules do not move hygroscopically, so an external force is needed for spore discharge, the most beneficial of which (for long-distance dispersal) would be air turbulence. Indeed, Stoneburner *et al.* (1992) showed that spores of *A. angustatum* become easily airborne in great numbers. The obvious bottleneck, however, is the dispersal of gametes from male to female plants, which was shown by Wyatt (1977) to act over very short distances only (observed maximum: 11.0 cm). At suitable locations, male and female plants of *A. angustatum* grow close to each other, resulting in considerable genetic variation both among and within colonies (Cummins & Wyatt 1981). But as Lansdown *et al.* (2016) rightly noted in their consideration of the species' decline in Britain, this dioecy also makes *A. angustatum* more vulnerable to extinction if numbers fall below a threshold density. (This is even more true if plants cannot form rhizoidal tubers, which is currently unclear.)

The lack of understanding of the species' favoured substrate and population dynamics also makes it hard for the managers of the Doeveren reserve to take the species into account in conservation planning. The current extensive cattle grazing in summer is expected to be beneficial to *A. angustatum* as it provides a modest degree of disturbance. Intriguingly, the respective zone of all *Atrichum* sightings has been subject to the intense removal of large, very dense stands of mature *Rhododendron ponticum* L. (± 2003-2022). It remains unclear whether *A. angustatum* has increased because of the clearing of *Rhododendron*; or to the contrary, whether the species was in a more favourable status among these shrubs, albeit more difficult to detect.

A. angustatum may be present in more areas in the western part of the Flemish (sand) district, which is relatively under-prospected by bryologists (Van Landuyt & Van Calster 2022). We therefore call to carefully scrutinize samples of *Atrichum* sp. during future inventories. In case of doubt, DNA barcoding can be considered.

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