Behaviour, morphology and molecular characterization of a *Monophadnus* sawfly species (Hymenoptera : Tenthredinidae) feeding on *Helleborus* spp. in Western Switzerland

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Abstract

Recently, an undescribed sawfly species (Hymenoptera : Symphyta) has been causing serious damage to the Helleborus *collections of the Conservatory and Botanical Garden of Geneva, destroying up to 80 % of some species populations. These sawflies were very aggressive towards* Helleborus *and were shown to be able to feed on 14* Helleborus *species and subspecies. This study aimed to identify this oligophagous sawfly species, which was morphologically identified as belonging to the genus* Monophadnus *(Hymenoptera : Tenthredinidae). The morphological description was supplemented with molecular identification using several genes, which showed this species to be genetically different from* M. monticola*, often considered as a synonym of* M. latus*, and of any species for which molecular data are available. Therefore, it is likely that this sawfly species could be* Monophadnus latus *sensu Lacourt 1999, which could be confirmed in a next future, at the occasion of a revision of the genus* Monophadnus *supported by genetic identifications.*

Keywords : *Molecular identification, Symphyta,* Monophadnus*, Switzerland, botanical garden, hellebores, Geneva, horticulture*

Résumé

Comportement, morphologie et caractérisation moléculaire d'une espèce symphyte de *Monophadnus* (Hymenoptera : Tenthredinidae) se nourrissant sur diverses espèces d'*Helleborus* en Suisse Occidentale. – *Depuis quelques années, une espèce non décrite de mouche à scie (Hymenoptera : Symphyta) cause d'importants dégâts aux collections d'hellébores des Conservatoire et Jardin botaniques de Genève, détruisant jusqu'à 80 % des plantes de certaines espèces. Ces mouches à scie, très voraces, ont été observées se nourrissant sur 14 espèces et sous-espèces d'hellébores. Cette étude avait pour objectif d'identifier cette espèce de mouche à scie laquelle a été morphologiquement identifiée comme appartenant au genre* Monophadnus *(Hymenoptera : Tenthredinidae). La description morphologique a été complétée par une identification moléculaire à plusieurs gènes, qui montre que cette espèce est génétiquement différente de*

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M. monticola*, souvent considérée comme un synonyme de* M. latus*, et de toutes les espèces pour lesquelles une description moléculaire est disponible. Il est probable que cette espèce soit* M. latus sensu *Lacourt 1999, ce qui pourrait être confirmé dans le futur par une révision du genre* Monophadnus *complétée par des identifications génétiques.*
Mots-clés : *Identification moléculaire, Symphytes,* Monophadnus*, Suisse, Jardin botanique, hellébores, Genève, horticulture*

Introduction

More than 8000 Symphyta (Hymenoptera) species distributed worldwide have been described, including important pest insects, which are major defoliators in the Northern hemisphere, in natural areas as well as in horticultural and agricultural production sites (Boevé 2004; Taeger et al. 2010). Symphyta include more than 1000 species, for which the phylogeny has been recently refined (Prous et al. 2014; Welz & Vilhelmsen 2014; Vilhemsen 2015), and new sawflies species are continuously being discovered and described (Haris 2015; Niu et al. 2015; Looney et al. 2016).

In the literature, several authors have reported *Monophadnus* spp. larvae as feeding on *Helleborus* spp., especially on *H. niger* L., *H. foetidus* L. and *H. viridis* L. (Fenili 1965; Jahn & Schedl 1992; Lacourt 1994). The *Helleborus* collections of the Conservatory and Botanical Garden of Geneva (CBG) have been particularly infested by sawflies for the last five years, with damage reaching 80% of individuals for some *Helleborus* species. For the purpose of developing biological control trials against this pest, it appeared important to first try to secure its identity. Additionally, the *Helleborus* collection found at the CBG provided an opportunity to observe this sawfly on 14 *Helleborus* species and subspecies.

Materials and Methods

Survey of Plant Species Hosting Sawflies in the CBG Collections

Observations were carried out in Spring 2015 in all *Helleborus* collections and isolated plants of the CBG in order to evaluate the range of species attacked by this sawfly. Specimens, both adults and larvae, were collected on *Helleborus* plants at the CBG and deposited at the Museum of Natural History of Geneva under voucher numbers MHNG ENTO 9805 to 9808.

Morphological Identification

The morphological description of the adult was initially achieved by using the determination keys for Hymenoptera Tenthredoidea by Berland (1947).

Molecular Identification

Samples of 3-4 larvae at the fourth stage were ground in liquid nitrogen with a mortar and pestle. Larvae were sampled and submitted to DNA extraction. Total nucleic acids were extracted from the resulting powder using the DNeasy Blood & Tissue kit (Qiagen AG, Switzerland), according the protocol « Purification of total DNA from insects ». Genomic DNA quality was checked and assayed with a Nanodrop ND-1000 spectrophotometer (WITEC AG, Switzerland). PCR amplifications were carried out for the 18S and 28SrDNA nuclear genes and the cytochrome oxidase subunit 1 mitochondrial gene (COI) with specific primers, whose sequences and sources are given in Table 1. PCR reactions were carried out in 50 µl final volume containing 50 ng DNA, using 1.25 u of MyTaq™ HS DNA Polymerase (Bioline, UK) in 1x MyTaq™ Reaction Buffer. PCR programs used for the amplification of the three genes are given in Table 2. Touchdown programs were used for 28S and COI genes. PCR products were checked for quality by gel electrophoresis, then purified with the Wizard® SV Gel and PCR Clean-up system (Promega AG, Switzerland) and submitted to Sanger sequencing. Result-

Table 1. Primers used for the phylogenetic study.

Genes	Primer	Sens	Sequence (5' - 3')	Source
18S	2880	F	CTG GTT GAT CCT GCC AGT AG	Von Dohlen & Moran, 1995
	Br	R	CCG CGG CTG CTG GCA CCA GA	Von Dohlen & Moran, 1995
28S	S3660	F	GAG AGT TMA ASA GTA CGT GAA AC	Dowton & Austin, 1998
	A335	R	TCG GAR GGA ACC AGC TAC TA	Whiting et al., 1997
COI	LepF1	F	ATT CAA CCA ATC ATA AAG ATA TTG G	Hebert et al., 2004
	LepR1	R	TAA ACT TCT GGA TGT CCA AAA AAT CA	Hebert et al., 2004

Fig. 1. Damage caused by Monophadnus *sawfly larvae on* Helleborus foetidus.

ing sequences were edited with FinchTV and registered in NCBI GenBank (Benson et al. 2013), where they were compared to deposited sequences with the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1990).

Outgroup sequences for the same genes were recovered from GenBank. Phylogenetic inference was based on a multigene dataset (28S and COI). Sequence alignment was constructed using Bioedit 7.1.9 (Hall 1999). Phylogeny was generated using maximum likelihood. The best-fitting nucleotide substitution model was selected via the Akaike information criterion (AIC), as implemented in the PhyML-SMS online execution program (http://www.atgc-montpellier.fr/phyml-sms/). The General Time Reversible model, with gamma-distributed among-site rate heterogeneity and invariant sites (GTR+G+I), was recovered as most appropriate. ML analysis was inferred using PhyML online execution platform (Guindon et al. 2010), applying model suggested by PhyML-SMS. Node support was quantified with 1000 rapid bootstrap replicates. The tree was visualised using Fig-Tree 1.4.2 (Rambaut 2009).

Results

Survey of Host Plant Species in CBG collection

The unknown sawfly species was observed feeding on all 14 *Helleborus* species and subspecies found at the CBG: *H. argutifolius* Viv., *H. cyclophyllus* Boiss., *H. dumetorum* ssp. *atrorubens* (Waldst. & Kit.) Merxm. & Podlech, *H. foetidus*, *H. lividus* Aiton, *H. multifidus* ssp. *bocconei* (Tenore) Mathew, *H. niger*, *H. odorus* Waldst. & Kit. ex Willd., *H. orientalis* Lam., *H. orientalis* ssp. *abchasicus* (A. Braun) B. Mathew, *H. purpurascens* Waldst. & Kit., *H. torquatus* Archer-Hind, *H. viridis and H. viridis* ssp. *occidentalis* (Reut.) Schiffn.. The damages were most important on *H. foetidus* (Fig. 1), while *H. niger* supported the least significant damages.

Morphological description

The morphological description was carried out with the descriptors of the determination key established by Berland (1947). Following Berland (1947), two characters enable this *Monophadnus* species to be identified as *Monophadnus longicornis* Har-

Table 2. PCR programs used to amplify the 3 genes used for the phylogenetic study.

Gene		Initial denaturation	30-35 cycles			Final extension
			Denaturation	Hybridation	Amplification	
18S	Temperature [°C]	94	94	55	72	72
	Time	4 min	1 min	50 s	45 s	7 min
28S	Temperature [°C]	95	95	49 - 61	72	72
	Time	4 min	1 min	50 s	50 s	7 min
COI	Temperature [°C]	95	95	48 - 60	72	72
	Time	5 min	1 min	1 min	1 min	10 min

tig, 1837: 1) simple tarsal claws and 2) third segment of antennae only one and a half length of fourth segment (Fig. 2). Other morphological characters of adults of this *Monophadnus* species are: body length 6.5-8.2 mm; antennae of nine articles and shorter than the abdomen; legs almost entirely black, but upper half of tibiae yellow-brown; dark brown to black veins and stigma. Our observations on the CBG sawfly are congruent with the morphological descriptions of *M. longicornis* given by Berland (1947), Fenili (1965) and Jahn and Schedl (1992). The general aspects of the adult and larva are shown in Figure 3. Given that the key of Berland (1947) is obsolete for identification purposes and that the *Monophadnus* genus includes many undescribed or wrongly named species (Lacourt 1999), our morphological and behavioural observations allow us to identify the CBG specimens as an undescribed species of the genus *Monophadnus,* close to *Monophadnus latus sensu* Lacourt 1999, labelled *Monophadnus* sp. MHNG ENTO 9805.

Molecular Identification

The DNA sequences obtained have been registered in NCBI GenBank under accessions numbers KU302717-KU302718 for the 18S rDNA gene, KU302719-KU302720 for the 28S rDNA gene, and KU302721-KU302722 for the COI gene. Genbank searches showed that genetic information on *Monophadnus* was very scarce. There are, for instance, no 18S rDNA sequences available, except the ones produced in this study for *Monophadnus* sp. MHNG ENTO 9805, and very few sequences registered for both 28S rDNA and COI genes. Sequence comparison allowed to confirm that the sequences for *Monophadnus* sp. MHNG ENTO 9805 were the first ones registered for this undescribed species. A phylogram built with the concatenated genes 28S and COI shows that *Monophadnus* sp. MHNG ENTO 9805 was very different from *M. monticola* Hartig 1837 (Fig. 4). A second phylogram, built only with COI sequences, clearly showed that these two species differ from each other and are also different from other *Monophadnus* species for which COI sequences are available (Fig. 5). A specimen labeled *Monophadnus* sp. A GM-2013 is the closest genotype to *Monophadnus* sp. MHNG ENTO 9805. Cited by Boevé et al. (2013), this specimen from an unidentified species was found on *H. foetidus* in Delémont, Northern Switzerland. The *Monophadnus* sp. B GM-2013 (Boevé et al. 2013) was sampled in Meride (Ticino), South East Switzerland, as was the *Monophadnus* sp. DEIGISHym11547 sampled on *H. viridis*. Though close to *Monophadnus* sp. MHNG ENTO 9805, at 99% similarity, these

Fig.2. Morphological criteria used for identification; third antennal segment only one and a half length of fourth segment (2A), simple tarsal claws (2B).

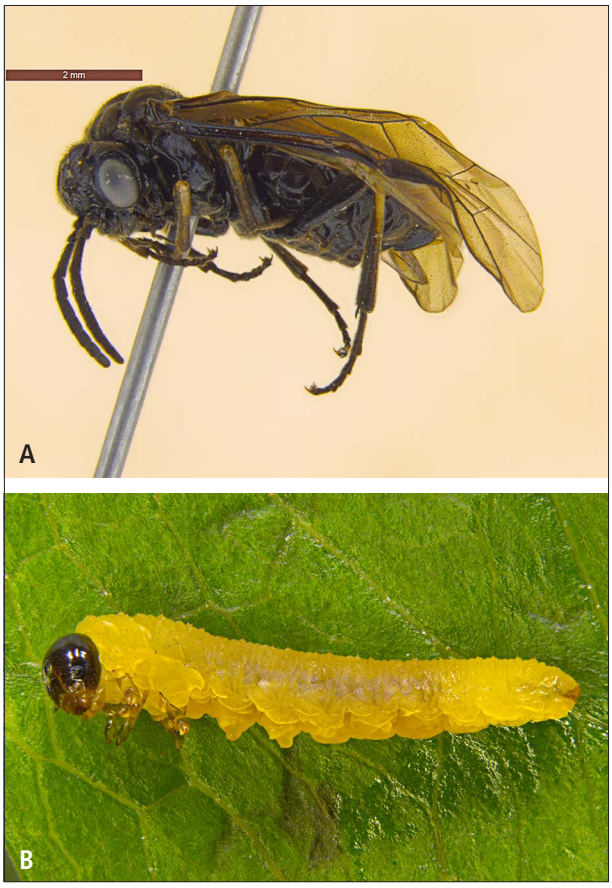

Fig.3. General aspects of the adult (3A) and the larva (3B).

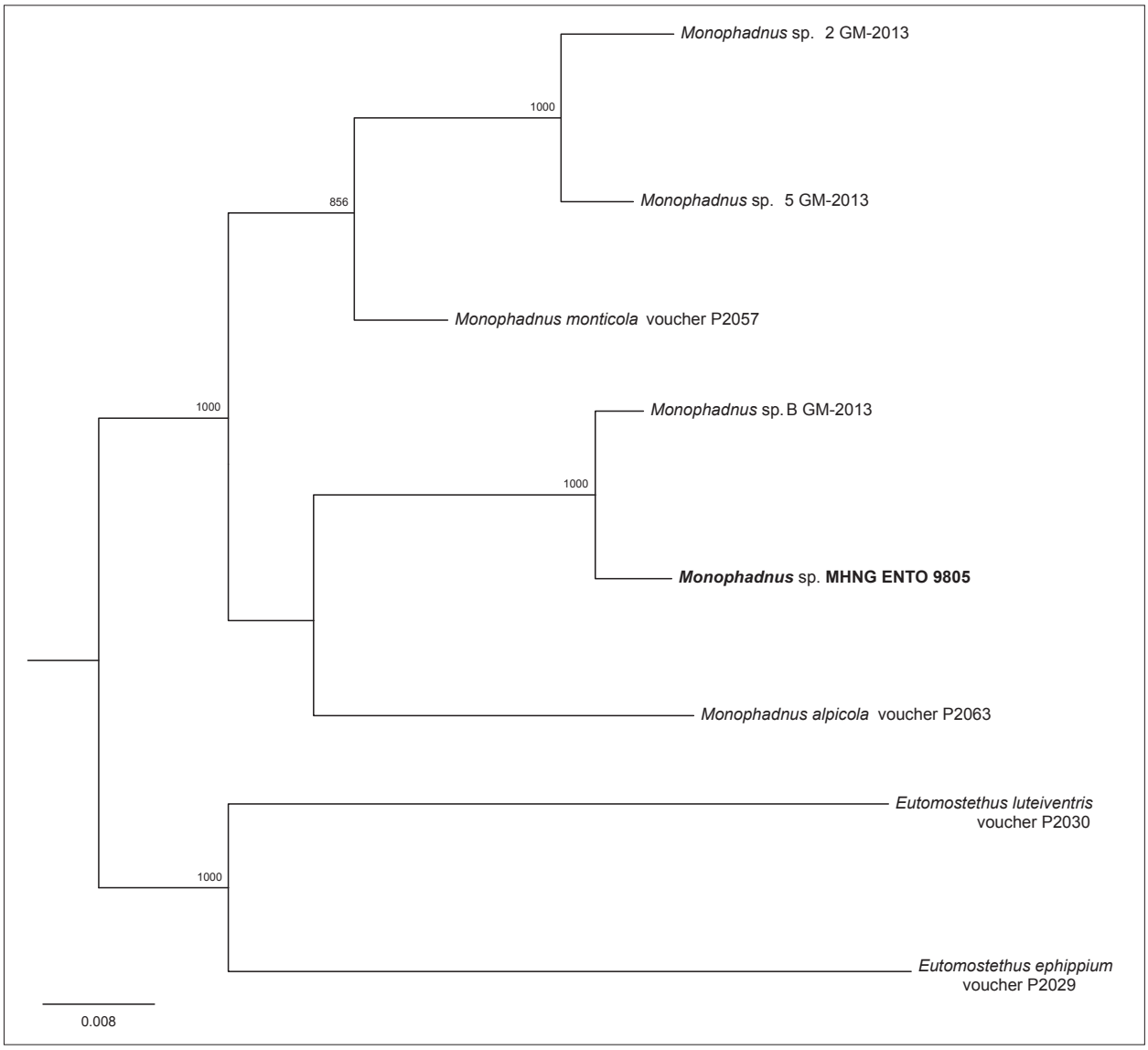

Fig. 4. ML tree topology derived from multigene analysis (28S and COI). Bootstrap support values are given at nodes. Only bootstrap values > 850 are shown. Blanks indicate values that were below the corresponding thresholds. Bar = 0.008 substitution positions.

three specimens, are nevertheless distinct genotypes. These first registered DNA sequences for three genes add to the morphological description of *Monophadnus* sp. MHNG ENTO 9805 and will help to place it in the phylogeny of the genus in the future.

Discussion and Conclusion

This study identified a *Monophadnus* species as the species causing damage to the *Helleborus* collections of the CBG. However, morphology as well as genetic data do not allow us to name this species, which could likely be *M. latus sensu* Lacourt 1999. Although the species described here was easily differentiated from the four other European species *Monophadnus alpicola* Benson 1954, *M. monticola, M. pallescens* Gmelin 1790, *and M. spinolae* Klug 1816, it has not been possible to name this species because of historical confusions, using three different names *Monophadnus latus, M. longicornis and M. monticola,* with no name being correct. The key of Berland does not allow *Monophadnus latus* sensu Lacourt 1999, to be distinguished from *M. monticola* and does not include *M. alpicola*. *M. monticola* is known to feed on *Ranunculus* spp. and *M. alpicola* on *Pulsatilla* spp., and for this reason, these specimens have not been considered in this study focusing on *Helleborus* spp. There is no morphological characters that would help distinguish whether it is *M. latus sensu* Lacourt 1999, or another undescribed species, and it is not clear, from the literature, if the aforesaid synonyms correspond to the same species. There is however a consensus that the *Monophadnus* species feeding

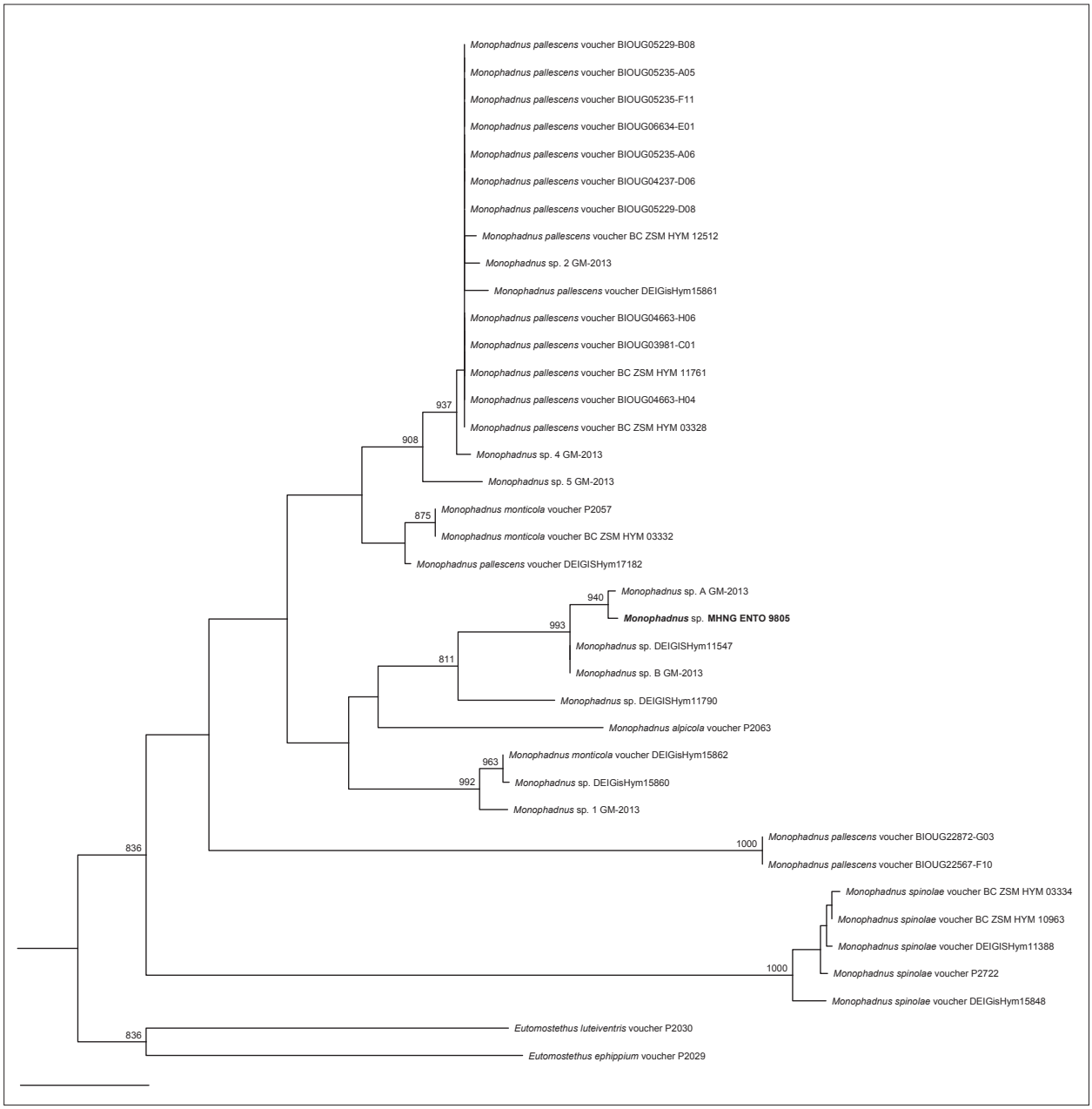

Fig. 5. ML tree topology for the COI gene. Bootstrap support values are given at nodes. Only bootstrap values > 850 are shown. Blanks indicate values that were below the corresponding thresholds. Bar = 0.05 substitution positions.

on *Helleborus* spp. could be named *M. latus sensu* Lacourt 1999. The species present in CBG, feeds on a large range of *Helleborus* spp. and seems to be genetically different from all referenced specimens. Concerning the behavior of *M. latus sensu* Lacourt 1999, females lay their eggs in the mesophyll of the leaves and petals of hellebores and are reported to feed only on this genus: they have been found feeding only on *H. viridis*, *H. foetidus* and *H. niger* (Fenili 1965; Jahn & Schedl 1992; Lacourt 1994). Concerning the host plants, Fenili (1965) stated that *M. longicornis* attacked *H. viridis* and *H. foetidus*. If Lacourt (1994) considered that *M. longicornis* could be restricted to *H. niger*, while *M. monticola* auct. *gallica* would be a specialist on *H. viridis* and *H. foetidus,* he proposed later that there was only one species, *Monophadnus latus sensu* Lacourt 1999 feeding on *Helleborus* spp. (Lacourt 1999, 2003). Jahn and Schedl (1992) reported that *M. longicornis* might also attack these three *Helleborus* species. Thus *M. latus sensu* Lacourt 1999 was reported to be limited to *H. niger*, *H. viridis* and *H. foetidus*, while our observations found that *Monophadnus* sp. MHNG ENTO 9805 is able to forage on 14 *Helleborus* species and sub-species, with *H. foetidus* being the most attacked among these species.

Concerning its distribution, *M. latus sensu* Lacourt 1999, is restricted to Europe and Taeger et al. (2006) have given the best inventory available at this point, completed with recent reports. The presence of *M. latus sensu* Lacourt 1999 has been recorded in Albania, Austria, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, Germany, Hungary, Italy and Sardinia, Romania, Spain, Switzerland and Ukraine, and previously recorded as *M. longicornis* in Austria, Italy, Romania and Ukraine. Since then it has also been reported in South West France (Savina & Chevin 2012; Chevin & Savina 2013) and North West France. Concerning Switzerland, *M. latus sensu* Lacourt 1999 was not, even recently, considered to be present in Geneva (Boillat 2010). There was only one report by Peter (2006), who mentioned a first report for South Eastern Switzerland in Ticino in 1987 on *H. niger* and another report by Pschorn-Walscher and Altenhofer (2000), in Northern Switzerland, who also used the name *M. longicornis*. The DNA sequences provided here are the first registered for this *Monophadnus* species, identified as potentially being *M. latus sensu* Lacourt 1999. Whether the specimens from CBG correspond to this species or another undescribed species remains unknown. For this reason, a revision of the genus *Monophadnus*, supported by genetic identification would be required in order to precisely name this species.

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