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New insights confirming the presence of *Myriostoma coliforme* in Italy

Claudia Perini[1], Elena Salerni[1]*, Diego Cantini[1], Daniele Antonini[2], Massimo Antonini[2], Giancarlo Bistocchi[3], Andrea Arcangeli[3], Rosalba Padula[4], Giancarlo Angeles Flores[3], Roberto Venanzoni[3], Paola Angelini[3]

[1] Department of Life Sciences, University of Siena, Via P.A. Mattioli 4, IT-53100 Siena, Italy
[2] Via Ferrucci 18, IT-51036 Larciano, Pistoia, Italy
[3] Department of Chemistry, Biology and Biotechnology, University of Perugia, Via Elce di sotto 8, IT-06123 Perugia, Italy
[4] Arpa Umbria, via Pievaiola Str. San Sisto, IT-06132 Perugia, Italy
*corresponding author: elena.salerni@unisi.it

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The genus *Myriostoma*, until recently regarded as monotypic, is nowadays considered to be a polytypic genus, in which six species with a distinct geographic distribution have been distinguished. *Myriostoma coliforme* has been demonstrated to be restricted to the Holarctic realm.

The main objective of the present study is to identify new finds from Umbria and Tuscany. Based on both macro- and micro-morphological features they were identified to be *M. coliforme* s. str., which was also confirmed by molecular analysis (ITS nrDNA).

Thanks to this investigation this rather rare saprotroph has shown to be present at a few sites and seems to be extinct at some others. A continuing decrease in the already small total population size is foreseen. An assessment at the regional level proposes the IUCN category Near Threatened.

Key words: *Basidiomycota*, gasteroid fungi, morphology, molecular characters, red list evaluation, central Italy.

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Rod *Myriostoma*, donedávna považovaný za monotypický, je nyní shledán polytypickým, přičemž v rámci tohoto komplexu je rozlišeno šest druhů se zřetelným zeměpisným rozšířením. Pravá *Myriostoma coliforme* se jeví být druhem holarktické oblasti.

Hlavním cílem aktuální studie je určit identitu nových nálezů z Umbrie a Toskánska. Podle morfologických znaků, makro- i mikroskopických, byly určeny jako *M. coliforme* s. str., což potvrdila i molekulární analýza (ITS nrDNA).

Na základě provedeného výzkumu lze konstatovat, že tento vzácný saprotrof se vyskytuje na několika lokalitách, zatímco z jiných vymizel a lze předpokládat postupné zmenšení celkové velikosti jeho populace (už tak dost malé). Dle vyhodnocení na regionální úrovni je navrženo jeho zařazení mezi téměř ohrožené druhy podle klasifikace IUCN.

INTRODUCTION

In recent years, fungal taxonomy has seen various changes and corrections. In this process, molecular techniques accompanying revisited morphological characters have been very valuable, often clarifying the position of cryptic and/or semi-cryptic taxa in species complexes (Angelini et al. 2017b, Peintner et al. 2019). Furthermore, this position is a prerequisite for reliable Red List assessments and functional conservation measures (Wagensommer et al. 2018, Angelini et al. 2021, Ceci et al. 2021). Current molecular studies indicate that the dimension of fungal taxonomic diversity is severely underestimated (Pecoraro et al. 2014).

Myriostoma Desv. is an example of this process of reviewing fungal systematics. *Myriostoma*, until recently represented by the only species *M. coliforme* (Dicks.) Corda, is a peculiar and easily to recognise gasteroid fungus with a starlike exoperidium and a globose endoperidium supported by numerous stalks. Until recently, it was reported from Europe, Asia, South Africa and America, growing in a wide variety of habitats, preferring low altitudes, often sandy soils, both in shady and sunlit places (Calonge 1998, Sarasini 2005, Sousa et al. 2017).

It was one of the candidates for the status of threatened species in Europe, proposed for Appendix I of the Bern Convention, document T-PVS (2001) n. 34 (Dahlberg et Croneborg 2006) and for assessment at the global level by the Global Fungal Red List Initiative.

Under the mapping project of the European Council for the Conservation of Fungi (ECCF), its distribution, ecology and conservation status at the European level was studied (Fraiture et Otto 2015). The number of known localities was established to be about 150, and the species appeared to be more common in the Mediterranean basin and absent in arctic and boreal regions, decreasing in some countries and regarded extinct in some others. In Italy, a few records were reported from the north and south, while it seemed lacking in the middle of the country (Onofri et al. 2005).

A study by Sousa et al. (2017) provided interesting facts and proved that this taxon is a complex of species. Based on morphological and molecular characters, four species were delimited: *M. areolatum* (Calonge et M. Mata) M.P. Martín, J.O. Sousa et Baseia, M. *calongei* Baseia, J.O. Sousa et M.P. Martín, *M. capillisporum* (V.J. Staněk) Suz, A.M. Ainsw., Baseia et M.P. Martín, and *M. coliforme*

sensu stricto. Later, in Sousa et al. (2019), a new species was described: *Myriostoma australianum* J.O. Sousa, Baseia et M.P. Martín. During the reviewing process of this paper, another new species from Mexico was published online: *Myriostoma herrerae* Guzm.-Dáv., Ram.-Cruz et Cabarroi-Hern (Guzmán-Dávalos et al. 2021).

Motivated by the work of Sousa et al. (2017, 2019), new find in central Brasil by Camilo-Cotrim et al. (2021), the recent contribution by Guzmán-Dávalos et al. (2021), and new records from Umbria and Tuscany (Central Italy), the aim of this contribution was (i) to revise samples conserved in the herbaria of the University of Perugia and the University of Siena observing morphological characters and testing our conclusions by molecular analyses; (ii) to expand our knowledge with specimens collected in the Italian peninsula but not mentioned in the above cited publications; (iii) to assign the correct name to our collections, confirming or not that the samples of central Italy belong to the type species *Myriostoma coliforme* sensu stricto; (iiii) to evaluate the conservation status at the regional level.

Material and methods

Myriostoma coliforme was sampled at different sites in Umbria and Tuscany (Central Italy), dried, codified by means of the on-line Anarchive database (www.anarchive.it) and conserved at the Herbarium of the University of Perugia (PeruMyc) and section Fungarium of the Herbarium Universitatis Senensis (SIENA). Moreover, at SIENA, one exsiccate gift of Prof. Rimoczi comes from Hungary. One historical collection is conserved at the Herbarium Centrale Italicum of the University of Florence. Herbarium ID, locality and habitat of the studied collections are given in Tab. 1.

Pictures were taken (digital video camera: True Chrome HD II S, TiEsseLab S.r.l., Milan, Italy) and some morphological observations were documented directly in situ, others at the laboratory by means of standard techniques using a Wild M3Z Leica stereomicroscope (Milan, Italy) and a Leitz Laborlux S optical microscope (Milan, Italy), with plan-achromatic 40× and 100× lenses (oil immersion) plus an extra lens 1.25×, reaching a total magnification of 500× and 1250×. Basidiospore measures including ornamentation were made on 36 spores per carpophore with distilled water or 4% KOH as mounting media and reported as minimum–maximum values. The colours of endoperidium and spore print are defined according to Edinburgh RBG (1969). A part of the spore was treated according to the acetolytic technique originally described by Hideux (1972), mounted on stubs and coated with gold (Angelini et al. 2008, 2009, Pagiotti et al. 2011). The observations and photos were made using a JCM-6000-plus scanning electron microscope, manufactured by JEOL S.p.A. (Milan, Italy).

Tab. 1. Herbarium and occurrence data of the studied *Myriostoma coliforme* specimens.

Herbarium ID	Locality	Habitat	Date	Legit	Determinavit	Revidit
PeruMyc1560	Collestrada (Perugia), Umbria	glades in *Quercus ilex* woods	29 Aug 2018	Arcangeli A., Bistocchi G.	Arcangeli A., Bistocchi G.	Angelini P. (July 2019)
PeruMyc2351	Isola Polvese (Castiglione del Lago, Perugia), Umbria	under *Pinus* spp., and glades in *Quercus ilex* woods	10 Nov 2018	Arcangeli A., Bistocchi G., Padula R., Angelini P.	Arcangeli A., Bistocchi G.	Angelini P. (July 2019)
PeruMyc2436	Cascata delle Marmore (Marmore, Terni), Umbria	under *Quercus ilex*	1 May 2019	Venanzoni R.	Angelini P.	Angelini P. (July 2019)
SIENA8151	Tempio di Diana, Maremma Regional Park (Grosseto), Tuscany	under *Pistacia lentiscus* and *Juniperus communis*	21 Oct 2014	Perini C., Cantini D.	Perini C., Cantini D.	Perini C. (July 2018)
SIENA8152	Monteferrato (Prato), Tuscany	under *Cupressus sempervirens*	13 Nov 2009	Alessandrini I.	Alessandrini I.	Perini C. (July 2018)
SIENA8153	Montalgeto near Carmignano (Pistoia), Tuscany	under *Quercus ilex*	10 Nov 2017	Brocchi I.	Brocchi I.	Perini C. (July 2018)
SIENA8154	Colle di Calenzano (Florence), Tuscany	under *Cupressus sempervirens*	14 Mar 2018	Alessandrini I.	Alessandrini I.	Perini C. (July 2018)
SIENA8442	Parco di Rimigliano (Livorno), Tuscany	evergreen Mediterranean forests	1 Jul 2018	Cantini D.	Cantini D.	Perini C. (July 2018)
Herbarium Centrale Italicum	Villa Altoviti-Avila, Lastra a Signa (Florence), Tuscany	park of the villa of Marquise Altoviti-Avila	Jun 1920	Marchioness Altoviti-Avila	Beccari O.	Chiarugi A. (1958)
SIENA5734	Hungary		1999	Rimoczi I.	Rimoczi I.	Perini C. (July 2018)

The identification was first carried out according to Calonge (1998) and Sarasini (2005), then with the new key by Sousa et al. (2017).

To test our identification based on morphological characters of two dry specimens, one from Umbria and one from Tuscany (PeruMyc-1560 and US8442 = SIENA8442), the total genomic DNA was extracted using the ZR Fungal/Bacterial DNA Kit (Euroclone S.p.A., Milan, Italy). The genomic DNA quality and quantity was evaluated with BIO-RAD (Milan, Italy) model 200/2.0 Power Supply gel electrophoresis [0.8% agarose gel in 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 7.6)] in the presence of SafeView Nucleic Acid Stain (NBS Biologicals, Huntingdon, United Kingdom) and a MassRuler DNA Ladder Mix (Thermo Scientific, Vilnius, Lithuania), and visualised with Safe ImagerTM 2.0 Blue-Light Transilluminator Invitrogen (Parma, Italy). DNA samples were subsequently diluted with up to 10 µg/µl nuclease-free water before PCR amplification. As described in Angelini et al. (2018, 2019, 2020), the internal transcribed spacer (ITS) region nrDNA was amplified through polymerase chain reactions (PCRs) with ITS1F

and ITS4 primers. SimpliAmp Thermal Cycler Applied Biosystems (Monza, Italy) was programmed as follows: one cycle of denaturation at 95 °C for 2.5 min; 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s and extension at 72 °C for 45 s; one final extension cycle at 72 °C for 7 min. Electrophoresis of PCR amplicons was carried out on 1.2% agarose gel as described above. The PCR-amplified ITS fragment was purified using the ExoSap-IT PCR Cleanup reagent (Thermo Fisher, Monza, Italy) and then sequenced by Macrogen Europe (Netherlands). The obtained sequences were submitted to GenBank under accession numbers MZ050117 and MZ049959, respectively.

Based on published and not published data (Chiarugi 1958, Angelini et al. 2016, 2017a, Cecchini et Narducci 2019, www.anarchive.it, Antonini et Antonini in verb., Cantini in verb.), a distribution map was compiled using the QGIS programme and an assessment at the regional level following Dahlberg et Mueller (2011) is proposed.

RESULTS

Myriostoma coliforme was collected at different sites in Umbria and Tuscany (Tab. 1) ranging from coastal evergreen Mediterranean oak-woods to inland deciduous forests, under *Quercus ilex*, *Q. cerris*, *Q. pubescens*, conifers like *Pinus* spp., *Cupressus arizonica*, *C. sempervirens*, and under *Eucalyptus camaldulensis*. Moreover, a specimen from Hungary, for a long time the only one of *Myriostoma* conserved at the Herbarium of the University of Siena, was revised. According to the new key and description given in Sousa et al. (2017), our collections correspond to *M. coliforme*, with the exoperidium typically subdivided into a star-like body of variable dimensions (6–12 cm diam.), possessing 7–10 rays of 1.5–4.5 cm free length, non-hygroscopic, revolute and hidden under the endoperidium in very old specimens. Endoperidium spheroidal, slightly vertically flattened, 2.5–6 cm in diameter, smoke grey (code 34) or milky coffee (code 28), flocculose, with 6–20 well-developed but non-delimited flat ostioles, slightly differing in size (around 1 mm in diameter), the apical part of very old carpophores often being disrupted. One specimen, instead of 3–10 stalks variable in dimensions, only had one very tough, wrinkled stalk, almost 1 cm thick. Gleba of mature specimens dark with snuff brown spore print (code 17). Basidiospores globose, 5.9–6.5 µm, to subglobose (4.7)5.9–7.8(8.2) µm including the dense ornamentation formed by convoluted, interconnected ridges and isolated warts 0.9–2.0 µm wide, spore wall and ornamentation cyanophilous. Capillitium constituted of very long hyphae (> 350 µm), up to 3.5 µm wide with thickened walls (up to 1.5 µm), smooth or with scattered spine-like bumps, sparsely branched, without pores, with long acuminate hyphal ends, often bifurcate, strongly cyanophilous (Fig. 1).

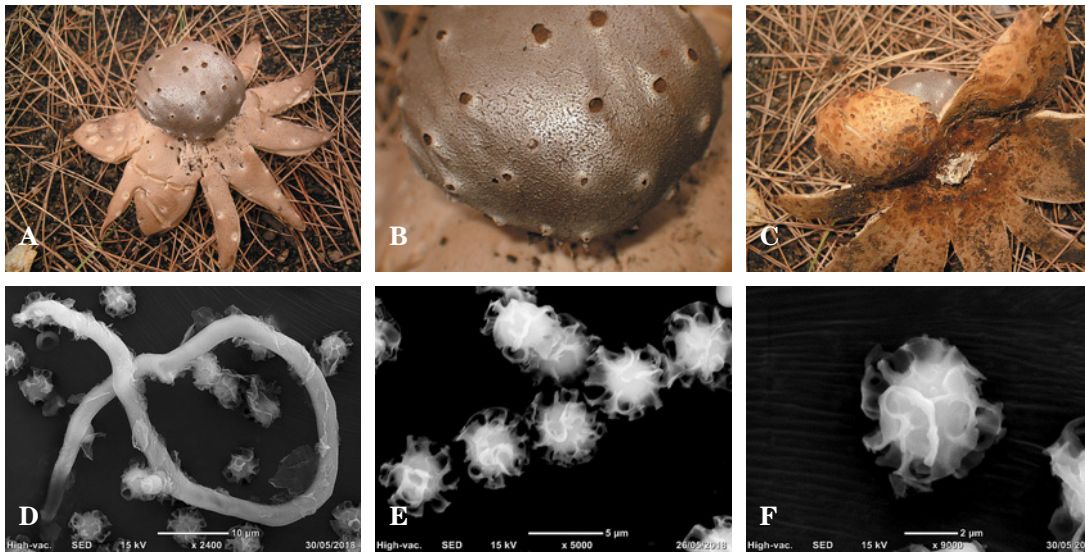

Fig. 1. *Myriostoma coliforme* (PeruMyc2351): **A** – carpophore in situ (Polvese Island, Umbria); **B** – surface of endoperidium with stomata; **C** – surface of exoperidium. Photos: Andrea Arcangeli. SEM: **D** – spores and capillitium; **E, F** – spores. Scale bars = 10 µm (D), 5 µm (E), 2 µm (F). Photos: Rosalba Padula.

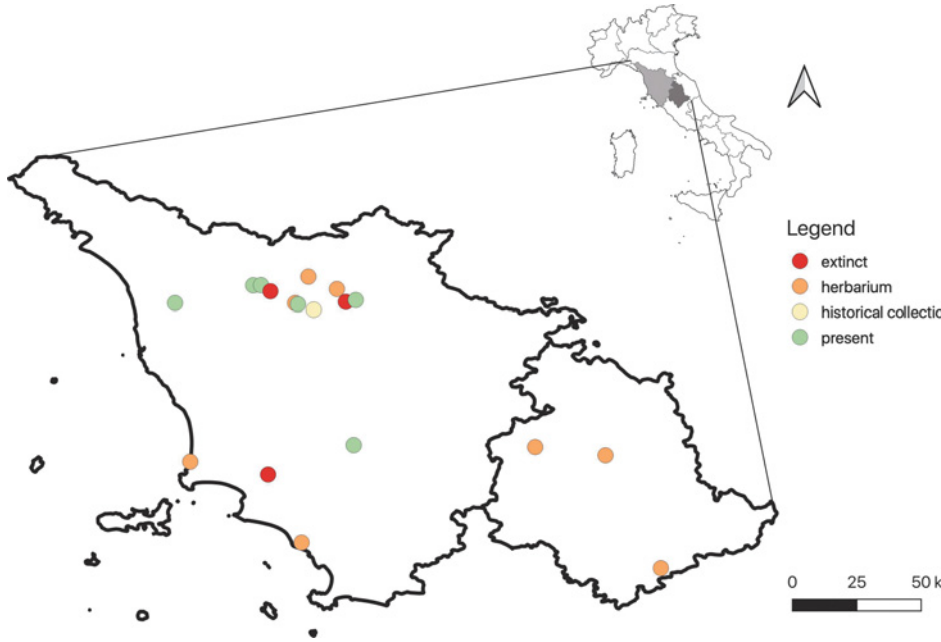

Fig. 2. Distribution map of known sites of *Myriostoma coliforme* in Umbria and Tuscany. Two close sites in municipality of Carmignano are visible as one dot.

The ITS dataset included 38 sequences, 2 generated in this study and 36 obtained from GenBank. Additionally, a BLAST search confirmed that our samples belong to *M. coliforme*, as it showed a close match with specimens of this species (Tab. 2).

Compiling all available data, published or not, a distribution map was compiled (Fig. 2). The species was found at 19 sites, 3 in Umbria and 16 in Tuscany, principally in the northern part of this region. The species has not been observed since the past 60 years in 2 parks near the city of Florence and is extinct at 2 other sites in northern and southern Tuscany.

DISCUSSION AND CONCLUSIONS

Until recently the genus was considered monotypic, represented only by *Myriostoma coliforme* (Dicks.) Corda and its various synonyms; we can find it under the name *Lycoperdon coliforme* Dicks. (1785) in the first description, some years later as *Geaster coliforme* (Dicks.) Pers. (1801) and *Myriostoma anglicum* Desv. (1809) (www.indexfungorum.org, accessed on 1st March 2021). This fungus was described as the largest earthstar, unique in its shape and easy to identify because of its size, multiple ostioles on the endoperidium, and stalks supporting it (Calonge 1998, Sarasini 2005).

With the research by Sousa et al. (2017, 2019), who analysed numerous collections from the whole world, and the recent publication by Guzmán-Dávalos et al. (2021), the genus appears to be a complex of six species with *M. coliforme* as the type species, justified by an iconotype (designated as a lectotype in Sousa et al. 2017) and an epitype from the UK designated by the same authors. *Myriostoma coliforme* seems to be restricted to Europe and North America, while *M. capillisporum* (V.J. Staněk) Suz, A.M. Ainsw., Baseia et M.P. Martín to South Africa, *M. areolatum* (Calonge et M. Mata) M.P. Martín, J.O. Sousa et Baseia to Costa Rica, *M. calongei* Baseia, J.O. Sousa et M.P. Martín to Argentina, Brazil, the USA and Mexico (Sousa et al. 2017, Camilo-Cotrim et al. 2021, Guzmán-Dávalos et al. 2021), *M. australianum* J.O. Sousa, Baseia et M.P. Martín to Australia (Sousa et al. 2019), and *M. herrerae* Guzm.-Dáv., Ram.-Cruz et Cabarroi-Hern to Mexico (Guzmán-Dávalos et al. 2021).

In the present study, collections from Italy were studied, a country not included in the above mentioned works. They have been principally collected in evergreen Mediterranean oak woods, close to *Cupressus sempervirens* or *Pinus pinaster*. From a morphological point of view, the Italian specimens show slightly smaller spores (length ranging between 5.9 and 7.8 µm instead of 6.1–8.0 µm, including ornamentation) and its colour is more olivaceous than yellow, when compared with the description by Sousa et al. (2017), which is somehow intermediate

Tab. 2. GenBank sequences and identity percentages with *Myriostoma coliforme* PeruMyc1561 and US8442 = SIENA8442 (two obtained in this study, the remaining published in Sousa et al. 2019).

Species	Accession no.	Voucher	% identity with PeruMyc1561	% identity with US8442
M. coliforme	MZ050117	PeruMyc1561	–	99.59
M. coliforme	MZ049959	US8442	99.59	–
M. coliforme	MG675909	L 3961241	100.00	99.55
M. coliforme	MG675910	L 3961244	99.78	99.78
M. coliforme	MG675911	L 3961247	99.78	99.55
M. coliforme	MG675912	L 3961237	99.78	99.78
M. coliforme	EU784376	K(M)37233	100.00	99.59
M. coliforme	KY096681	K(M)61641	100.00	99.55
M. coliforme	MG675913	L 3961239	99.78	99.55
M. coliforme	MG675914	L 3961240	99.56	99.78
M. coliforme	MG675915	L 3961242	99.78	99.78
M. coliforme	MG675916	L 3961243	99.78	99.55
M. coliforme	KC582020	M. Jeppson 8714	99.60	99.59
M. coliforme	MG675917	L 3961251	99.78	99.78
M. coliforme	KY096685	MA-Fungi 31316	99.78	99.78
M. coliforme	KY096687	K(M)154620	100.00	99.55
M. coliforme	MG675918	L 3961238	99.60	100.00
M. coliforme	KY096689	MA-Fungi 60898	99.56	99.55
M. coliforme	KF988337	J.C. Zamora 496	100.00	99.55
M. coliforme	JN845203	TNS: TKG-GE-50801	98.47	98.44
M. coliforme	MG675919	L 3961250	99.78	99.55
M. calongei	KF988467	MA-Fungi 83759	91.03	90.42
M. calongei	MG675905	L 3961249	92.35	92.05
M. calongei	KY096674	UFRN-Fungos 386	91.03	90.42
M. calongei	KY096675	UFRN-Fungos 990, paratype	91.25	91.09
M. calongei	KY096676	UFRN-Fungos 2019, holotype	91.25	91.09
M. calongei	KY096677	UFRN-Fungos 2020, isotype	91.25	91.09
M. calongei	MG675906	ICN 175617	91.47	90.65
M. calongei	MG675907	ICN 177080	91.47	90.85
M. calongei	MG675908	URM 31433	91.47	90.85
M. capillisporum	KY096678	K(M)205482	90.99	90.83
M. capillisporum	KY096679	K(M)205483	90.99	90.83
M. capillisporum	KY096680	K(M)205540	90.99	90.83
M. australianum	MG675902	MEL 2060796	90.24	89.93
M. australianum	MG675903	MEL 2091620	90.47	90.16
M. australianum	MG675904	MEL 2095275	90.24	89.93
M. australianum	MG675901	MEL 2305388	90.47	90.16
M. areolatum	KY096673	MA-Fungi 36165	84.68	84.91

between *M. areolatum* and *M. coliforme*. On the other hand, the ostioles are flattened, not well delimited, looking like damaged holes, which is typical of *M. coliforme*. The molecular analysis confirmed the species as *Myriostoma colifome* s. str.

The rarity and peculiarity of this species was highlighted just a century ago. As Chiarugi (1958) describes in his manuscript, in July 1920 his professor, Mr Beccari, showed him excitedly various carpophores collected near Florence (Tuscany). The following years, Chiarugi searched for this gasteroid fungus and found it only after nearly 40 years in another park near Florence, at Villa Stibbert, in a mixed forest with *Laurus nobilis*, *Quercus ilex* and *Cupressus sempervirens*. In his manuscript, he wrote enthusiastically: "Soltanto il 23 febbraio 1957 la mia costante speranza è stata inaspettatamente coronata da successo" (Not until on 23 February 1957 my constant hope was unexpectedly crowned with success). No other collections have been reported for these two sites. The original layout of the parks has changed and so for instance the one around Villa Stibbert is now an English landscape garden.

Only few other reports are known from Tuscany (Antonini in verb., Cantini in verb., Cecchini et Narducci 2018). They include two localities, known from 1987 and 2016, respectively, where the species disappeared due to human activities. The site at Parco di Rimigliano (Livorno Province) underwent a clearcutting of the woods to make way for a new built area. The site at Lago dell'Accesa (Grosseto Province) underwent a reduction in size of a little forested area near a lake in a Site of Community Importance (SCI). It is safe to conclude that *Myriostoma coliforme*, being an easily observable and distinct species, is a rare fungus. The known number of sites in the two regions is 19 (4 of which no longer exist or their disappearance is hypothesised). According to Dahlberg et Mueller (2011) and because the ecology of the species is not very restricted, the number of sites could be estimated at 10 times higher, with approximately 20 mature individuals per site. In conclusion, the total population size is estimated to consist of 3800 mature individuals and a decline is observed, qualifying the species in category VU, criterion C1. At the regional level *Myriostoma coliforme* is proposed as NT (near threatened).

In this context it is clear that protection of a habitat can provide protection of the majority of organisms living at that site, but the proposed management is not always beneficial for fungi. Different approaches, such as key sites for fungi and important areas from the mycological perspective, should be considered. They can sometimes be the only way to conserve fungal species (Perini et al. 2011).

Fortunately, the species is under assessment globally and can become part of conservation measures. With the addition of these records in central Italy, we can confirm the presence, although threatened, in different habitats of the whole peninsula.

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