THE DIVERSITY OF LICHENIZED TRENTEPohlIOID ALGAL (ULVOPHYCEAE)
COMMUNITIES IS DRIVEN BY FUNGAL TAXONOMY AND ECOLOGICAL FACTORS

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Trentepohliales are a group of both free-living and lichenized algae, with most diversity occurring in tropical regions. Recent studies showed that the abundance of lichens with a trentepohlioid photobiont has been increasing in temperate habitats, probably because of global warming, which makes them an interesting study case. A detailed molecular study of the diversity of lichenized Trentepohliales, epiphytic as well as epilithic, was performed in three forests of north-western Europe. Additional samples of lichens of the Arthoniales order (associating essentially with a trentepohlioid photobiont) from other European regions and from other continents were also sequenced. A total of 195 algal sequences were obtained. Phylogenetic analyses with \( \text{rbcL} \) and ITS loci were performed and associations between phylogenetic distances of photobionts and ecological factors (substratum, climate or Wirth indices, mycobiont taxonomy, and geographic location) were tested by variation partitioning and phylogenetic signal analyses. The high number of \( \text{rbcL} \) algal haplotypes found in some lichens or on different substrata revealed that the Trentepohliales diversity in extratropical regions was underestimated. The phylogenetic patterns showed selectivity of some photobionts in their fungal partner choice and vice-versa, while others were linked with several haplotypes. Photobionts seemed to be less selective than mycobionts. The main factors influencing lichenized algal community were climate and mycobiont species. Coevolution between mycobionts and photobionts as well as switching between free living and lichenized lifestyles appeared to drive the evolution of Trentepohliales and might explain the high cryptic diversity observed, which might be changing in some regions due to climate change.

Key index words: Climate change; ITS; mycobiont; photobiont; phylogenetic signal; \( \text{rbcL} \); selectivity; symbiosis; variation partitioning

Abbreviations: AICc, Akaike; ARD, all-rate different model; BR, Meise; ER, equal rates model; ML, maximum-likelihood; PCNM, principal coordinates of neighbourhood matrix; PCoA, principal coordinates analysis; RDA, redundancy analysis; SYM, symmetric model

Lichens can be considered as chimerical organisms or even complex ecosystems (Hawksworth and Grube 2020), as they are composed of a main fungus, one or two photosynthetic partners (a cyanobacterium, a trebouxioid, or a trentepohlioid alga), and other microorganisms, such as endolithic fungi (e.g., Arnold et al. 2009, Stribeille et al. 2016). Around 20–30% of lichen species have a trentepohlioid alga as photobiont (Nelsen et al. 2011, Kosecka et al. 2020), and most of them belong to Arthoniales, Ostropales, and Pyrenulales orders (Lücking et al. 2017, Nelsen et al. 2020). Trentepohliales consist of filamentous green algae and are an important cosmopolitan order of the class Ulvophyceae (Leliaert et al. 2012). They are all terrestrial, either lichenized, plant parasitic or free-living (Nelsen et al. 2011) and colonize all kind of substrata (bark, rock, leaf, and also artificial substrata, such as metal and plastic; Brooks et al. 2015). The majority of Trentepohliales are tropical (Rindi et al. 2010, Kosecka et al. 2020), but a substantial number of species is also found in temperate regions (Hametner et al. 2014a). Although Trentepohliales consist of a single family (Trentepohliaceae), they were traditionally divided into 19
genera and 113 species (Guiry and Guiry 2020), but
only five genera have been recognized with confi-
dence (Skaloud et al. 2018). Taxonomic revision of
the order based on molecular and phylogenetic
studies has only recently begun (e.g., Rindi et al.
2009, Nelsen et al. 2011, Johnston et al. 2018),
revealing an unexpected high number of genetic
lineages (e.g., Zhu et al. 2018, Kosecka et al. 2020,
Nelsen et al. 2020). Traditionally, trentepohlioid
algae were distinguished by morphological vegeta-
tive and reproductive characters, such as filament
shape, branching type, gametangia, sporangia, and
sporangiate-lateral shape (Brooks et al. 2015). How-
ever, different morphologically based groups were
shown to be polyphyletic (morphological conver-
gence; Rindi et al. 2009, Nelsen et al. 2011, Zhu
2020, Fang et al. 2021). Moreover, lichenized Tre-
tepohliaceae are subjected to morphological modifi-
cations (e.g., branching pattern; Hametner et al.
2014b) compared to the free-living forms (Hamet-
ner et al. 2014b). In addition, photobionts, which
normally have a digenetic and isomorphic life cycle,
only reproduce asexually when lichenized (Chap-
Hence, reproductive structures (gametangia and
sporangia), which are fundamental for a morp-
ho logical recognition, are absent. Therefore, molecu-
lar analyses are essential to distinguish lichenized
Trentepohliaceae species (Klimešová et al. 2019).

Four of the five recognized genera of
Trentepohliales include lichenized species: Cepha-
leuros, Phycolpetis, Printzina, and Trentepohlia
(Kosecka et al. 2020). Lichenization independently
originated several times, resulting in different
clades, always closely related to free-living lineages
(Nelsen et al. 2011, Hametner et al. 2014a, Kosecka
et al. 2020). All Cephalleuros and Phycolpetis (either
free-living or lichenized) are strictly folicolous,
whereas the other genera are found on other, more
diverse substrata (Grube et al. 2017, Kosecka et al.
2020). Our knowledge on ecological factors that
influence the distribution of Trentepohliales is still
limited. Lichenized photobionts are often known to
have environmental preferences in humidity, sun
exposure, or substratum (Rikkinen et al. 2002,

However, not all fungi have the same degree of
selectivity and specificity (Beck et al. 2002) regard-
ing the choice of photobiont and vice-versa. The
more a mycobiont associates with phylogenetically
close photobionts, the more the mycobiont is selec-
tive, and vice-versa; and the more both partners are
selective, the more the association is specific (Beck
et al. 2002). Algae are generally less selective than
mycobionts (e.g., Kroken and Taylor 2000, Ruprecht
et al. 2012), with often one algal species occurring
in different lichens (e.g., in two to eight species of
Leparia and Stereocaulon; Peksa and Skaloud 2011,
Peksa et al. 2022).

While some fungi show a moderate (e.g., Rocella)
or high selectivity (e.g., some Bryoria and Cladonia
species, Fulgensia fulgida), others (e.g., the wide-
spread Graphis scripta and Xanthoria parietina) are
known to switch among several species of photo-
bionts, even sometimes from different families or
classes (e.g., Beck et al. 2002, Yahr et al. 2004,
Hametner et al. 2014a, Lindgren et al. 2014, Nyati
et al. 2014, Ranft et al. 2018). A change in lichen
thallus morphology can be observed when there is a
switch of phylogenetically distant photobionts
(Tonsberg and Holtan-Hartwig 1983, Heidmarsson
2013), and the switch can influence reproduction of
the lichen (vegetative propagules vs ascospores; Ertz
et al. 2018).

The ability to switch among different photobiont
species enables some lichenized fungi to increase
their ecological niche amplitude, allowing them to
colonize different substrata or habitats, with differ-
ent selective pressures and environmental condi-
tions, possibly influencing lichens evolution (e.g.,
Magain and Sérusiaux 2014, Voytskekovich and
Beck 2016, Williams et al. 2017, Ertz et al. 2018,
Rolshausen et al. 2020). The distribution range of
the lichen taxon can also be influenced (or even deter-
mined) by the alga (Kosecka et al. 2020, Rolshausen
et al. 2020). For instance, two Umbilicaria species
and Cetraria aculeata increased their altitudinal and
latitudinal range, respectively, by switching among
photobiont species that have different altitudinal
and latitudinal preferences (Fernández-Mendoza
et al. 2011, Rolshausen et al. 2020). Some lichens
were also shown to have more than one species of
photobiont in the same thallus (e.g., Pleurosticta
acetabulum): the additional photobiont may not only
increase the distribution range of the mycobiont,
but could also compensate a possible scarcity of the
primary alga (Voytskekovich and Beck 2016).

Previous studies suggest that Trentepohliales usu-
ally occur in shaded, moist and tropical evergreen
forests (Sipman and Harris 1989, Wolseley and
Aguirre-Hudson 1997, Cáceres et al. 2008, Rivas-
Plata et al. 2008, Nelsen et al. 2011). Some Trente-
phliales species are generalists, while others are
specialized to particular substrata and ecological
conditions (Rindi and Guiry 2002, Rindi and López-
Bautista 2008, Suutari et al. 2010, Nelsen et al.
2011) such as folicolous trentepohlioid lichens of
the genus Porina, which are exclusively associated
with the folicolous algae of the genus Phycolpetis
(Baloch and Grube 2009, Grube et al. 2017, Kosecka
et al. 2020). However, the abundance of lichens
with a trentepohlioid alga in temperate ecosystems
seems to have gradually increased, probably due to
climatic change (Van Herk et al. 2002, Aptom
and Van Herk 2007, Van Herk 2009, Van Den
Broeck et al. 2010, Aptom et al. 2021). Little is known
about the diversity of trentepohlioid photobionts in
temperate regions although they obviously represent
an important indicator of global warming and influence the distribution of their mycobiont (in case of lichenized algae).

This study aims to deepen the knowledge of the free-living and lichenized trentepohlioid photobiont community (epiphytic and epilithic) in temperate forests in Belgium and northern France, and along a wide longitudinal and latitudinal span. More specifically, using plastid and ribosomal molecular markers, (i) we analyzed the diversity and phylogenetic patterns of Trentepohliae as photobionts, within a lichen, within lichens growing on the same tree, and within lichens from different trees and in different forests; (ii) we investigated the degree of selectivity between myco- and photobionts; and (iii) we examined the association between phylogenetic patterns of photobiont communities and ecological factors (substratum, climate or Wirth indices, mycobiont taxonomy, and geographic location) at a local scale and over a wide latitudinal and longitudinal span. We discuss whether these factors influence photobionts' distribution in a context of climate change.

**MATERIALS AND METHODS**

Study sites, sampling, and sample ecological characterization. Lichens with trentepohlioid algae were sampled on various substrata (bark and rock) during two excursions in June and July 2020, in three temperate forests known for their high diversity in lichens and located in three different phytogeographic districts, with different temperature and rainfall regimes (Tables 1 and 2, Tables S1 and S2; Fig. S1 in the Supporting Information). The first two study sites (S1 and S2) were located in southern Belgium at 200 and 160 km from the sea, respectively, and characterized by a relatively continental climate: the Bohan-Membre park (S1) in Bohan (Vresse-sur-Semois, Belgium) in the Ardennes phytogeographic district, which mainly consisted of acidic siliceous rock outcrops (schists), and a relatively cold and moist climate, and the forest of Matignon (S2), in Treignes (Viroinval, Belgium) in the Meuse phytogeographic district, which was mainly characterized by the presence of calcareous rock outcrops (limestone) and by warmer and drier climate conditions than in the Ardennes (Lambinon and Verloove 2012). The Boulogne Forest study site (S3), in La Capelle-lès-Boulogne (France) in the Boullonais phytogeographic district, was located at c. 10 km from the sea and was characterized by an oceanic climate and clayey (marlous) soils. One specimen of free-living trentepohlioid alga was also collected (specimen LB20; Table S1). Substratum (tree species or siliceous or calcareous rock type, and surface shape), local ecological conditions (slope, tree circumference, light exposure, and humidity exposure) and height from the soil were recorded for each sample (Tables S1 and S2). Geographic coordinates and altitude per substratum were recorded with a GPS (GPSMAP 64s, Garmin). Meteorological data (minimal, maximal and average temperature, precipitations and relative humidity) were obtained from the Ourthes and Humain meteorological stations (provided by the Royal Belgian Institute of Meteorology) for study sites S1 and S2, and from the Rainghen and Bouloigne-sur-Mer-SEM meteorological stations (Météo-France publibl) for study site S3 (Table S3 in the Supporting Information). Sampled specimens were also classified following the Wirth climatic indices (i.e., light, temperature, continentality, and moisture; Wirth 2010).

Fresh material without any sign of superficial contamination was identified, stored at −20°C and used for chemical and molecular analyses. To enrich our dataset, 63 lichen specimens collected from previous excursions and stored in Meise Botanic Garden (BR), and sequences from GenBank for 399 accessions, from geographic regions covering a wide latitudinal and longitudinal span in all continents were added to the 88 specimens sampled in the three study sites (Tables S4 and S5 in the Supporting Information). All specimens were characterized, when possible, by the substratum (bark, rock, leaf, artificial), climatic variables (climatic zone, continentality, and moisture; Wirth 2010).

**pH measurements.** As the photobiont lives inside the mycobiont, the mycobiont can be considered as a separate microenvironment, different from the bark where it grows.

**Table 1.** Detail of the three study sites.

<table>
<thead>
<tr>
<th>Code</th>
<th>Country</th>
<th>Location</th>
<th>Toponym</th>
<th>Phytogeographic district</th>
<th>Soil type</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Belgium</td>
<td>Bohan</td>
<td>Parc de Bohan-Membre</td>
<td>Ardennes</td>
<td>Siliceous soil</td>
</tr>
<tr>
<td>S2</td>
<td>Belgium</td>
<td>Treignes</td>
<td>Bois de Matignon</td>
<td>Meuse</td>
<td>Calcareous soil</td>
</tr>
<tr>
<td>S3</td>
<td>France</td>
<td>La Capelle-lès-Boulogne</td>
<td>Forêt de Boulogne</td>
<td>Boulonais</td>
<td>Marlous soil</td>
</tr>
</tbody>
</table>

**Table 2.** List of the sampled substrata (tree species, rock) and geographic coordinates in the three study sites (S1, S2, S3). See Table S2 for more details.

<table>
<thead>
<tr>
<th>Substratum code</th>
<th>Geographic coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 Siliceous rock 1</td>
<td>49°53'0.5&quot; N; 4°53'18.0&quot; E</td>
</tr>
<tr>
<td>S2 Siliceous rock 3</td>
<td>49°53'0.0&quot; N; 4°53'18.9&quot; E</td>
</tr>
<tr>
<td>S3 Siliceous rock 5</td>
<td>49°53'0.5&quot; N; 4°53'18.5&quot; E</td>
</tr>
</tbody>
</table>
Therefore, the pH of selected samples of corticolous lichens (Coniocarpus cinnabarinum, Enterographa crassa, Graphis scripta, and Opegrapha vulgata, which were the most sampled species) was directly measured (Table 3). For this purpose, a small sample piece of ca. 1 cm³ was detached and moistened with a 25 mM of KCl solution for 5 min (Farmer et al. 1990). The pH was then measured with a HI99171 pH-meter, with a HI1141 flat-head electrode (HANNA) in contact with the moistened thallus.

**DNA extraction and amplification.** A method of direct-PCR was used (see Erz et al. 2015 who used this method successfully for the amplification of mycobionts) in order to avoid contaminations by co-occurring non-symbiotic algae (occasionally covering lichen thalli) that might be frequent when using standard DNA isolation protocols on large parts of lichen thalli. Hand-made sections of the lichen thallus were performed using a sterile razor blade. The lichen material was washed with a few drops of acetone on a sterile glass slide, then rinsed with distilled water to remove lichen secondary metabolites. The material was then added to a tube containing the PCR reaction mixture and amplified directly. Amplification reactions were prepared for a 50 µL final volume containing 31.75 µL of distilled water, 5 µL of 10× DreamTaq Buffer (Thermo Scientific, Waltham, MA, USA), 0.5 µL of each of the 20 µM primers, 4 µL of 2.5 mg · mL⁻¹ bovine serum albumin (#B14; Thermo Scientific), 4 µL of 2.5 mM each dNTPs (Thermo Scientific), 4 µL of MgCl₂ and 0.25 µL DreamTaq DNA polymerase (Thermo Scientific). Direct-PCRs were run in a T100 thermocycler (Bio-Rad, Hercules, CA, USA). Chloroplastic rbcL and ribosomal ITS algal loci were amplified and sequenced using primers rbcL-203F and rbcL-991R (Nelsen et al. 2011), under the GTR-GAMMA model. Node support was calculated using bootstrap replications with 1,000 bootstrap replications. A clade and its internal relations were assumed to be significantly supported if the bootstrap values (added near the internal branches) ≥ 70% (Mason-Gamer and Kellogg 1996). Only significantly supported lineages with at least three specimens were considered as clades. Specimens outside clades were considered as isolated haplotypes. Due to editing reasons, 38 excessive numbers of specimens from GenBank with an identical sequence were removed from the trees, but their clade and haplotype numbers were registered to be used for statistical analyses. These specimens are marked with an asterisk in Table S3.

**Phylogenetic analyses.** The sequences were aligned, together with sequences from GenBank (Table S5), using MAFFT v7.305 (Katoh et al. 2002) on the CIPRES Web Portal (Miller et al. 2011), and manually edited using Mesquite 3.61 (Madison and Maddison 2015). Terminal ends of sequences and ambiguously aligned regions were manually delimited and excluded from the datasets.

**Phylogenetic analysis was carried out using maximum likelihood (ML) inference on rbcL single-locus matrices.** The program RAxML has been used on the CIPRES Web portal (Miller et al. 2011), under the GTR-GAMMA model. Node supports of the maximum likelihood analyses were estimated by 1,000 bootstrap replications. A clade and its internal relations were assumed to be significantly supported if the bootstrap values (added near the internal branches) ≥ 70% (Mason-Gamer and Kellogg 1996). Only significantly supported lineages with at least three specimens were considered as clades. Specimens outside clades were considered as isolated haplotypes. Due to editing reasons, 38 excessive numbers of specimens from GenBank with an identical sequence were removed from the trees, but their clade and haplotype numbers were registered to be used for statistical analyses. These specimens are marked with an asterisk in Table S3.

### Table 3. pH of selected lichen specimens in the three study sites.

<table>
<thead>
<tr>
<th>Collection number</th>
<th>Lichen species</th>
<th>Study site</th>
<th>Substratum (Tree species)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB32</td>
<td>Coniocarpus cinnabarinum</td>
<td>S1</td>
<td>Corylus avellana 1</td>
<td>5.87</td>
</tr>
<tr>
<td>LB35</td>
<td>Opegrapha vulgata</td>
<td>S1</td>
<td>Corylus avellana 1</td>
<td>5.90</td>
</tr>
<tr>
<td>LB37</td>
<td>Coniocarpus cinnabarinum</td>
<td>S1</td>
<td>Corylus avellana 1</td>
<td>5.94</td>
</tr>
<tr>
<td>LB39</td>
<td>Graphis scripta</td>
<td>S1</td>
<td>Acer pseudoplatanus 1</td>
<td>5.27</td>
</tr>
<tr>
<td>LB42</td>
<td>Graphis scripta</td>
<td>S1</td>
<td>Acer pseudoplatanus 1</td>
<td>5.30</td>
</tr>
<tr>
<td>LB51</td>
<td>Coniocarpus cinnabarinum</td>
<td>S1</td>
<td>Corylus avellana 2</td>
<td>6.11</td>
</tr>
<tr>
<td>LB53</td>
<td>Graphis scripta</td>
<td>S1</td>
<td>Corylus avellana 2</td>
<td>6.04</td>
</tr>
<tr>
<td>LB54</td>
<td>Graphis scripta</td>
<td>S1</td>
<td>Corylus avellana 2</td>
<td>5.72</td>
</tr>
<tr>
<td>LB57</td>
<td>Opegrapha vulgata</td>
<td>S1</td>
<td>Carpinus betulus 3</td>
<td>5.89</td>
</tr>
<tr>
<td>LB59</td>
<td>Graphis scripta</td>
<td>S1</td>
<td>Carpinus betulus 3</td>
<td>5.49</td>
</tr>
<tr>
<td>LB62</td>
<td>Graphis scripta</td>
<td>S2</td>
<td>Fraxinus excelsior 1</td>
<td>5.40</td>
</tr>
<tr>
<td>LB73</td>
<td>Graphis scripta</td>
<td>S2</td>
<td>Carpinus betulus 1</td>
<td>5.15</td>
</tr>
<tr>
<td>LB84</td>
<td>Coniocarpus cinnabarinum</td>
<td>S3</td>
<td>Carpinus betulus 1</td>
<td>5.29</td>
</tr>
<tr>
<td>LB86</td>
<td>Graphis inustuloides</td>
<td>S3</td>
<td>Carpinus betulus 1</td>
<td>4.89</td>
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<tr>
<td>LB89</td>
<td>Graphis inustuloides</td>
<td>S3</td>
<td>Carpinus betulus 1</td>
<td>5.69</td>
</tr>
<tr>
<td>LB94</td>
<td>Graphis scripta</td>
<td>S3</td>
<td>Corylus avellana 1</td>
<td>5.69</td>
</tr>
<tr>
<td>LB96</td>
<td>Enterographa crassa</td>
<td>S3</td>
<td>Corylus avellana 1</td>
<td>5.76</td>
</tr>
<tr>
<td>LB97</td>
<td>Opegrapha vulgata</td>
<td>S3</td>
<td>Corylus avellana 1</td>
<td>6.00</td>
</tr>
<tr>
<td>LB100</td>
<td>Enterographa crassa</td>
<td>S3</td>
<td>Quercus robur 1</td>
<td>5.34</td>
</tr>
<tr>
<td>LB103</td>
<td>Enterographa crassa</td>
<td>S3</td>
<td>Carpinus betulus 2</td>
<td>5.38</td>
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<td>Enterographa crassa</td>
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<td>Carpinus betulus 2</td>
<td>5.73</td>
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<tr>
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<td>S3</td>
<td>Quercus robur 2</td>
<td>4.74</td>
</tr>
<tr>
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<td>Graphis scripta</td>
<td>S3</td>
<td>Fagus sylvatica</td>
<td>5.54</td>
</tr>
<tr>
<td>LB120</td>
<td>Graphis scripta</td>
<td>S3</td>
<td>Fagus sylvatica</td>
<td>4.76</td>
</tr>
<tr>
<td>LB124</td>
<td>Coniocarpus cinnabarinum</td>
<td>S3</td>
<td>Corylus avellana 2</td>
<td>5.65</td>
</tr>
<tr>
<td>LB125</td>
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<td>S3</td>
<td>Corylus avellana 2</td>
<td>5.46</td>
</tr>
<tr>
<td>LB127</td>
<td>Graphis scripta</td>
<td>S3</td>
<td>Corylus avellana 2</td>
<td>5.58</td>
</tr>
<tr>
<td>LB129</td>
<td>Opegrapha vulgata</td>
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<td>Corylus avellana 2</td>
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</tr>
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<td>Enterographa crassa</td>
<td>S3</td>
<td>Corylus avellana 2</td>
<td>5.32</td>
</tr>
<tr>
<td>LB134</td>
<td>Opegrapha vulgata</td>
<td>S3</td>
<td>Corylus avellana 3</td>
<td>5.73</td>
</tr>
<tr>
<td>LB135</td>
<td>Opegrapha vulgata</td>
<td>S3</td>
<td>Corylus avellana 3</td>
<td>5.74</td>
</tr>
</tbody>
</table>

(continued)
TABLE 3. (continued)

<table>
<thead>
<tr>
<th>Collection number</th>
<th>Lichen species</th>
<th>Study site</th>
<th>Substratum (Tree species)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB136</td>
<td>Enterographa crassa</td>
<td>S3</td>
<td>Corylus avellana 3</td>
<td>5.63</td>
</tr>
<tr>
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<td>Enterographa crassa</td>
<td>S3</td>
<td>Quercus robur 3</td>
<td>5.62</td>
</tr>
<tr>
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<td>Enterographa crassa</td>
<td>S3</td>
<td>Quercus robur 3</td>
<td>5.31</td>
</tr>
<tr>
<td>LB145</td>
<td>Opegrapha vulgata  crassa</td>
<td>S3</td>
<td>Carpinus betulus 3</td>
<td>6.10</td>
</tr>
<tr>
<td>LB149</td>
<td>Enterographa crassa</td>
<td>S3</td>
<td>Carpinus betulus 3</td>
<td>5.17</td>
</tr>
<tr>
<td>LB92</td>
<td>Opegrapha vulgata  crassa</td>
<td>S3</td>
<td>Corylus avellana 1</td>
<td>5.81</td>
</tr>
<tr>
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<td>Opegrapha vulgata  crassa</td>
<td>S3</td>
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<td>S3</td>
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<tr>
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<td>Enterographa crassa</td>
<td>S3</td>
<td>Corylus avellana 3</td>
<td>5.33</td>
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</table>

Some clades, mostly containing taxa from other studies, were collapsed using FigTree v. 1.4.2 (Rambaut 2012). The complete tree is available in supplementary material (Fig. S2 in the Supporting Information). All trees obtained with FigTree v. 1.4.2 (Rambaut 2012) were edited with Adobe Illustrator v. 25.41 (Adobe Inc. 2019). A bipartition network graph was constructed by using the Bipartite Network Analysis program (Greenville 2018) to visualize the associations between mycobionts and photobionts.

In addition to the rhlL phylogenetic tree, ITS single-locus subtrees were built (based on a maximum-likelihood analysis) for symbionts of specific fungal clades to elucidate the phylogenetic position of specimens for which only one ITS sequence was available. In these trees, only sequences from S1, S2, and S3 study sites were used (Figs. S3–S5 in the Supporting Information). A general ITS tree was not built due to the excessive number of ambiguous regions across the whole ITS dataset.

Rarefaction analyses. For rhlL clades with more than 10 specimens (including specimens from ITS trees with a certain correspondence in the rhlL tree), we performed rarefaction analyses on mycobiont abundance (counts) as a function of the number of samples per clade, at species, genus, and family levels using iNEXT online (Chao et al. 2016; Hsieh et al. 2016), to determine whether the sampling effort was enough to estimate selectivity in clades. A non-asymptotic approach based on extrapolation and extrapolation was applied with default options and 50 bootstrap replications to calculate 95% confidence intervals to obtain rarefaction curves.

Effect of lichen pH on photobiont distribution. Two-tailed Mann–Whitney U tests (McKnight and Najab 2010) were carried out to compare pH values between lichen species (Table S6 in the Supporting Information), tree species, and algal haplotypes from the different phylogenetic clades to which the selected species belonged.

Variation partitioning. To identify the relative contributions of substratum, climate, mycobiont (at order, family, genus, and species levels), and geographic location on the photobiont phylogeny, we performed variation partitioning in redundancy analysis in R (R Core Team 2018) using the varpart function in vegan R package version 2.5.7 (Oksanen et al. 2020). This analysis was made separately on the total and local (study sites S1, S2, and S3) datasets. The phylogenetic distances of the total and local datasets were calculated and a distance matrix was made using the online program PATRISTIC (Fournier and Gibbs 2006). These were transformed into principal coordinate analysis (PCoA) components using the pcoa function in ape R package version 5.5 (Paradis 2012, Paradis and Schliep 2019). Two hundred and twenty-one and 12 PCoA axes that showed positive eigenvalues were used for the RDA analyses. Principal component analyses (PCA) were performed using the rda function in Vegan, separately on substratum, climate, and mycobionts (either at the order, family, genus, or species level), coded as presence/absence dummy variables. For the local dataset, Wirth climatic indices were used as climate data. The number of resulting PCA axes per variable is included in Table S7 in the Supporting Information. The geographic distance matrices were transformed into principal coordinates of neighbour- hood matrix (PCNM) components using penn function in Vegan (Borcard and Legendre 2002; Borcard et al. 2011), the 31 and 6 PCNM components showing positive eigenvalues, respectively, were kept for the RDA analyses. Samples with missing ecological, geographic, or mycobiont data were excluded from the analyses, resulting in a dataset of 332 and 72 samples for the total and local datasets, respectively.

Phylogenetic signal. The degree of phylogenetic signal for ecological traits (substratum and climate) was measured on the total dataset by calculating Pagel’s lambda (λ) index (Pagel 1999) using the fitDiscrete function in the geiger R package version 2.0.7, which was designed for categorical trait variables (Pennell et al. 2014). The λ value can vary from 0 (lack of phylogenetic signal in the trait) to 1 (strong phylogenetic signal; Münkemüller et al. 2012). The best model (ER, SYM or ARD) was selected using the lowest corrected Akaike Information Criterion (AICc) value (Akaik 1973). To test for significance of the λ value, we performed a likelihood ratio test (Münkemüller et al. 2012), approximated by a χ² distribution that compared the negative log likelihood value with no phylogenetic signal (i.e., λ = 0, calculated using the “white” transformation of the fitDiscrete function) with negative log likelihood value of the λ value obtained for the original tree topology, using the lr.test function in the extRemes R package version 2.0 (Gilleland and Katz 2016).

RESULTS

Phylogenetic trees. In total, 195 algal sequences were generated using 151 samples. The rhlL phylogenetic tree was based on 427 sequences and 883 unambiguously aligned sites. One hundred and twenty-six of these sequences were from this study (74 from S1, S2, and S3 study sites and 52 from samples of previous excursions; Tables S1, S4, and S5). Thirty-two well-supported clades based on ML were distinguished (Fig. 1). The main clades, with the majority of our specimens, are shown in detail in Figures 2–4 (for the complete phylogenetic tree, see Fig. S2).

From the three ITS trees focusing on particular groups, the first one included specimens from Arthoniaceae and Graphis scripta (Fig. S3); the second one contained representatives of clade 31, Enterographa and Opegrapha and Porina, and another lineage with three other Porina specimens (Fig. S4); the last one was dedicated to specimens from Mediterranean-type ecosystems (Fig. S5). A total of
CLADE FIGURE(S)

1. 2, S2, S3
2. S2
3. 2, S2, S3
4. S2
5. S2
6. S2
7. S2
8. S2
9. S2
10. S2
11. S2
12. S2
13. S2
14. S2
15. 2, S2, S3
16. S2
17. S2
18. S2
19. S2
20. S2
21. S2
22. S2
23. S2
24. S2
25. 4, S2, S5
26. S2
27. 3, S2, S5
28. S2
29. 4, S2
30. S2
31. 4, S2, S4
32. S2

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69 sequences were used (24, 22, and 23 sequences, excluding the outgroups, and 1252, 1295 and 1416 unambiguously aligned sites in the three trees, respectively). The sequences allowed to include 26 additional specimens for which only ITS sequences were available (Figs. S3–S5). These results allowed to refer 17 specimens to clades of the rbcL tree, while the position of nine others did not permit to determine to which rbcL group they belong. Seven of these sequences formed two independent lineages in the ITS trees, composed of three and four photobionts from Porina leptalea and Alyxia sp., respectively.

**Photobiont diversity.** The rarefaction analyses on the 16 clades comprising more than 10 specimens showed that sampling effort was not enough (curve not asymptotic) for four clades (4, 11, 12, and 13) at the species level, and one clade (4) at the genus level (Fig. S6 in the Supporting Information), so that the number of specimens could be considered as representative of mycobiont host richness for most of the clades (Fig. S6). Among the 32 algal clades, clades 29 and 31 represented unknown lineages of algae associated with Enterographa crassa, *E. hutchinsiae*, *E. pitardii*, Mazosia carnea, *Opegrapha vernicellifera*, *O. vulgata*, and *Porina leptalea* (Figs. 4 and S4 and S5). Clade 25 (Figs. 4 and S5) included an unknown small group composed of sequences from the photobionts of two *Divina fallax*, one *Arth-onia meridionalis* and one *A. trifurcata*. In addition, clade 27 revealed two additional haplotypes (27-1 and 27-2; Tables S1 and S4), reported in several European countries, suggesting a wide geographic distribution in Europe (Fig. 3). Two additional haplotypes could be found in clades 15 and 24, exclusively composed of specimens from France (Figs. 1 and 2 and S2). Other haplotypes are in isolated positions (Figs. 1–4 and S2).

Only 10 haplotypes were found in study site S2 (belonging to clades 1, 25, 27, and 28; with two additional isolated haplotypes), while in S1 there were 21 haplotypes (belonging to clades 1, 3, 27, and 31; with three additional isolated haplotypes). The richest study site was S3, with 28 haplotypes (belonging to clades 1, 3, 15, 24, and 29). Some haplotypes (like some specimens from clade 1, 3, and 25, and the saxicolous *Roccella*’s of clade 27) could be found on both calcareous and siliceous rocks and on bark of several tree species, suggesting a wide ecological amplitude.

Lichenized photobionts tended to be separated from free-living algae (e.g., clade 28, with only free-living algae; clades 7, 18, 27, and 31 with exclusively lichenized algae; Figs. 1–3 and S2). Other clades were composed of both free-living and lichenized algae: five of them (1, 3, 10, 21, 25) contained haplotypes with both lifestyles, while in the remaining clades (2, 6, 9, 11, 12, 15, 19, 22, 24, 32) free-living algae did not belong to the same haplotype than lichenized ones (Fig. S2).

The analyses of the trentepohlioid lichen communities of one single tree trunk in the three study sites revealed the presence of several Trentepohliales species, generally belonging to at least two different clades (Table 4), which illustrated that fungal species living in close proximity were not all associated with the same trentepohlioid taxon. The highest diversity was found on the trees *Carpinus betulus* 1 and *Fagus sylvatica* 1 in study site S3, where four different haplotypes of photobiont were detected (Table S1). On the sampled trunks or rocks, there was no visible evidence of the presence of free-living algal colonies. Several algal haplotypes could also be found within a single lichen species, from two in *Opegrapha vulgata*, to five in *Coniocarpon cinnabarinum*. The phylogenetic distances between photobionts in a single lichen species were also variable, as algae from *O. vulgata* belonged to the same clade (31), while those from *Arthonia atra*, *Graphis scripta*, and *C. cinnabarinum* belonged to different groups (see below). Moreover, in our localities, we found three cases where two specimens of the same mycobiont species occurring on the same trunk contained different algal haplotypes from different clades: for *Alyxia varia*, *C. cinnabarinum*, and *G. scripta* (Figs. 2 and S2; Table S1). In addition, within the same clade, several haplotypes were associated with the same mycobiont (Figs. 2, 5 and Fig. S7 in the Supporting Information), like in *C. cinnabarinum* (clade 1). Contrary to *A. atra*, *C. cinnabarinum*, and *G. scripta*, all *Roccella* haplotypes were grouped in the same clade (27; Figs. 3, 5, and S7); it was also the case for *Enterographa crassa* and *O. vulgata* haplotypes (clade 31; Figs. 4, 5, and S7).
Mycobiont and photobiont selectivity. No reciprocal monophyly was observed between algal and fungal orders, families, or genera. However, in 14 clades, the photobiont was associated with mycobionts from only Ostropales or Arthoniales orders, or even from one single genus (e.g., Astrothelium; Fig. S2, Table 5). Trentepohliales showed, as expected, a low selectivity, as each lineage was able to associate with several mycobiont species. The only few exceptions concerned photobionts associated with sterile thalli such as Dichosporidium nigrocinctum, with the majority of Herptheallaminae species, which formed very close and well supported clades (clades 7, 18, and 32; Fig. S2). Photobionts of clade 12 were also selective, as they only occurred in the lichen genus Astrothelium (Nelsen et al. 2011, Kosecka et al. 2020). Clade 27 contains algae previously considered to be exclusively in symbiosis with the Roccellaceae family (Dendrographha, Divina, Diromnia, Roccella; Hametner et al. 2014a, Kosecka et al. 2020). These haplotypes were also associated with lichens belonging to other families of Arthoniales (Fig. 3; Table S1), such as Opegraphaceae (Opegrapha, Sparria), Lecanographaceae (Alyxia, Lecanographa), and Roccelloraphaceae (Roccellographa). Only photobionts from Arthoniaceae were absent from clade 27 (Fig. 3). Other clades (e.g., clades 5 and 30) were composed of photobionts associated with only one fungal genus (Herptheallan and Porina), but were represented by few individuals and could therefore be under-sampled.

Several species of mycobionts showed a high selectivity: all lichenized algae from Enterographra cressa, E. hutchinsiae, Opegrapha vermicelliifera, and O. vulgata belonged to clade 31 (Fig. 4). Moreover, clade 27 contained several photobionts, with fungal species not found elsewhere in the phylogenetic tree: Lecanographa amylacea, all Roccella, and all Synesia; other species were also only present in that clade, but were represented by only one specimen.

Other mycobionts hosted different algal species (Table 6; Figs. 5 and S7). Clade 1 included the majority of the photobionts of Graphis scripta, Arthonia atra, and Coniocarpon cinnabaratum from all study sites (Table S1), from previous excursions (Table S4), and other studies (Table S5). Some of them were not in clade 1, but could be found in other clades (clades 3 and 15, or isolated position; Figs. 2 and S2), showing that the mycobiont could associate with several algae, even phylogenetically distant ones. As for specimens of A. atra, C. cinnabaratum, and G. scripta, specimens from Dendrographha decolorans and A. varia were not in the same group (clade 27 vs. clade 25 for the firsts; clade 25 vs. isolated position for the seconds; Figs. 3 and S2, Table S1). The same was observed with specimens from Divina (clade 27 vs. clade 25; Figs. 3 and 4 and Tables S1 and S4), but from different countries.

In clade 1, photobionts associated with mycobionts belonging to Arthoniales and Ostropales orders were closely related (Kosecka et al. 2020). Two Arthoniales (Arthonia atra and Coniocarpon cinnabaratum) and most of the specimens of Graphis scripta (Ostropales) from Belgium clustered with algal specimens of G. scripta, Graphis elegans, and C. cinnabaratum from other countries (UK and Austria). This tendency was also visible in other clades (clades 2, 3, 4, 5, 7, 8, 11, 15, 16, 18, 20, 24, 25, 27, 29, 31, and 32). Specimens of Sperodophoron cretaceum, A. atra, and G. inustuloides were in the same group of algae from other Ostropales and Arthoniales (clade 15). Moreover, two specimens of Reichlingia leopoldii, one specimen of Diarthonis spadicea and one of G. scripta were in clade 3, together with other Arthoniales (Fig. 2; Table S1). Algae in symbiosis with mycobionts from other orders (especially Pyrenulales) were also present in these clades.

Fungal specimens from Arthoniaceae tended to be separated from other Arthoniales families. No representative of Arthoniaceae was observed within the seven clades (22, 23, 25, 27, 29, 31, and 32) where algal partners from other families of Arthoniales were essentially found. Only four exceptions were noted: (i) in clade 24, where three specimens of alga from Lecanactis ahietina shared the same haplotype with one photobiont from Arthonia radiata from the United Kingdom (Fig. S2); (ii) in clade 25, where two algal specimens of A. meridionalis and A. trifascata were close to two Divina photobionts (Figs. 4 and S5); and (iii-iv) photobionts from two Sagenidiopsis specimens, which were found in clades 18 and 2, respectively, together with other algae from Arthoniaceae mycobionts (Fig. S2). In addition, in clades with photobionts from both Arthoniales and Ostropales, algal host from Arthoniaceae
Figure 3. Majority-rule consensus tree of trentepohlioid algae of clade 27, based on a dataset of rbcL sequences that resulted from a RaxML analysis under a GTR-GAMMA model. Bootstrap support values were estimated using 1000 replications and are shown near the nodes. Each branch considered strongly (bootstrap ≥ 70%) is represented by a thick line. For symbols and specimen names description, see Figure 2 legend. [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 4. Majority-rule consensus tree of trentepohlioid algae of clades 25, 29, and 31, based on a dataset of \textit{rbcL} sequences that resulted from a RaxML analysis under a GTR-GAMMA model. Bootstrap support values were estimated using 1000 replications and are shown near the nodes. Each branch considered strongly (bootstrap \(\geq 70\%\)) is represented by a thick line. For symbols and specimen names description, see Figure 2 legend. [Color figure can be viewed at wileyonlinelibrary.com]
were linked to photobionts from *Graphis* s. l. (clades 1, 15, or 18), while in clades with algae from other Arthoniales families (clades 31 and 32), only one *Ocellularia* (thelotremoid Graphidaceae) and one Porinaceae were found.

**Climatic, substratum, and geographic trends.** Venn diagrams obtained by variation partitioning showed a clear climatic, rather than geographic, pattern shaping the phylogenetic structure (explaining up to 42% of the variance), at wide and local scales (Fig. 6), with some clades only including sequences either from various tropical regions of the world (e.g., clades 7, 18, and 32; Figs. 1 and S2) or from Mediterranean-type regions (clades 25 and clade 27; Figs. 3 and 4) or from temperate zones (e.g., clades 7, 18, and 32; Figs. 1 and S2) or from tropical regions of the world (e.g., clades 3, 15, 25, 27, or 31; Figs. 2–4, S2 and S4 and S5). Some algal haplotypes also occurred in both corticolous and saxicolous lichens, like, for example, in *Roccella* (clade 27; Fig. 3) or in *Enterographa hutchinsiae* (clade 31; Figs. 4 and S4). The mycobiont host (especially at species, genus, and family levels of the fungal host) appeared to be determinant in explaining the phylogenetic structure of the algae, but also its ecological patterns, given the high proportion of phylogenetic variance explained by mycobiont together with climate for both scales, and a substratum effect only substantial when co-varying with mycobiont, especially at local scale.

### pH measurements

The pH of selected lichen species ranged from 4.74 to 6.11 and significantly differed between some lichens (*Enterographa vulgaris-Enterographa crassa*: $U = 13$, $ddl = 10$ and 11, $P < 0.005$; *O. vulgaris-Graphis scripta*: $U = 21$, $ddl = 10$ and 12, $P < 0.02$). However, no significant difference ($U = 168.5$, $ddl = 18$ and 21, $P > 0.2$) was found between phylogenetic clades (i.e., clades 1, 3, 7, 15, 25, 27, and 31).

### Table 4. Number of haplotypes per substratum (tree species, rock) and in the three study sites (S1, S2, S3). For each substratum, the number of sampled fungal specimens (column 3), the number of sampled mycobiont species (column 4), the number of algal clades to which photobionts belong (column 5), and the number of algal haplotypes found in fungal specimens (column 6) is indicated. The two substrata in grey show the highest number of haplotypes. See Table S2 for more details on substrata.

<table>
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<th>Locality</th>
<th>Substratum</th>
<th>Number of specimens</th>
<th>Number of mycobionts</th>
<th>Number of algal clades</th>
<th>Number of haplotypes</th>
<th>Total number of haplotypes/Locality</th>
</tr>
</thead>
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</table>

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**LICHENIZED TRENTEPOHIOID DIVERSITY**
FIGURE 5. Interaction network structure between photobiont haplotypes and mycobiont species using a Bipartition network analysis and showing the degree of selectivity of mycobionts from some Arthoniaceae and Graphidaceae (a), other families from Arthoniales (b) and species from the new clade 31 (c). The algal haplotypes are on the bottom, the mycobionts on the top. The two numbers of each algal haplotype are the clade and the haplotype numbers, respectively. For each clade, each algal haplotype was counted from the top to the bottom in the phylogenetic tree (Figs. 2–4 and S2). Mycobionts linked with an additional isolated haplotype are marked with an asterisk (*). The width of the links is proportional to the number of specimens forming the association. The interaction network showing the totality of specimens found in the phylogenetic tree is in Figure S7. [Color figure can be viewed at wileyonlinelibrary.com]
and 15 with Coniocarpon cinnabarinum and G. scripta on one hand, and clade 31 with E. crassa and O. vulgaris on the other hand; Tables 3 and S6).

**DISCUSSION**

**Photobiont diversity.** The phylogenetic diversity of photobionts in Belgium and northern France was surprisingly high, despite the restricted studied geographic areas, even at the tree scale (up to four haplotypes on one single tree), and likely underestimated as only the lowest part of the tree trunk has been sampled and a considerable proportion of the lichen diversity is usually present in the upper part (e.g., Aptroot 1997, Komposch and Hafellner 2000, Kaufmann et al. 2019). The only other study that reported several trentepohlioid haplotypes on a single tree was Nelsen et al. (2011), who found six algal haplotypes from seven Trypetheliaceae species on one tree in the tropics.

The discovery of still unknown lineages in our study confirms, as in other recent studies (e.g., Zhu et al. 2018, Klimešová et al. 2019, Kosecka et al. 2020), that algal diversity in Trentepohliales is cryptic and hence still largely underestimated. Several haplotypes belonging to different main lineages coexist in the three study sites and are shared by different groups of mycobionts. This means that several lichen “guilds” (as defined by Rikkinen et al. 2002; i.e., a group of lichens that share a common pool of photosynthetic partners from a determined substratum) are present in corticolous lichen communities.

Our results indicate that photobiont diversity in lichen thalli depends on the mycobiont species. While some of them (e.g., Enterographa crassa, Opegrapha vulgaris) associate with only one algal haplotype (or two very similar haplotypes), other lichen species show a higher photobiont diversity and associate with different and phylogenetically distant algae (e.g., Alyxia varia, Anthoxania atrata, Coniocarpon cinnabarinum, Dendrographa decolorans, Graphis scripta). This switch did not only occur in one study site (e.g., photobionts from G. scripta belonging to clades 1 and 3, or photobionts from C. cinnabarinum belonging to clades 1 and 15), but also on the same trunk, such as for A. varia, C. cinnabarinum and G. scripta. The ability of lichens to switch between several, sometimes phylogenetically distant, photobionts, has been widely demonstrated in cyanolichens and lichens with a trebouxioid photobiont (e.g., White and James 1988, Fernández-Mendoza et al. 2011, Magain and Sérusiaux 2014, Voytek and Beck 2016, Ertz et al. 2018, Ranft et al. 2018, Peksa et al. 2022).

**Mycobiont and photobiont selectivity.** Lichenized trentepohlioid photobionts form a polyphyletic group, suggesting that association with lichens occurred multiple times, as reported in previous

**Table 5. Number of haplotypes per clade (see also Fig. S2), with degree of selectivity of photobionts, that is, the range of fungal order, family, genus, or species to which alga associates (– = not selective)**

<table>
<thead>
<tr>
<th>Clade number</th>
<th>Number of haplotypes</th>
<th>Fungal taxa present in the clade</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>Ostropales</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>Herpoliophillum</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>Ostropales (one haplotype</td>
</tr>
<tr>
<td></td>
<td></td>
<td>exclusively with Diorygma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>antillarum)</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>Ostropales</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>11</td>
<td>All lichenized algae with</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Coenogonium</td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>Ostropales</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>Astrethelium (except free-living</td>
</tr>
<tr>
<td></td>
<td></td>
<td>algae)</td>
</tr>
<tr>
<td>13</td>
<td>9</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
<td>11</td>
<td>–</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>Graphidaceae</td>
</tr>
<tr>
<td>17</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>18</td>
<td>24</td>
<td>–</td>
</tr>
<tr>
<td>19</td>
<td>11</td>
<td>–</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>Graphidaceae</td>
</tr>
<tr>
<td>21</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>22</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>23</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>24</td>
<td>30</td>
<td>–</td>
</tr>
<tr>
<td>25</td>
<td>7</td>
<td>Arthoniales (except free-living</td>
</tr>
<tr>
<td></td>
<td></td>
<td>algae)</td>
</tr>
<tr>
<td>26</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>27</td>
<td>15</td>
<td>Arthoniales</td>
</tr>
<tr>
<td>28</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>29</td>
<td>2</td>
<td>Roccellaceae</td>
</tr>
<tr>
<td>30</td>
<td>3</td>
<td>Porina</td>
</tr>
<tr>
<td>31</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>32</td>
<td>8</td>
<td>Dichosporidium (except free-living</td>
</tr>
<tr>
<td></td>
<td></td>
<td>algae)</td>
</tr>
<tr>
<td>Mean</td>
<td>6.7</td>
<td>–</td>
</tr>
</tbody>
</table>

and 15 with Coniocarpon cinnabarinum and G. scripta on one hand, and clade 31 with E. crassa and O. vulgaris on the other hand; Tables 3 and S6).

**Table 6. Number of algal haplotypes in selected lichen species, with their position in the phylogenetic tree (Fig. S2). The last two columns indicate the number of clades to which haplotypes belong and how many haplotypes are in isolated position.**

<table>
<thead>
<tr>
<th>Lichen species</th>
<th>Number of haplotypes</th>
<th>Number of clades</th>
<th>Isolated position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arthonia atra</td>
<td>5</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Alyxia varia</td>
<td>3</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Coniocarpon</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>cinnabarinum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dichosporidium</td>
<td>6</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>nigrocinenum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diorygma antillarum</td>
<td>1</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Enterographa crassa</td>
<td>2</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Enterographa</td>
<td>1</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>hutchinsiae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Graphis scripta</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Opegrapha vulgaris</td>
<td>2</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Roccella sp.</td>
<td>8</td>
<td>1</td>
<td>–</td>
</tr>
</tbody>
</table>
studies (Nelsen et al. 2011, 2020, Hametner et al. 2014a,b, Kosecka et al. 2020). This pattern is quite similar to the fungal partners for which lichenization also appeared several times during evolution. However, contrary to the algae, lichenized lifestyle is usually irreversible in mycobionts (Lutzoni and Miadlikowska 2009, Meeßen and Ott, 2013), although some mycobionts in the genera *Stictis* and *Conotrema* (Stictidaceae; Ostropales) easily switched between lichenized and saprotrophic lifestyles (Wedin et al. 2004). Interestingly, lichenized and free-living algae tend to cluster in separate clades. Coevolution between mycobionts and photobionts as well as switching between the free-living and lichenized lifestyles might have driven the evolution of Trentepohliales and explain the high cryptic diversity observed. Indeed, the less the photobionts switch between free-living and lichenized lifestyles, the more the lichenized algae will differentiate from exclusively free-living algae; and the less the mycobionts/photobionts switch between photosynthetic/fungal partners, the more their symbiosis is specific and the more they will differentiate from other closely related haplotypes or species. Similar observations were made for the role of lifestyle switching in the evolution of mycobionts, like in Stictidaceae (Thiyagaraja et al. 2021).

Our results show that the degrees of selectivity and specificity (according to Beck et al. 2002) of mycobionts and photobionts are variable, which

![Venn’s diagrams showing variation partitioning results on complete and local datasets, at mycobiont order, family, genus, and species level.](wileyonlinelibrary.com)

**Figure 6.** Venn’s diagrams showing variation partitioning results on complete and local datasets, at mycobiont order, family, genus, and species level. [Color figure can be viewed at wileyonlinelibrary.com]
Several selectivity and specificity patterns were observed in our study. First, the high number of clades including photobionts from both Arthoniales and Ostropales lichens indicates that their mycobionts have a low selectivity at high taxonomic levels. Even one fungal species might associate with several algal haplotypes belonging to more than one clade, like for *Arthonia atra*, *Graphis scripta*, and *Coniocarpon cinnabarinum*. However, the majority of specimens of *C. cinnabarinum* and *G. scripta* are in one clade, which means that the algal species of that clade should be the primary photobiont of these species. Some photobiont lineages are not selective, like the one from Clade 27 that includes a wide array of Roccellaceae (*Dendrographa*, *Dirina*, *Diromma*, *Roccella*, *Synecia*), but also of other lichen families of Arthoniales (Lecanographaceae, Opegraphaceae, Roccellographaceae). Those samples also covered a wide geographic range (mainly Europe and its Atlantic islands, but also the Caribbean, Bolivia, Panama, and Madagascar). Interestingly, all *Roccella* and *Sparria* share two very similar algal haplotypes (differing only by two nucleotides) and are therefore selective for these haplotypes; but the other genera of lichenized fungi of Arthoniales and Ostropales lichens show a larger flexibility for some fungal families: photobionts from *Roccella s. l.* are more closely related than algae from thelotreloid Graphidaceae (*Hametner et al. 2014b*).

Finally, some algal strains have a nearly exclusive selectivity to one single fungal genus (*Astrothelium*) or species (*Diorygma antillarum* and *Dichosporidium nigroinctum*, respectively), which are also exclusively selective of these photobiont haplotypes. This makes their association nearly specific (following *Beck et al. 2002*). These species mainly have sterile thalli, which may explain why the algal diversity is low compared to fertile thalli: photobionts are conserved from the mother thallus when lichens reproduce with vegetative propagules (but exceptions were found; *Nelsen and Gargas 2008*). The probability of photobiont switching is therefore higher in fertile thalli (*Lücking et al. 2009, Peksa and Skaloud 2011, Voytsekhovich and Beck 2016, Kosecka et al. 2020*). The mycobiont flexibility allows the fungus to associate with secondary photobionts when the primary one is absent or scarce (*Peksa and Skaloud 2011, Voytsekhovich and Beck 2016*), as partly shown by some of our *Dirina* specimens (clade 25).

If the alga has a limited geographic distribution, the mycobiont may have a different primary photobiont in other localities (*Rolshausen et al. 2020*). These evidences of the contribution of the mycobiont phylogeny to the shaping of the photobiont phylogenetic structure are highly supported by the variation partitioning analyses.

*The photobiont phylogenetic patterns are driven by climate.* The overall phylogenetic structure of the studied algae shows a climatic pattern at a wide geographic scale, with climatically highly differentiated clades, likely related to the combination of temperature and humidity (*Helms 2003, Blaha et al. 2006, Fedrowitz et al. 2012, Nyati et al. 2014, Kosecka et al. 2020, Rolshausen et al. 2020*). It can be noted that *Dirina massiliensis*, which occurs in Mediterranean xero-thermophilous calcareous substrata, was found in Belgium (temperate region), and share the same photobiont as specimens from the Mediterranean region. Similarly, *Graphis scripta* and *Coniocarpon cinnabarinum* were found in both temperate and continental regions (*Hametner et al. 2014a*).

Climatic patterns in alga phylogeographic structure were also observed at a local scale. However, only three study sites were sampled, so that additional sites within this geographic range are needed to separate climate from geographic effects on these haplotypes (some of them were only found in the oceanic S3 study site), and to detect eventual local differentiations and endemism, as highlighted in trebouxoid photobionts (*Ruprecht et al. 2020*). Another study showed that trentepohlioid lichens were influenced by local humidity conditions in Spain (*Prieto et al. 2017*).

*Effect of substratum.* Substratum (bark, rock, or plant) may be expected to influence the distribution of Trentepohliales (*Brooks et al. 2015, Grube et al. 2017, Kosecka et al. 2020*). Surprisingly, our
results showed no evidence of direct substantial contribution of substratum to the phylogenetic structure of Trentepohliales when taking other factors (climate, mycobiont) into account. The only significant exception was a shared proportion of variance with the mycobiont at genus and species levels (9%), which can be expected, as the mycobiont is strongly linked to the substratum. However, the phylogenetic signal linked to the substratum is significant, even if weaker than the one linked to the climate. This is consistent with the specialization of foliolicous Trentepohliales, as previous studies showed that the genera *Phycopeltis* and *Cephaloeca* are essentially restricted to leaves (Printz 1939, Thompson and Wujek 1997, Grube et al. 2017, Kosecka et al. 2020). In contrast, there was no clear separation between saxicolous and corticolous Trentepohliales algae, unlike Cyanobacteria on bark and soil substrata (Rikkenen et al. 2002), or mycobionts themselves (Resl et al. 2018). Some lichens of this study were able to colonize both bark and rock substrata, without changing algae, like the crustose lichen *Enterographa hitchiniae* or some species of the fruticose genus *Roccella*. In our study area, several trentepohlioid algae were found indifferently on trees and on calcareous and siliceous rocks. The studies of Helms (2003) and Peksa et al. (2022) on saxicolous lichenized trebouxoid photobionts showed that algae from calcareous rocks formed a separate lineage from acidophillus algae (on siliceous rock), suggesting that rock type may play a role in the differentiation. However, our saxicolous samples are probably too limited to show a difference between calcareous and siliceous rocks in the present study. Some clades are composed of exclusively corticolous algae, but there should be a bias in the sampling, with much more corticolous specimens collected. Therefore, additional sampling of saxicolous and foliicolous free-living and lichenized algae might be carried out to verify if these clades are really substratum-specific.

The substratum definition might also be too imprecise, and might be rather characterized by its microclimate conditions (e.g., light or rain exposure), as some lichen species are known to have a preference for microhabitats sheltered from rain (e.g., *Dirina, Roccella*), contrary to others (e.g., *Enterographa, Opegrapha*; Smith et al. 2009), which could also be the case for photobionts. Other ecological variables might also play a role in algal distribution such as light availability and inputs of acidic and nitrous substances, as demonstrated for mycobionts (Kaufmann et al. 2019). Besides, the lichen itself can be considered as a micro-ecosystem (Hawkworth and Grube 2020), with its local internal conditions, which can be different from the bark substratum. Corticolous photobionts are not influenced by the pH of the lichen thallus. This tolerance to support significantly different pH ranges could be a consequence of living inside lichens.

**CONCLUSIONS AND PERSPECTIVES**

Our findings enlighten the interest of conducting a very detailed study for taxonomical purposes. Indeed, our study has permitted to discover a highly diverse alga community at very restricted scales, with still unknown lineages, and has deepened the knowledge of the selectivity of photobionts in their fungal partner choice (and vice-versa) and of the ecological factors influencing photobiont phylogenetic structure. Future research should therefore concentrate on areas rich in trentepohlioid lichens (e.g., oceanic islands, Mediterranean-type, and tropical regions), with exhaustive sampling of rocks, trees, and other plant surfaces, to improve our knowledge of Trentepohliales diversity and of the geographic and climatic ranges of each haplotype. A second approach could consist in sampling targeted widespread species (e.g., *Graphis scripta*, *Opegrapha vulgata*) throughout their geographic distribution to understand their evolution in function of changing ecological (e.g., climate) conditions. Finally, the absence of morphological traits in the asexual photobiont species makes their identifications difficult. Therefore, an accurate revision of the phylogeny of the Trentepohliales at genus level is absolutely necessary, using the phylogenetic species concept (Leliaert et al. 2014) to give a scientific name to unnamed lineages. This is particularly important for implementing measures for the conservation of this high genetic diversity.

Resilience to climate change represents a challenge for many species, including in lichen communities (e.g., Aptroot et al. 2021). The trentepohlioid algae and their fungal partners clearly show a phylogenetic structure shaped by climatic conditions at wide scale. Our study at local scale is based on a too restricted geographic range to make the same conclusion, but given the increasing number of lichens with trentepohlioid photobiont recorded in temperate regions (Aptroot and Van Herk 2007, Van Den Broeck 2010, Nelsen et al. 2011), we may expect evolutionary processes and conservation issues related to global warming.

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The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

L. Borgato: Conceptualization (equal); Data curation (lead); Formal analysis (equal); Funding acquisition (equal); Investigation (lead); Methodology (equal); Resources (supporting); Supervision (equal); Visualization (lead); Writing – original draft (lead); Writing – review & editing (equal). D. Ertz: Conceptualization (equal); Data curation (supporting); Formal analysis (supporting); Funding acquisition (equal); Investigation (supporting); Methodology (equal); Project administration (supporting); Resources (lead); Supervision (equal); Validation (equal); Writing – review & editing (equal). F. Van Rossum: Formal analysis (equal); Resources (supporting); Validation (equal); Writing – review & editing (equal). A. Verbeke: Conceptualization (equal); Funding acquisition (equal); Methodology (equal); Project administration (lead); Supervision (equal); Validation (equal); Writing – review & editing (equal).


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**Figure S5.** Majority-rule consensus tree of Trentepohlioid algae of clades 25 and 27, and another clade not represented in the *rbcL* tree, from ML analysis based on ITS data set with bootstrap support values from RaxML analysis presented near the nodes.

**Figure S6.** Rarefaction curves of the 16 clades with more than 10 specimens (including specimens from ITS trees), showing the sampling coverage of mycobiont host at species (a), genus (b), and family (c) level.

**Figure S7.** Interaction network structure between photobiont haplotypes and mycobiont species using a Bipartition network analysis.

**Table S1.** List of specimens collected in the three sampled localities (S1–S3).

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**Table S2.** List of sampled substrata.

**Table S3.** Meteorological data from the four meteo-stations.

**Table S4.** List of specimens collected in previous excursions.

**Table S5.** List of *rbcL* sequences retrieved from GenBank.

**Table S6.** Results of Mann–Whitney tests on lichens’ pH (U = Mann–Whitney critical value; df = degrees of freedom of the two datasets).

**Table S7.** Number of principal components axes used in the PCA.