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The co-dispersal strategy of *Endocarpon* (Verrucariaceae) shapes an unusual lichen population structure

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Abstract

The reproduction and dispersal strategies of lichens play a major role in shaping their population structure and photobiont diversity. Sexual reproduction, which is common, leads to high lichen genetic diversity and low photobiont selectivity. However, the lichen genus *Endocarpon* adopts a special co-dispersal model in which algal cells from the photobiont and ascospores from the mycobiont are released together into the environment. To explore the dispersal strategy impact on population structures, a total of 62 *Endocarpon* individuals and 12 related Verrucariaceae genera individuals, representing co-dispersal strategy and conventional independent dispersal mode were studied. Phylogenetic analysis revealed that *Endocarpon*, with a large-scale geographical distribution, showed an extremely high specificity of symbiotic associations with their photobiont. Furthermore, three types of group I intron at 1769 site have been found in most *Endocarpon* mycobionts, which showed a high variety of group I intron in the same insertion site even in the same species collected from one location. This study suggested that the ascospore-alga co-dispersal mode of *Endocarpon* resulted in this unusual mycobiont-photobiont relationship; also provided an evidence for the horizontal transfer of group I intron that may suggest the origin of the complexity and diversity of lichen symbiotic associations.

Keywords: group I intron, mycobiont, photobiont, symbiosis

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1. Introduction

Lichens are defined as highly specific mutualistic symbiotic associations between lichen-forming fungi (the mycobiont) and their algal partners (the photobiont, usually green algae and/or cyanobacteria) (Nash, 2008). The patterns of association between the mycobionts and photobionts are described by the degree of specificity, i.e. the phylogenetic range of associated partners, and of selectivity, i.e. the frequency of association among partners (Yahr et al., 2004). There are varying degrees of specificity for photobionts to maintain the co-evolution of lichen symbioses. While some mycobiont species can associate with a wide range of photobiont species, showcasing algal diversity, different fungal species may share the same algal partner, allowing for the possibility of algal partners switching among different lichen-forming fungi across different species, genera, and families (Ahmadjian & Jacobs, 1987; Beck et al., 1998; Hauck et al., 2007; Hawksworth, 1988). Various factors may influence the selectivity and specificity of lichen-forming fungi and their photobionts, including stress and extreme environmental conditions, which have been found to reduce photobiont selectivity in both green algae and cyanobacteria (Vargas Castillo & Beck, 2012; Wirtz et al., 2003).

Reproductive and dispersal strategies play significant roles in shaping photobiont diversity and population structure in lichens (Otalora et al., 2013; Steinova et al., 2019). Lichen reproduction occurs through sexual, asexual, or vegetative means (Frohlich, 2003). The sexual reproduction structure of ascomycete lichens takes place in a structure called ascomata, which is divided into four types based on its morphological structure: apothecia, perithecia, locules, and patsches (some lichens lack a true ascomata). In nature, most lichen-forming fungi reproduce sexually by producing meiospores that are dispersed independently into the environ-

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ment, where they must find compatible photobiont cells to re-establish symbiosis under challenging conditions. This process carries a high risk of failure due to the limitations of timing and partner compatibility, although some lichenized fungi have been shown to temporarily associate with non-preferred partners or exist in a free-living state to prolong their survival time (Etges & Ott, 2001; O'Brien et al., 2013). On the other hand, vegetative reproduction does not face the same restrictions, as cloning does not depend on encountering symbiotic algae in the environment. However, this type of reproduction has its own limitations, with clonal propagules being limited to short dispersal distances, unlike sexual reproduction which can facilitate long-distance dispersal (Ronnas et al., 2017; Scheidegger & Werth, 2009; Walser, 2004).

Previous studies revealed that lichen-forming fungi that use sexual reproduction strategies tend to have low selectivity for photobionts, and lichen-forming fungi with low photobiont selectivity demonstrated stronger adaptability to different ecological niches, enabling them to establish symbiotic relationships in a wide range of habitats (Muggia et al., 2014). In the order Verrucariales, vegetative reproduction through structures such as soredia, isidia, or blastidia is very rare (Geiser et al., 2006). *Endocarpon* Hedw. (Verrucariaceae, Verrucariales, Ascomycota) is a famous genus in this order that has a unique co-dispersal mode of reproduction. Its perithecium contains both ascospores and hymenial algal cells, and both partners are released together into the environment during spore maturation (Geitler, 1938; Shukla et al., 2014). This differs from other sexually reproducing lichen species. The sexual reproductive structures of *Endocarpon* are critical to this co-dispersal strategy and appear to facilitate successful lichenization in various ecological conditions, although this possibility has not yet been reported. To investigate the photobiont selectivity in this co-dispersal strategy of *Endocarpon* and how this strategy shapes lichen population structures, we collected a large number of thalli from *Endocarpon* and three closely related genera with different dispersal modes: *Placidiopsis* Beltr., *Placidium* A. Massal., and *Verrucaria* Schrad. The samples were collected on a large geographical scale across China, and we explored the dispersal mode and performed phylogenetic analysis of the mycobionts and photobionts to address these questions.

2. Materials and Methods

2.1. Materials

A total of 74 individuals were collected from six geographic regions that spanned a distance of over 2000 km, all within China (Fig. 1). Among the individuals, 62 *Endocarpon* were collected from Diqing in Yunnan province, Guoluo in Qinghai province, Helan Mountain in Ningxia Hui Autonomous Region, Linzhi in Tibet Autonomous Region and Yanchi in Ningxia Hui Autonomous Region. Twelve other individuals belonged to three related genera: *Verrucaria, Placidiopsis*, and *Placidium*. Three were *Placidiopsis* sampled from Helan Mountain, four were *Verrucaria* from Diqing and Helan Mountain, and five were *Placidium* from Linzhi and Duolun. The information of all the specimens in this study is listed Table 1.

2.2. DNA extraction and Sanger sequencing

Total DNA from mycobionts and photobionts was extracted using a modified CTAB method (Zhou et al., 2006). The mycobiont ITS segments were then amplified using the fungal specific primer

Fig. 1 Schematic diagram of collection sites. Lichens were collected from six localities in China. .

Table 1. Information of all individuals and GenBank accession numbers for the taxa sequenced in this study. GenBank accession numbers include mycobionts and photobionts. The types and the length of group I intron at 1769 site on SSU rDNA are listed.

No.	No. of thallus	Species name	Locality	GenBank no.		Group I Intron (site 1769)	
				No. of lichen-forming fungi	No. of photobionts	Length (bps)	Type
1	HL12Y023	*Endocarpon adsurgens*	Helan Mountain, Ningxia, China	OQ361512	OQ361402	235	Short
2	HL12Y033-1	*Endocarpon adsurgens*	Helan Mountain, Ningxia, China	OQ361513	OQ361406	235	Short
3	HL12Y033-2	*Endocarpon adsurgens*	Helan Mountain, Ningxia, China	OQ361514	OQ361407	235	Short
4	HL12Y045	*Endocarpon adsurgens*	Helan Mountain, Ningxia, China	OQ361515	OQ361408	234	Short
5	HL12Y071	*Endocarpon adsurgens*	Helan Mountain, Ningxia, China	OQ361517	OQ361411	235	Short
6	HL12Y073	*Endocarpon adsurgens*	Helan Mountain, Ningxia, China	OQ361518	OQ361412	235	Short
7	HL12Y078	*Endocarpon adsurgens*	Helan Mountain, Ningxia, China	OQ361520	OQ361415	235	Short
8	HL12Y134	*Endocarpon adsurgens*	Helan Mountain, Ningxia, China	OQ361524	OQ361426	235	Short
9	HL12Y187	*Endocarpon adsurgens*	Helan Mountain, Ningxia, China	OQ361528	OQ361439	236	Short
10	HL12Y215	*Endocarpon adsurgens*	Helan Mountain, Ningxia, China	OQ361535	OQ361450	235	Short
11	HL12Y216	*Endocarpon adsurgens*	Helan Mountain, Ningxia, China	OQ361536	OQ361451	235	Short
12	YC12Y015	*Endocarpon adsurgens*	Yanchi, Ningxia, China	OQ361511	OQ361399	237	Short
13	YC12Y131	*Endocarpon adsurgens*	Yanchi, Ningxia, China	OQ361523	OQ361425	237	Short
14	DQ12Y003	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361509	OQ361393	228	Short
15	DQ12Y010	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361510	OQ361396	234	Short
16	DQ12Y066	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361516	OQ361409	235	Short
17	DQ12Y076	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361519	OQ361414	-	-
18	ZD12Y091	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361521	OQ361421	235	Short
19	ZD12Y095	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361522	OQ361422	235	Short
20	ZD12Y173	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361525	OQ361434	234	Short
21	ZD12Y178	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361526	OQ361437	235	Short
22	ZD12Y185	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361527	OQ361438	222	Short
23	ZD12Y187	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361529	OQ361440	234	Short
24	ZD12Y188	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361530	OQ361441	234	Short
25	ZD12Y189	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361531	OQ361442	234	Short
26	ZD12Y192	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361532	OQ361443	234	Short
27	ZD12Y193	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361533	OQ361444	234	Short
28	ZD12Y201	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361534	OQ361448	234	Short
29	ZD12Y223	*Endocarpon adsurgens*	Diqing, Yunnan, China	OQ361537	OQ361453	234	Short
30	XZ12Y366	*Endocarpon adsurgens*	Linzhi, Tibet, China	OQ361538	OQ361456	234	Short
31	XZ12Y409	*Endocarpon adsurgens*	Linzhi, Tibet, China	OQ361539	OQ361457	-	-
32	XZ12Y424	*Endocarpon adsurgens*	Linzhi, Tibet, China	OQ361540	OQ361459	-	-
33	HL12Y013	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361470	OQ361397	235	Short
34	HL12Y014	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361471	OQ361398	366	Long
35	HL12Y017	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361472	OQ361400	488	Long
36	HL12Y019	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361473	OQ361401	338	Long
37	HL12Y028	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361474	OQ361404	235	Short
38	HL12Y029	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361475	OQ361405	251	Medium
39	HL12Y076	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361476	OQ361413	234	Short
40	HL12Y083	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361477	OQ361416	253	Medium
41	HL12Y085	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361478	OQ361417	253	Medium
42	HL12Y089	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361479	OQ361419	251	Medium
43	HL12Y118-1	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361480	OQ361423	502	Long
44	HL12Y120	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361481	OQ361424	231	Short
45	HL12Y147	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361483	OQ361428	235	Short
46	HL12Y213	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361486	OQ361449	-	-
47	HL12Y227	*Endocarpon pusillum*	Helan Mountain, Ningxia, China	OQ361487	OQ361454	235	Short
48	YC12Y146	*Endocarpon pusillum*	Yanchi, Ningxia, China	OQ361482	OQ361427	233	Short
49	YC12Y155	*Endocarpon pusillum*	Yanchi, Ningxia, China	OQ361484	OQ361430	233	Short
50	ZD12Y176	*Endocarpon pusillum*	Diqing, Yunnan, China	OQ361485	OQ361435	252	Medium
51	YC12Y153	*Endocarpon. deserticola*	Yanchi, Ningxia, China	OQ361488	OQ361429	392	Long
52	YC12Y157	*Endocarpon. deserticola*	Yanchi, Ningxia, China	OQ361489	OQ361431	529	Long
53	HL12Y222	*Endocarpon. nigromarginatum*	Helan Mountain, Ningxia, China	OQ361469	OQ361452	232	Short
54	Q11Y198	*Endocarpon. nigromarginatum*	Guoluo, Qinghai, China	OQ361467	OQ361445	231	Short
55	Q11Y200	*Endocarpon. nigromarginatum*	Guoluo, Qinghai, China	OQ361468	OQ361447	232	Short
56	HL12Y087	*Endocarpon. petrolepideum*	Helan Mountain, Ningxia, China	OQ361502	OQ361418	237	Short
57	Q11Y269	*Endocarpon. petrolepideum*	Guoluo, Qinghai, China	OQ361503	OQ361455	221	Short
58	HL12Y067	*Endocarpon. sinense*	Helan Mountain, Ningxia, China	OQ361504	OQ361410	240	Short
59	HL12Y091	*Endocarpon. sinense*	Helan Mountain, Ningxia, China	OQ361505	OQ361420	232	Short
60	YC12Y159	*Endocarpon. sinense*	Yanchi, Ningxia, China	OQ361506	OQ361432	233	Short
61	ZD12Y177	*Endocarpon. unifoliatum*	Diqing, Yunnan, China	OQ361507	OQ361436	234	Short
62	YC12Y026	*Endocarpon sp.*	Yanchi, Ningxia, China	OQ361508	OQ361403	235	Short
63	HL12Y046	*Placidiopsis sp.*	Helan Mountain, Ningxia, China	OQ361490	OQ361460	-	-
64	HL12Y166	*Placidiopsis sp.*	Helan Mountain, Ningxia, China	OQ361491	OQ361461	-	-
65	HL12Y172	*Placidiopsis sp.*	Helan Mountain, Ningxia, China	OQ361492	OQ361463	-	-
66	XZ12Y360	*Placidium sp.*	Linzhi, Tibet, China	OQ361494	OQ361466	-	-
67	XZ12Y411	*Placidium sp.*	Linzhi, Tibet, China	OQ361495	OQ361458	-	-
68	DL12Y004	*Placidium sp.*	Duolun, Inner Mongolia, China	OQ361496	OQ361394	-	-
69	DL12Y008	*Placidium sp.*	Duolun, Inner Mongolia, China	OQ361497	OQ361395	-	-
70	DL12Y030	*Placidium sp.*	Duolun, Inner Mongolia, China	OQ361493	OQ361465	-	-
71	HL12Y169	*Verrucaria sp.*	Helan Mountain, Ningxia, China	OQ361499	OQ361462	-	-
72	ZD12Y168	*Verrucaria sp.*	Diqing, Yunnan, China	OQ361498	OQ361433	-	-
73	ZD12Y196	*Verrucaria sp.*	Diqing, Yunnan, China	OQ361500	OQ361464	-	-
74	ZD12Y199	*Verrucaria sp.*	Diqing, Yunnan, China	OQ361501	OQ361446	-	-

Note: "-" means that no group I introns can be found at 1769 site in mycobionts, which does not mean that mycobionts do not contain other group I introns.

pairs ITS5 (5'-TCCTCCGCTTATTGATATGC-3') and ITS4 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al., 1990).

For the photobiont ITS sequences, algal-specific primer pairs nr-SSU-1780-5' (5'-CTGCGGAAGGATCATTGATTC-3') and nr-LSU-0012-3' (5'-AGTTCAGCGGGTGGTCTTG-3') were used (Piercey-Normore & DePriest, 2001). The PCR reaction was performed as follows: initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles of 95 °C for 30 s, 53 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min.

Then, dideoxy-mediated chain-termination sequencing reaction electrophoresis was conducted using the 3730XL DNA Sequencing Instrument (Applied Biosystems, CA, USA). The sequencing primers were the same as those described for PCR. Double-directional sequence data were obtained for the mycobionts and photobionts. These data were checked and assembled using the SEQMAN program within the Lasergene v7.1 software package (DNAStar Inc.,-WI, USA).

2.3. Data analysis and phylogenetic tree construction

All sequences generated in this study have been submitted to NCBI, and their accession numbers are listed in Table 1. We checked the sequence length of all data and found that the large variation in sequence length (582–1066 bps) of lichenized fungi indicated the presence of group I intron. To determine the insertion sites of these introns, we retrieved the 18S rRNA gene sequence of *Saccharomyces cerevisiae* (Desm.) Meyen (GenBank no. Z75578) and the 16S rRNA sequence of *Escherichia coli* (Migula) Castellani and Chalmers from GenBank, and aligned these sequences with our data.

The sequences analyzed in this study were divided into three datasets: the mycobiont ITS dataset, the group I intron dataset, and the photobiont ITS dataset. The group I introns, small subunit region (SSU) and large subunit (LSU) rDNA have been removed from the mycobiont matrix before undertaking the phylogenetic analysis (Supplementary Fig. 1). For the mycobiont and the photobiont ITS datasets, preliminary alignment was performed using the ClustalW algorithm included in MEGA11 software (Tamura et al., 2021). In the alignment of group I introns from mycobionts, the secondary structures were constructed prior to performing the alignment and the alignment was conducted using ClustalW embedded in MEGA with default parameters (Tamura et al., 2021; Thompson et al., 1994) and then made manual adjustments to ensure that the conserved elements remained properly aligned within the sequences. Alignment gaps were treated as missing data. The phylogenetic trees for the mycobionts, photobionts, and group I introns were constructed using the minimum evolution (ME) method with MEGA11. The Kimura two-parameter model was used to estimate the number of nucleotide substitutions. Pairwise deletion was used for the gaps and missing data treatment, while default settings were used for other parameters. The support values for the phylogenies were assessed with 1000 bootstrap replicates.

2.4. Group I intron secondary structure prediction

We used DNAMAN software V9.0 (Lynnon Biosoft, Quebec, Canada) to convert the group I introns into RNA sequences. Subsequently, thermodynamically stable structures were calculated using RNAStructure 3.5 (Mathews et al., 1999). Finally, the secondary structures of the group I introns were prepared using Adobe Illustrator 2021 (Adobe Systems Inc., CA, USA).

3. Results

3.1. Phylogenetic analyses of mycobiont ITS rDNA

The ME tree of mycobiont based on the ITS rDNA sequences exhibit four distinct monophyletic groups: *Endocarpon, Verrucaria, Placidiopsis,* and *Placidium* (Fig. 2). The *Endocarpon, Placidiopsis*, and *Placidium* groups are powerfully supported with a 99% bootstrap value. The *Verrucaria* group exhibits slightly less support, with a 63% bootstrap value.

In this study, phylogenetic analyses were conducted on 62 *Endocarpon* individuals, with 61 of them being identified as belonging to seven species. These species manifest as follows: *E. adsurgens* Vain., *E. deserticola* T. Zhang, X.L. Wei and J.C. Wei, *E. unifoliatum* T. Zhang, X.L. Wei and J.C. Wei, *E. petrolepideum* (Nyl.) Hasse, *E. nigromarginatum* H. Harada, *E. sinense* H. Magn., and *E. pusillum* Hedwig. The remaining individual was marked as *Endocarpon sp.* which was considered to be an unidentified lineage or unreported species. Out of these species, *E. adsurgens* is monophyletic and comprises 32 individuals collected from various locations including Helan Mountain in Ningxia, Yanchi in Ningxia, Linzhi in Tibet, and Diqing in Yunnan. This species can be further divided into several sub-groups; however, most of these sub-groups lack strong support. There is no clear association between the clustering of individuals and their geographic origins, although some individuals collected from the same location did show a tendency to cluster together.

Phylogenetic analysis shows that *E. pusillum* forms a well-supported monophyletic group with 98% bootstrap support, consisting of 18 individuals collected from three locations: 15 from Helan Mountain, two from Yanchi, and one from Diqing. The *E. pusillum* group can be further divided into three distinct monophyletic subgroups. The first sub-group comprises individuals from Helan Mountain and is supported by 68% bootstrap, while the second subgroup includes individuals from Helan Mountain and Yanchi that are geographically close (separated by dozens of kilometers) and has a high bootstrap support of 99%. The third sub-group is composed of individuals from Helan Mountain and Diqing, which are located about 1300 km apart, and has a bootstrap support of 60%. The *E. pusillum* individuals collected from Helan Mountain are assigned to the three sub-groups described above, indicating that the species is polyphyletic in this geographic area. Furthermore, similar to *E. adsurgens*, the clustering of *E. pusillum* is not strictly based on collection location. Among the other *Endocarpon* species, *E. deserticola*, *E. unifoliatum*, *E. petrolepideum*, *E. nigromarginatum,* and *E. sinense* groups consist of fewer individuals, but each group is monophyletic.

The ME tree of the mycobiont shows that individuals sharing identical ITS genotypes were likely to originate from the same habitat. We have found evidence across different species; for example, HL12Y028F *E. pusillum* and HL12Y147F *E. pusillum*, HL12Y078F *E. adsurgens* and HL12Y216F *E. adsurgens*, Q11Y198F *E. nigromarginatum* and Q11Y200F *E. nigromarginatum*. Additionally, in many other branches, individuals tend to cluster with other individuals from the same or nearby locations.

3.2. Phylogenetic analyses of photobiont ITS rDNA

In the present study, we obtained 74 ITS rDNA sequences of photobionts and conducted molecular analysis by constructing ME tree. The analyses demonstrated that the majority of the photosynthetic partners were classified into two well-supported monophyletic groups with high bootstrap support (99%). These two groups

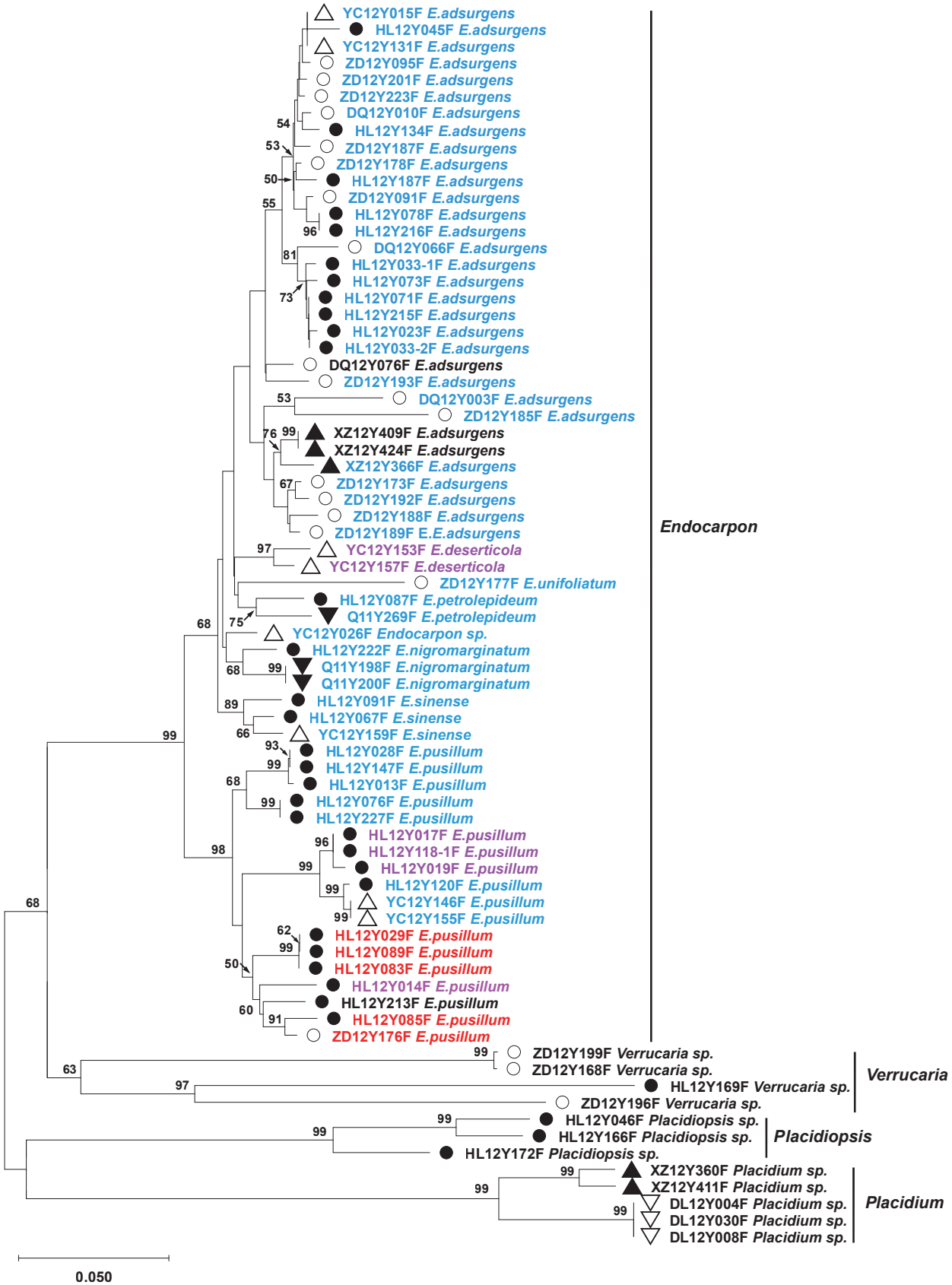

Fig. 2 Minimum evolution (ME) tree of mycobiont based on ITS rDNA sequences. The ME tree displays bootstrap support values (1000 replications) and numbers <50 are not shown. Different symbols are used to represent different locations. ● Helan Mountain (Ningxia), △ Yanchi (Ningxia), ▲ Linzhi (Tibet),▽ Duolun (Inner Mongolia),▼ Guoluo (Qinghai), ○ Diqing (Yunnan). Different font colors indicate the type of 1769 introns found in the sequences of mycobionts, categorized as follows: black font indicates no 1769 introns, light blue font indicates the short 1769 group, red font indicates the medium 1769 group, and purple font indicates the long 1769 group.

were recognized as distinguishable taxa at the species level, namely the *Diplosphaera chodatii* Bialosuknia and *Stichococcus mirabilis* Lagerheim (Fig. 3).

Sixty-seven individuals constituted the *Diplosphaera chodatii* group, of which 62 individuals of *Endocarpon* were collected from Helan Mountain, Yanchi in Ningxia, Linzhi in Tibet, and Diqing in Yunnan, two individuals of *Verrucaria* were collected from Diqing in Yunnan, and three individuals of *Placidium* were collected from Linzhi in Tibet and Duolun in Inner Mongolia, which suggests that the distribution of *D. chodatii* occurred across a large geographical range. Furthermore, *D. chodatii* exhibited low genetic diversity according to the phylogenetic analysis. No evidence was found that the genotypes of photobionts were related to geographical locations or even the host mycobiont species.

Both the *Verrucaria* and *Placidium* genera exhibited photobiont diversity. For example, *Verrucaria* from Diqing form associations with two algal partners, namely the green algae *D. chodatii* and *S. mirabilis*. *Placidium* from Linzhi and Duolun harbored *D. chodatii* and *Pseudochlorella* spp. as their photosynthetic partners. Although the data showed that three photobiont individuals from *Placidiopsis* sampled from Helan Mountain were identified as *S. mirabilis*, a previous study indicated that it could also form lichen symbiosis with *D. chodatii* (Thüs et al., 2011). Mycobionts from all species of *Endocarpon* collected in different locations exhibited high selectivity toward *D. chodatii,* which was also shared with other genera of lichenized fungi in the same habitat. This finding suggest the existence of algal pools in these habitats.

3.3. Group I intron analyses

Through sequence alignment, it was found that 58 out of 62 *Endocarpon* individuals harbor a group I intron (Table 1; Fig. 2). The insertion site was calibrated at 1769 based on the SSU rDNA sequence of *S. cerevisiae* (GenBank no. Z75578) as reference (Cao et al., 2011), and this site is 1506 based on the SSU rDNA sequence of *E. coli* (Bhattacharya et al., 2002; Del Campo et al., 2009; Friedl et al., 2000; Nyati et al., 2013).The group I introns were classified into three groups based on their lengths (Gutierrez et al., 2007), which are marked as the “short 1769 group” (221–240 bps), the “medium 1769 group” (251–253 bps), and the “long 1769 group” (338–529 bps), as shown in Table 1. The ME tree of group I introns shows three well-supported clades (Fig. 4). Clade I contains 47 introns from *E. pusillum*, *E. adsurgens*, *E. unifoliatum*, *E. petrolepideum*, *E. nigromarginatum*, *E. sinense*, and *Endocarpon* sp., while Clade II contains six introns, all from *E. pusillum*, and Clade III is composed of five introns from *E. deserticola* and *E. pusillum*. The group I introns in Clade I all belongs to the short 1769 group and form monophyletic sub-clades according to their host mycobiont species. In addition, similar to the analysis in photobionts and mycobionts, the introns do not show a correlation between intron genotypes and geographical locations. Five out of six introns in Clade II belong to the medium 1769 group, which form a separate sub-clade with 71% support, while the final intron belonged to the long 1769 group, and introns in Clade III all belong to the long 1769 group. It is particularly striking that the group I introns from *E. pusillum* collected in Helan Mountain are polyphyletic, and the short, medium, long, and non-intron types can all be found in *E. pusillum* from Helan Mountain.

Previous analyses show that the secondary structure of group I introns from the same insertion site is conserved, and most of the conserved nucleotides are located in the elements P, Q, R, and S (Burke et al., 1987; Del Campo et al., 2009). Group I introns generally have base pairing regions named P1 to P9, and the P4 region is formed by P and Q, while the P7 region consists of R and S. However, in this study these elements are not completely consistent in the three clades, as shown in Table 2 and Figure 5. The P7 element is the most conserved, while P3 and P4 showed high similarity in the medium and long groups, but not in the short group. The results show that introns from the short 1769 group harbor a larger P6 (Fig. 5A). Figure 5B illustrates the secondary structure of the medium 1769 group, whose P8 elements are similar to those in the short 1769 group in length but the sequence in P3/P4 elements are similar to those in the long 1769 group. In addition, the long 1769 group has a particularly large P8 region, but some members of this group lack the P5 element (Fig. 5C–E). Among the long group, the intron from HL12Y014F is rather special because sequences of its P3–P6 elements are identical with those from the medium group (Fig. 5C).

4. Discussion

The ribosome ITS rDNA is a critical tool in lichen research for investigating phylogenetic relationships and species diversity in fungi and green algae at both the interspecific and intraspecific levels (Begerow et al., 2010; Moniz & Kaczmarska, 2010; Schoch et al., 2012). The ITS region is a crucial DNA barcode for species identification, evolutionary relationship analysis, and fungal diversity research in mycology. Phylogenetic analysis based on ITS markers has revealed the evolutionary relationship among closely related species of *Rhizoplaca* Zopf in China (Dal-Forno et al., 2016; Zhou et al., 2006). In the study of algae, the ITS rDNA marker is also used to reveal the diversity of algae and their environmental adaptability. For example, the photobiont diversity of *Dermatocarpon*, a member of Verrucariaceae, was analyzed using the ITS rDNA marker, which showed that mycobionts in different habitats exhibited the ability to capture the same photobiont (Fontaine et al., 2012). The ITS rDNA marker is also applicable in studying the relationship between mycobionts and photobionts in lichen symbiosis. In a previous study, the ITS rDNA-barcode was used to assess the degree of photobiont selectivity and specificity in thalli from multiple genera, including *Carbonea* Hertel, *Austrolecia* Hertel, *Lecanora* Ach., *Lecidella* Körb., *Caloplaca* Th. Fr., *Umbilicaria* Hoffm., collected from extreme ecosystems in Antarctica (Perez-Ortega et al., 2012). In this present study, ITS rDNA was used to reveal the relationships between *Endocarpon* and three closely related genera and their photobionts. The study demonstrates the ability of the ITS rDNA marker to distinguish different species or approximate genera in both lichenized fungi and their photosynthetic partners. Thus, the findings of this study suggest that ITS rDNA, as a classical marker, still plays an essential role in revealing the ascospore-alga co-dispersal mode of *Endocarpon*.

4.1. Unusual population structures shaped by co-dispersal strategy in Endocarpon

The relationship pattern between mycobionts and their photobionts is one of the core research hotspot of lichen biology, and is influenced by the specificity and selectivity of mycobionts toward their photosynthetic partners. Numerous studies on photobiont selectivity reveal the complexity of symbiotic relationships, with research focusing on species differentiation, environmental factors, reproductive strategies, and dispersal modes. For instance, researchers have conducted studies on photobiont selectivity, exploring the topic from different perspectives (De Carolis et al., 2022; Hauck et al., 2007; Merinero et al., 2017; Muggia et al., 2014; Steinova et al., 2019; Vargas Castillo & Beck, 2012). In our previous study on two green-algae-harboring lichens from *Umbilicaria esculenta*

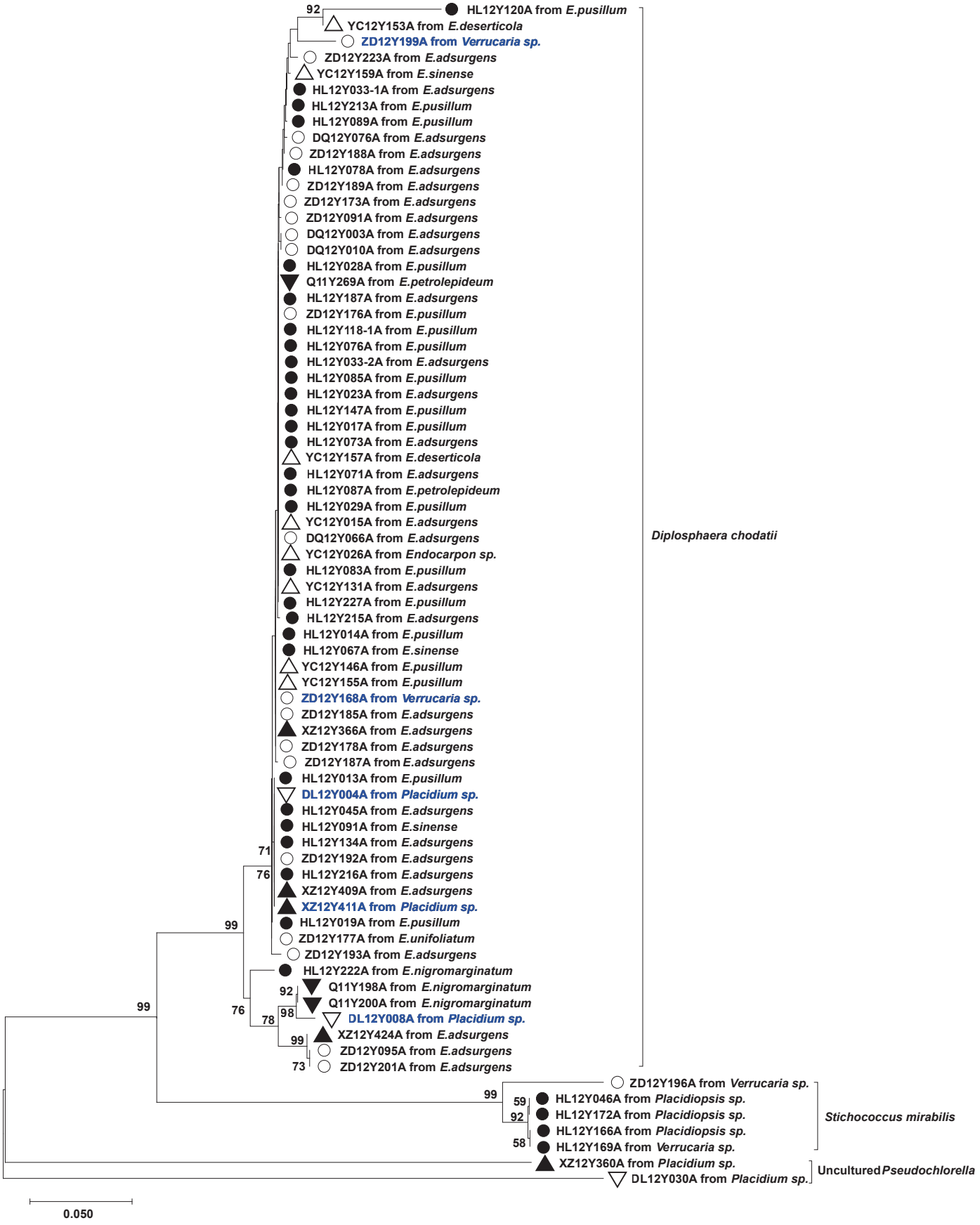

Fig. 3 Minimum evolution (ME) tree of photobiont based on ITS rDNA sequences. Photobionts are marked with the name of the corresponding lichen-forming fungus and its collection number, and the last letter "A" represents algae. The ME tree displays bootstrap support values (1000 replications) and numbers <50 are not shown. Symbols representing locations are consistent with those in Figure 2. Dark blue font represents non-*Endocarpon* lichens whose photosynthetic partner is also *Diplosphaera chodatii*.

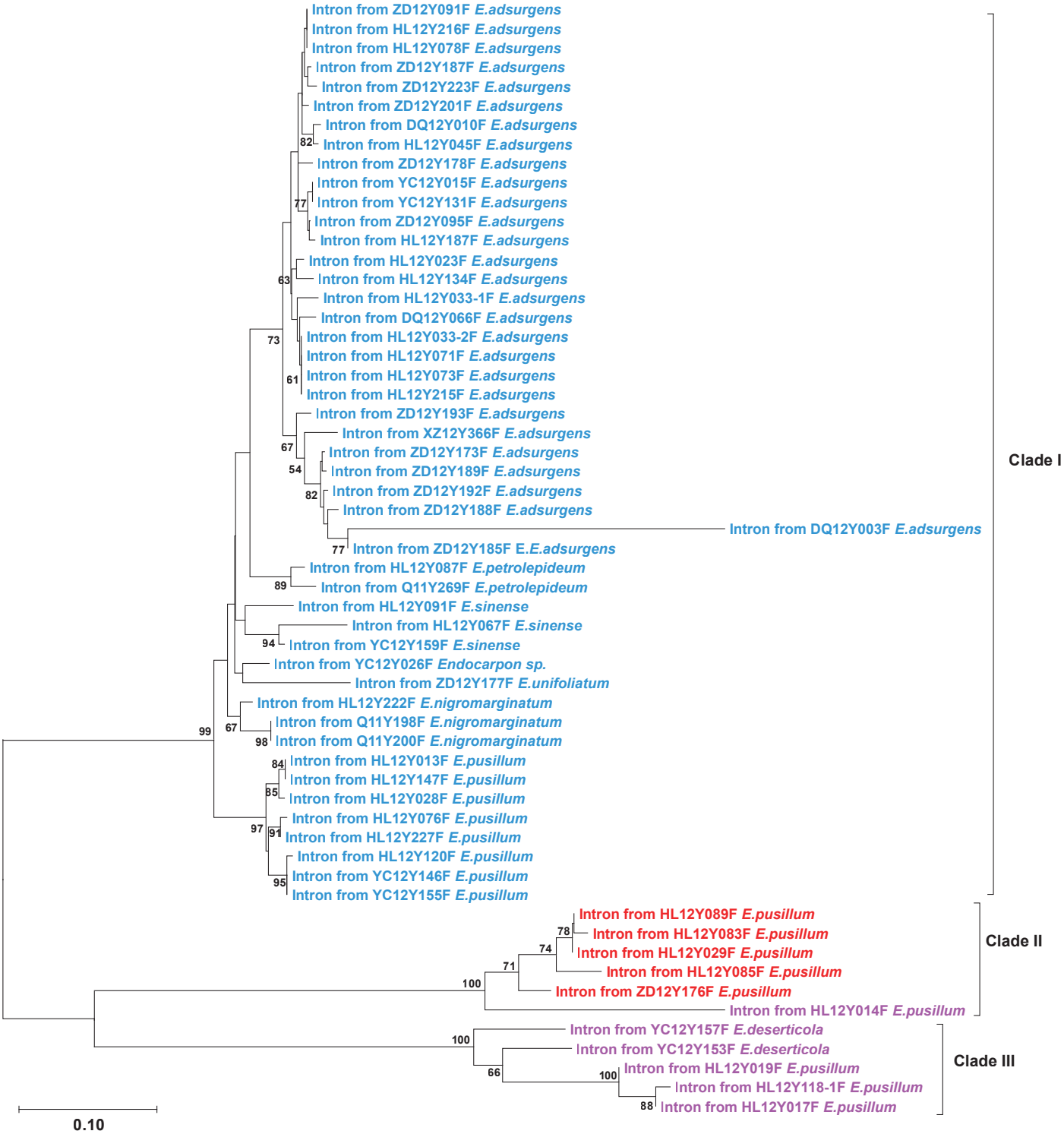

Fig. 4 Minimum evolution (ME) tree of group I intron sequences from *Endocarpon*. The insertion position at 1769 is relative to the SSU rDNA sequence of *Saccharomyces cerevisiae* (GenBank no. Z75578). Light blue font indicates the short 1769 group, which corresponds to Clade I, red font indicates the medium 1769 group, and purple font indicates the long 1769 group, consistent with the mycobiont tree.

Table 2. Conserved core sequences of *Endocarpon* group I introns at 1769 site on SSU rDNA.

	P3	P4 P	P4' Q	P7 R	P3'	P7' S
Clade I	TAACCA	GCGTC	GACGT	CAGATTA	TGGTGG	TAATCG
Clade II	CGTCACT	TGCTGG	TCAGCA	CAGATTA	AGTGACG	TAATCG
Clade III	CGTCACT	CTGCTGG	CCAGCAG	CAGATTA	GGTGACG	TAATCG

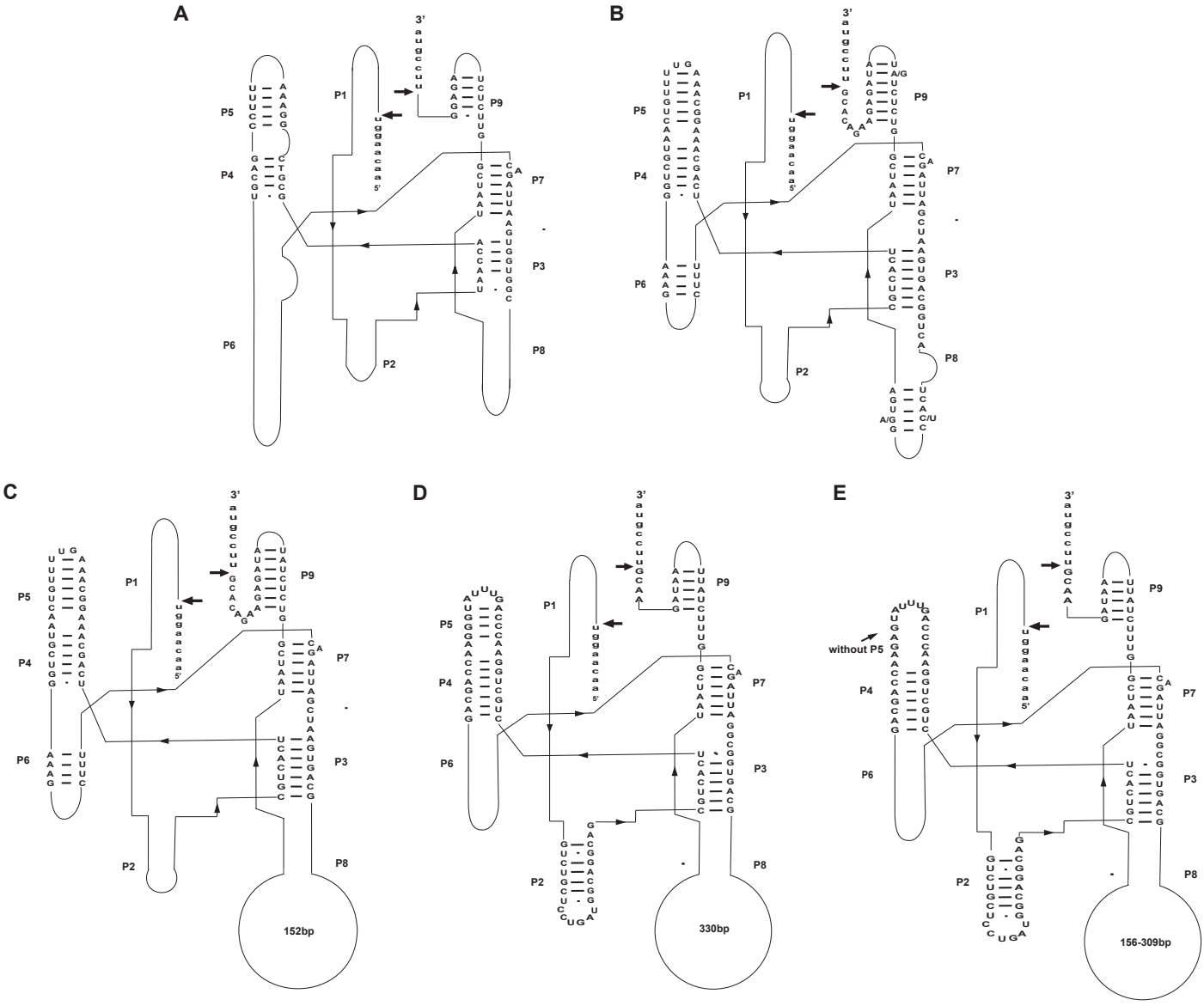

Fig. 5 Schematic diagram of secondary structure for group I introns at the SSU rDNA 1769 site from *Endocarpon*. The splicing sites are indicated by bold black arrows, and the conserved sequences in the helix region are shown with five bases. A: The secondary structure of the short 1769 group, with a larger P6 region. B: The secondary structure of the medium 1769 group. C: The secondary structure of the intron from HL12Y014F belonging to the long 1769 group. D: The secondary structure of the intron from YC12Y157F belonging to the long 1769 group. E: The secondary structure corresponds to belonging to the long 1769 group without P5.

(Miyoshi) Minks and *U. muehlenbergii* (Ach.) Tuck., which used different reproductive strategies, it was found that species using sexual reproduction exhibited low levels of photobiont selectivity compared to that using a vegetative reproduction strategy (Cao et al., 2015). A similar study on other green-algae-harboring lichen *Cladonia* P. Browne, also obtained a consistent conclusion (Steinova et al., 2019). Likewise, a study on the cyanobacteria-harboring-lichen *Degelia* Arv. and D.J. Galloway also showed that sexual species exhibited higher genetic diversity than asexual species, and asexual species formed high-specificity relationships with cyanobionts (Otalora et al., 2013). These findings suggest that photobiont selectivity is low in lichens with the strategy of sexual reproduction in the mycobiont because fungal spores must capture a variety of photobionts to stabilize the symbiotic association, or the mycobiont will not survive.

The present research investigated 62 individuals of *Endocarpon* that were collected from six regions in China, with a distance range between collection sites of 100–2000 km (Fig. 1; Table 1). The ME tree of mycobiont based on the ITS rDNA sequences revealed that these individuals could be divided into seven independent species with high support, and there was no difference in algae selectivity among species. The study further revealed that *Endocarpon*, a genus that strictly relies on sexual reproduction, exhibited an unusual mycobiont-photobiont selectivity pattern, which was contrary to the results of previous studies on lichens whose mycobionts used sexual reproduction. By contrast, individuals from the other three genera; *Verrucaria*, *Placidiopsis*, and *Placidium*, which are also in Verrucariaceae, showed low photobiont selectivity.

Endocarpon showed extremely high specificity for their photosynthetic partners, with all thalli from different species in *Endocarpon* establishing symbiotic relationships with only one green alga, *D. chodatii*, as shown in Figure 3. Hence, this close association between *Endocarpon* fungi and the green alga *D. chodatii* was fixed before the divergence of *Endocarpon*, and the present distribution pattern was shaped under long-term co-evolution and environmental adaptation.

The constructed ME trees of mycobiont and photobiont, based on the ITS rDNA sequences, indicate that neither mycobionts nor photobionts were divided according to geographical locations. The lack of distance isolation suggests that there is frequent gene flow or dispersal across a wide range of environments. During the development of lichen, the photobionts associated with mycobionts can change, a process called algae switching or photobiont switching (Piercey-Normore & DePriest, 2001). In some lichenized fungi, photobiont switching can lead to changes in morphology and reproduction strategies (Ertz et al., 2018). The phylogeny obtained in this study revealed that, in addition to *Endocarpon*, the genera *Verrucaria* and *Placidium* in the same habitat also selected *D. chodatii* to establish symbiotic relationships. However, *D. chodatii* were not divided into different lineages according to the different genera of host lichens or geographical regions (Fig. 3). This lichen family, Verrucariaceae, is characterized by a different trend in photobiont diversity and often forms associations with *Stichococcus*-like green algae, but the predominant photobionts *Trebouxia* Puymaly and *Asterochloris* Tschermak-Woess in most other lichens are only rarely or are never reported. In this family, *Diplosphaera*, *Stichococcus* Naegeli, and *Protococcus* C. Agardh are the most common lichen photobionts, and *D. chodatii* is shared among multiple mycobionts (Thüs et al., 2011). The present study suggests that there is an algal pool of *D. chodatii* in the sampled habitats. Considering the highly specific relationship between *Endocarpon* and *D. chodatii* we speculate that algae switching or photobiont stealing may occur in non-*Endocarpon* species because the unique co-dispersal mechanism of *Endocarpon* can provide abundant compatible algae in the habitat. The individuals from the other three genera in this study may establish an association with *D. chodatii* through re-lichenization or trans-lichenization, as described in a recent review (Pichler et al., 2023). Overall, the results suggest that there is a complex relationship between lichenized fungi and their photobionts, with frequent gene flow or dispersal of photobionts across a wide range of environments, and potential mechanisms of algae switching or photobiont stealing in non-*Endocarpon* species.

This high level of photobiont selectivity at the genus level is exceptionally rare among sexually reproducing lichens in nature. Typically, spores are dispersed independently, and it is necessary to find compatible algae cells in the habitat for successful lichenization to occur (Macedo et al., 2009). During this period, it can be challenging for fungi to find their compatible algal partners. Even if they do encounter them by chance, re-lichenization can take several stages to succeed. This is why sexual reproduction is often accompanied by low photobiont selectivity, which increases ecological tolerance, and improves the chances of successful symbiosis between widely distributed fungi and locally available photobionts (Muggia et al., 2014).

However, as a genus that relies on sexual reproduction, *Endocarpon* adopts a special co-dispersal model in which algal cells from the photobiont and ascospores from the mycobiont are sprayed into the habitat at the same time, which may explain the special relationship between *Endocarpon* and *D. chodatii*. This co-dispersal strategy seems to be more convenient for re-lichenization compared to single-dispersal sexually reproductive species. Moreover, chemical communication between mycobionts and photobionts plays an essential role from the early stages of lichenization up to the formation of thalli (Pichler et al., 2023). Therefore, for *Endocarpon* ascospores, there are compatible and identifiable symbiotic partners in the habitat, creating extremely favorable conditions for re-lichenization. This relationship may have been strengthened during evolutionary history of *Endocarpon*. Overall, the high photobiont selectivity of *Endocarpon* at the genus level is a fascinating finding that challenges our understanding of lichen evolution and adaptation.

In previous studies, co-dispersal in lichens was reported to occur mainly through vegetative reproduction, which is limited to short distances due to low evolutionary flexibility and selective specialization (Dal Grande et al., 2012; Ronnas et al., 2017; Steinova et al., 2019). However, the use of ascospores for dispersal in *Endocarpon* has allowed for long-distance transmission, providing a significant ecological advantage over lichens that rely on vegetative reproduction. This advantage is reflected in the widespread distribution of *Endocarpon* species across different regions and environments worldwide since they have been reported in many countries and regions ranging from terrestrial to marine and fresh water environments to arid environments, especially in biological soil crusts (Gueidan et al., 2007; Mead & Gueidan, 2020). Moreover, some *Endocarpon* species, such as *E. pusillum*, exhibit strong drought tolerance and both bionts in the symbiotic association play an important role in adaptation to harsh environmental conditions (Medwed et al., 2021; Wang et al., 2014). The special co-dispersal mode of the mycobiont and photobiont in *Endocarpon* may also improve its stress tolerance, expanding its ability to survive and thrive in a wider range of environments. In general, the findings of this study suggest that *Endocarpon* has advantage on long-distance transmission of sexual reproduction and avoids the disadvantages in re-lichenization through the use of a special co-dispersal strategy. Furthermore, this co-dispersal strategy likely contributes to the adaptability and success of *Endocarpon* in diverse ecological niches. *Endocarpon* was the first lichen whose genome was sequenced(Wang et al., 2014), and recently, the genome of the photobiont *D. chodatii* has also been made available online (Gueidan et al., 2023). This will greatly facilitate research into this co-dispersal strategy of *Endocarpon*. In future studies, we can anticipate using a wider range of tools and methods to explore the symbiotic relationship between *Endocarpon* and *D. chodatii*.

4.2. Evolutionary history reflected by group I introns in Endocarpon

Group I introns were first discovered in the LSU of *Tetrahymena thermophilia* Nanney and McCoy by Cech et al. (1981). Since then, numerous studies have shown that group I introns are widely distributed among diverse organisms, including protists, plants, eubacteria, archaea, and even lichenized fungi and photobionts (Bhattacharya et al., 2000; Del Hoyo et al., 2018; Depriest & Been, 1992; Gargas et al., 1995; Nawrocki et al., 2018). The splicing site of group I introns is characterized by the conservative nucleotide 5'-U↓... ...G↓ 3', and its secondary structure is more conservative compared to the primary structure. Studies have shown that group I introns are divided into three domains: the P1–P2 domain, P4–P6 domain, and P3–P9 domain (including P3, P7, P8, and P9), of which the latter two constitute the catalytic center of the intron ribozyme. As mobile genetic elements, group I introns have the characteristics of frequent loss and gain in the genome. Analyses of rRNA sequences have confirmed that the SSU and LSU are the most common regions where self-splicing group I introns insert (Harris & Rogers, 2011; Yokoyama et al., 2002). Furthermore, the SSU rDNA has multiple non-random positions that are acceptable for group I intron insertion (Gargas et al., 1995; Simon et al., 2005; Xu et al., 2013). In lichenized fungi, multiple group I introns may exist in the SSU rDNA of an individual (Bhattacharya et al., 2000). Additionally, sometimes group I introns are useful for taxonomic studies at the genus or intra-genus level (Leavitt et al., 2011).

According to the data obtained in the present study, the group I

intron at position 1769 was found in more than 90% of *Endocarpon* individuals, and these introns were divided into three clades, as shown in Figure 4. The ME trees of mycobiont and group I intron confirmed the similarity between the intron and mycobiont trees and suggested that group I intron could be used to identify species in Clade I, but not in Clade II and III. These findings confirm that group I introns can be applied to phylogenetic analysis, but their application has some limitations, which is consistent with previous studies (Mattsson et al., 2009). This work predicted the RNA secondary structure to reveal the conserved domain and its characteristics more clearly, as shown in Figure 5A–E. The main difference among introns was the P8 element; where the long type had a larger P8 region ranging in length from about 180–330 bps. However, the P8 element is not a conservative domain in group I introns and is more likely to undergo mutation or loss, without affecting the function of group I introns. It is particularly noteworthy that a 6 bp nucleotide sequence (AAGATA) at 3' in the P8 element was extremely conserved, which may provide important information for further research on the polymorphism and evolution of the 1769 intron.

Moreover, the P3/P4 elements, along with other helix regions, also exhibited differences in different group I introns, as shown in Table 2. While medium-long groups exhibited similarity, the short groups showed significant differences. In a previous study of group I introns from 39 species in 27 genera belonging to the family Parmeliaceae, the position 1769 intron showed length variability at the family or genus level, with long, medium, and short groups being employed to distinguish them (Gutierrez et al., 2007). This study found length polymorphisms not only at the genus level but also at the species level, as shown in *E. pusillum*.

The study identified a particular group I intron from sample HL12Y014F that exhibited similarities in sequence conservation and clustering to other medium individuals, but shared similarities in intron length and secondary structure with the long group, which had a large P8 element (Fig. 5C). Since the secondary structure of the group I intron was more conservative than the primary structure, the group I intron from sample HL12Y014F was classified as the long type. This case may represent a transitional state in the evolution of the group I intron at 1769 in *Endocarpon*, providing insight into the evolution of group I introns in lichenized fungi. Furthermore, the 1769 intron of *E. pusillum* from Helan Mountain showed polymorphism, including all states (the long, medium, short, long-medium transition, and "none" states), making it the first report that group I introns are non-conservative at the same insertion site from the same species in the same habitat. This finding implies that the group I intron at this site underwent relatively fast evolution or horizontal transfer, undergoing a process of rapid gain and loss. This case undoubtedly provides a good model for the evolution of group I intron in lichenized fungi.

5. CONCLUSION

In summary, this study has uncovered a distinct co-dispersal strategy in lichens that differs from the conventional dispersal modes of sexual and asexual reproduction. The research highlights the influence of this co-dispersal strategy on shaping lichen population structure. Future investigations will delve into the evolutionary history of lichens, exploring the origin and molecular mechanisms underlying this unique co-dispersal strategy, as well as its impact on lichenization.

DATA AVAILABILITY STATEMENT

The sequences generated for this study were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/; accession numbers are listed in Table 1).

AUTHOR CONTRIBUTIONS

CY analyzed the data, constructed the figures, and drafted the manuscript. QZ and SC gathered the specimens, performed the biological assays and took part in the data analysis. YS and LL helped to construct the figures. YC and HT participated in drafting the manuscript. CL and QZ initiated and designed the study, participated in the data analysis, and finalized the manuscript. All authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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