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RESEARCH ARTICLE

Using Chloroplast Regions *accD, matK, rbcL*, and *ycf-1* for Phylogeny Construction in *Polyspora huongiana* Orel, Curry & Luu

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ABSTRACT

Objective: Theaceae is a commodity with high economic value. The diversity of Theaceae species present in Southeast Asia, especially Vietnam, provides an excellent supply source for promoting the development of related industries. Nearly 130 species of Theaceae, many of which are highly endemic, were discovered in Vietnam, including 13 *Polyspora* species. *Polyspora huongiana* was discovered in Bidoup-Nui Ba National Park, Vietnam, on January 7, 2010. Because they are native species and lack data, studies are needed to provide genetic data for ecological assessment and original identification of the plant.

Materials and Methods: Sanger sequencing, data collection, and nucleotide analysis of the genetic data of *accD, matK, rbcL*, and *ycf1* in *P. huongiana* were provided.

Results: The results showed that combinations of two sequences could separate the *Polyspora* genus, whereas at least three sequences were necessary to identify *P. huongiana*, which was genetically closely related to *Polyspora axillaris* and *Polyspora hainanensis*.

Conclusion: *P. huongiana* is closely related to *Polyspora axillaris* and *Hainanensis*. The combination of 3–4 sequences allowed reliable identification of *P. huongiana*.

Keywords: *Polyspora huongiana*, Phylogeny, *matK, rbcL, accD, ycf1*.

INTRODUCTION

The tea family (Theaceae) comprises approximately 460 species, which are mainly distributed in East and Southeast Asia. *Camellia* is the largest tea genus, comprising more than 300 species worldwide.[1,2] Thus far, nearly 130 Theaceae species belonging 5 genera have been found in Vietnam, namely 95 species of *Camellia*, 13 species of *Polyspora*, 8 species of *Pyrenaria*, 7 species of *Schima*, and 3 species of Stewartia.[1] Many endemic *Polyspora* species have recently been discovered and described for the first time, such as *Polyspora congii, Polyspora bidoupensis*, and *Polyspora huongiana*.[2-4] The number of species and endemism reflect the biodiversity of tea species in Vietnam.[5] Conservation and sustainable exploitation of tea species is crucial in both the economy and the ecology.[6]

Theaceae species often share the same physical characteristics, especially *Polyspora* and *Camellia*.[7] The similarity of the two genera makes it difficult to distinguish them.[8,9] Ecologists can rely on winged seed morphology to differentiate if this plant is *Polyspora* or *Camellia*, and the result is hardly conclusive based on limited information.[10] The development of technology has provided new approaches to solving problems using molecular biology techniques. The DNA fingerprint method is considered an independent tool in forensic investigations, research, plant identification, and many other fields.[11] DNA Barcoding was born after the emergence of PCR and Sequencing methods and quickly became a promising aid in classifying species and individuals.[11-13] In addition, NGS techniques are currently widely used in classifying and studying the ecology of species, currently.[11,13] DNA barcoding was first described in 2003, and it refers to short gene sequence elements that can be used for species identification.[11] Since its inception, DNA barcoding has become a robust technique that has been

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dramatically developed to replace DNA fingerprints in ecological research. In 2004, The Consortium for the Barcode of Life (CBOL) was founded to develop orientations and standards in the barcode research and management system of living things. In 2007, the DNA barcode standard for terrestrial plants was announced by CBOL, and plant identification became easier.

In recent years, genetic analysis studies on *Polyspora* species have gained attention; comparative research and genetic analysis among the species have been conducted. The chloroplastic genome is weighed up a sequence region that is highly conserved throughout evolution. Hence, it is a source for comparing and identifying plant species. The chloroplastic structure of *Polyspora* was analysed and included 132 genes encoding 87 proteins, 37 tRNS, and 8 rRNA. Among them, *matK* (metabolite of the maturase K) and *rbcL* (Ribulose-bisphosphate carboxylase) are the two main regions suggested by CBOL for plant identification; *accD* (Acetyl-CoA carboxylase beta subunit) and *ycf1* (ycf1 is the second largest gene in the plastid genome) are potential sequences with high nucleotide diversity.[9] In this study, marker regions, including *rbcL* and *matK*, were used to indicate the genetic relationship between *P. huongiana* and *Polyspora* and *Camellia* published DNA data.[14] *accD* and *ycf1*, regions of inconsistency in plants, were also evaluated as species-identifying factors.[15,16]

MATERIALS AND METHODS

Plant Sample and Sample Preparation

Polyspora huongiana Orel, Curry & Luu were collected from TK89, Đạ Chais village, Lạc Dương district, Lạm Đỗng province, Vietnam, by Truong Quang Cuong. The plant was identified and tagged voucher number 210622PHU. The leaves were double-washed with distilled water before being stored at -20°C until analysis.

DNA Extraction and Amplification

The leaves were ground in liquid nitrogen, and DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method.[17] Amplicons were amplified using MyTaq mix (Meridian, USA) with the chloroplast primers listed in Table 1.[13] The PCR reactions were prepared as follows: 10 µL MyTaq mix 2X, 400 nMol primers (PhuSaBiochem, Vietnam), 50 ng template, and nuclease-free water (Promega, AUS) up to 20 µL. The PCR cycles were conducted using a DTlite instrument (DNA-Technology, Russia) with the temperature programme of 95°C for 2 min, 35 cycles of 10 sec at 95°C, 10 sec at 60°C, 60 sec at 72°C, and an additional 2 min of 72°C for once. A human beta-globin template (Medick Ltd Co., Vietnam) was used as a positive control (PCR using Beta globin primers) and negative control (PCR using chloroplast primers).

Product Purification and Sequencing

Electrophoresis in 1% agarose (Bio-Helix, Taiwan) for 45 min at 90 V confirmed the PCR products. An ExactMark 100 bp DNA ladder (1st BASE, Malaysia) was used for product length determination. The PCR products were purified by adding ExoSAP IT (ThermoFisher, USA) at a ratio of 5:2 and incubating for 15 min at 37°C and 15 min at 80°C. The purified products were subjected to PCR with BigDye Terminator Cycle Sequencing (Applied Biosystem, USA) and sequenced using an ABI 3500 analyser (Applied Biosystem, USA). The obtained sequences were trimmed for 20–50 nucleotides on both sides for noise signal elimination and analysed using Snapgen V5.3.2. The forward and reverse sequences were aligned and united.

Reducing Power Measurement

The DNA sequences of *accD, matK, rbcL*, and *ycf1* were checked using the NCBI database's BLAST (Basic local alignment search tools) tool. Each sequence's 100 most related sequences were chosen for further analysis (Table 2). DNA sequences were aligned in the Mega11 programme with the Clustal W algorithm.[18] Sequences were randomly assembled to form sequence combinations using Mega11 and DnaSP version 6.12.03.[18,19] The phylogeny of *P. huongiana* was determined by maximum likelihood estimation with 1000 bootstrap replications and was considered strong (frequency above 85%), moderate (50 - 85%), and weak (below 50%).[20] The neighbour-joining algorithm built the phylogenetic trees.[21]

Statistical Analysis

The data were stored and statistically analysed using GraphPad Prism version 9.0.0 software. Data expressed as mean ± standard deviation. The unpaired Student's t-test and One-way ANOVA followed by Tukey's post hoc test were applied for statistical comparisons with an alpha value of 0.05.

RESULTS

Estimation of Sequence Divergence

The DNA polymorphisms of the *accD, matK, rbcL*, and *ycf1* regions within *Polyspora* were computed on 10 random sequences, which further clarified the DNA divergence compared with the *Camellia* genus (Figure 1 and Table 3). The nucleotide diversity (π) and the average number of nucleotide differences of the regions are presented in Table 4, which varied from 0.00164 to 0.00531 for *Polyspora* and from 0.0004 to 0.10650 for

Table 1. The primers used for amplified the marker regions.

Target	Forward primer	Reverse primer
accD	CAGAGCGAGGCCAGTGAAAGTGAAGATC CG	GCCACTGGTACAGCCTCGGTTAATC CTG
matK	CAGAGCGAGGCGTACAGTACTTTTGTGTT TACGAG	GCCACTGGTAACCCAGTCCATCTGG AAATCTTGGTTC
rbcL	CAGAGCGAGGATGTCACCACAAACAGAG ACTAAAGC	GCCACTGGTAGTAAAATCAAGTCCA CCRCG
ycf1	CAGAGCGAGGTCTCGACGAAAATCAGATT GTTGTGAAT	GCCACTGGTACGATGGAATCGACCG TTGCG
Beta globin	ATGCCTCTTTGCACCATTCT	CAGTTTAGTAGTTGGACTTAG

Table 2. Sequence region locations on the chloroplast genome of gene regions were analyzed.

Accession No.	**Species**	**Nucleotide position**			
		accD	***matK***	***rbcL***	***ycf1***
NC_061599.1	*Camellia limonia*	58626 - 60367	1948 - 3647	56669 - 58296	125931 - 131552
NC_057956.1	*Camellia achrysantha*	102947 - 104688	46278 - 47977	100090 - 102617	13691 - 19412
NC_041672.1	*Camellia renshanxiangiae*	58647 - 60388	1947 - 3646	56694 - 58321	125959 - 131754
NC_039645.1	*Camellia nitidissima*	59123 - 60864	2034 - 2733	57165 - 58792	126423 - 132238
NC_035652.1	*Camellia elongata*	58719 - 60460	1947 - 3646	56759 - 58386	126052 – 131840
OP580978.1	*Camellia euryoides*	59134 - 60824	2020 - 3719	57126 - 58753	126393 - 132214
OQ630970.1	*Camellia cordifolia*	58751 - 60441	1953 - 3652	56744 - 58371	126067 - 131861
ON208849.1	*Camellia pingguoensis*	58956 - 60697	1947 - 3646	56998 - 58625	126196 - 132002
ON208848.1	*Camellia ptilosperma*	58934 - 60675	1947 - 3652	56976 - 58603	126244 - 132065
NC_069227.1	*Camellia tamdaoensis*	58628 - 60369	1941 - 3640	56671 - 58298	111129 - 116944
ON755230.1	*Polyspora axillaris*	58628 - 60369	1944 - 3637	56673 - 58300	126004 - 131825
NC_035645.1	*Polyspora axillaris*	58624 - 60325	1945 – 3638	56669 - 58296	126034 - 131855
MW801387.1	*Polyspora chrysandra*	58290 - 60031	1944 - 3637	56334 - 57961	125695 - 131516
NC_035648.1	*Polyspora dalgleishiana*	58360 - 60092	1945 - 2638	56404 - 58031	125657 – 131394
MK994520.1	*Polyspora hainanensis*	58229 - 59919	1945 - 3638	56221 - 57848	125578 – 131390
ON755225.1	*Polyspora longicarpa*	59057 - 60798	1980 - 3673	57101 - 58728	126321 - 131936
NC_059950.1	*Polyspora penangensis*	58976 - 60717	1946 - 3639	57028 - 58655	126240 - 132061
ON755229.1	*Polyspora speciosa*	59004 - 60745	1945 - 3645	57047 - 58674	126334 - 132155
NC_053889.1	*Polyspora tiantangensis*	59057 - 60798	1980 - 3673	57101 - 58728	126321 - 132136
NC_067734.1	*Polyspora tonkinensis*	59020 - 60761	1946 - 3639	57064 - 58691	126377 - 132186

Camellia, respectively. *ycf1* was the most variable chloroplastic sequence region compared within the genus in both *Polyspora* and *Camellia*.

P. huongiana Chloroplast DNA Markers

The selected regions for analysis included sequences with high nucleotide diversity values and were limited to two conserved regions of primers hybridising. The initial half sequences of *accD* and *rbcL*, the central *matK* gene, and the second half of *ycf1* were selected for analysis of *P. huongiana*. The sequences were submitted to NCBI (National Centre for Biotechnology Information, USA) with accession numbers OR525840, OR52584, OR52584, and OR525843 for *accD, matK, rbcL*, and *ycf1* of *P. huongiana*, respectively. The dynamic DNA quick response coding (DDQR) algorithm was provided by the Central China Normal University and Institute of Medicinal Plant Development, Chinese Academy of Medical Science, China (www.1kmpg.cn/ddqr/), which created DDQR for the sequences obtained in this work (Figure 2).[22]

A Phylogenetic Tree Built from a Single Sequence

The sequences of *accD* and *ycf1* showed the greatest similarity to *Camellia weiningensis* (MK820035.1) and *Polyspora axillaris* (ON755230.1); the sequences of *matK* and *rbcL* showed the greatest resemblance to *Polyspora hainanensis* (NC_035693.1).[23-25] The 100 most related sequences were collected for further analysis. The branches show the clustered associated taxa. The initial trees for the heuristic search were generated automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the fixed model. The topology obtained the highest log likelihood value. The sequences set up codon positions, including 3 frames and noncoding. The best-fixed models for *accD, matK*, and *ycf1* were the Tamura 3-parameter

Table 3. *Polyspora huongiana*-related sequences obtained by the BLAST tool and corresponding accession numbers.

Accession No.	Species	Marker gene regions			
		accD	***matK***	***rbcL***	***ycf1***
KJ197940.1	*Apterosperma oblata*		x		
NC_035641.1	*Apterosperma oblata* voucher YangSX 4361			x	
NC_057956.1	*Camellia achrysantha*	x			
KX216453.1	*Camellia albogigas*		x		
MK994520.1	*Polyspora hainanensis*		x	x	x
NC_050354.1	*Camellia anlungensis* voucher CANLU20191106	x			
MN078085.1	*Camellia vietnamensis*		x	x	
KY406793.1	*Camellia reticulata*	x			x
KY626042.1	*Camellia luteoflora*	x			x
MZ618349.1	*Camellia chrysanthoides*	x	x	x	
NC_024663.1	*Camellia reticulata*	x			x
MW256435.1	*Camellia brevistyla*				x
NC_052752.1	*Camellia brevistyla*				x
NC_072329.1	*Camellia caudata*				x
NC_058854.1	*Camellia confusa*				x
ON000202.1	*Camellia confusa*			x	
NC_067053.1	*Camellia connata*	x		x	
MN078088.1	*Camellia meiocarpa*			x	x
NC_061904.1	*Camellia costata*				x
NC_024541.1	*Camellia crapnelliana*				x
NC_022459.1	*Camellia cuspidata* voucher HKAS:S.X.Yang3159	x			
MN078090.1	*Camellia oleifera*		x	x	
MT123282.1	*Camellia suaveolens*		x	x	
NC_035643.1	*Polyspora speciosa* voucher YXQ145	x	x	x	x
NC_035645.1	*Polyspora axillaris* voucher YXQ099	x	x	x	x
KU669077.1	*Camellia chrysanthoides* voucher Y.Q.Liufu 1529				x
MT663341.1	*Camellia chuongtsoensis*				x
MZ151355.1	*Camellia oleifera* var. oleifera			x	x
NC_035652.1	*Camellia elongata* voucher YangSX 5065	x			
OL405564.1	*Camellia euphlebia*	x			
MZ151356.1	*Camellia oleifera* var. oleifera			x	x
NC_053896.1	*Camellia fascicularis*	x			
OM868265.1	*Camellia fascicularis*	x		x	
KU669083.2	*Camellia fascicularis* voucher S.X.Yang 93527				x
MZ189740.1	*Camellia oleifera* var. oleifera			x	x
MZ665482.1	*Camellia meiocarpa*			x	x
NC_050388.1	*Camellia fraterna*				x
MW801387.1	*Polyspora chrysandra* voucher FZF		x	x	x
NC_038181.1	*Camellia granthamiana*		x		
MZ054232.1	*Camellia gigantocarpa*				x
NC_058879.1	*Camellia gigantocarpa*				x
KX216461.1	*Camellia handelii*		x		
MZ151357.1	*Camellia oleifera*		x	x	
KY626040.1	*Camellia huana*	x			
NC_022461.1	*Camellia impressinervis* voucher	x			
MT157620.1	*Camellia indochinensis*	x			
NC_035693.1	*Polyspora hainanensis* voucher YXQ097	x	x	x	x
OK135162.1	*Camellia indochinensis* var. tunghinensis	x			
NC_058646.1	*Camellia micrantha*	x	x	x	x
NC_059950.1	*Polyspora penangensis* voucher SING2015-178	x	x	x	x
LC679291.1	*Camellia japonica* voucher TF<JPN>:TW021201		x		
LC678549.1	*Camellia japonica* voucher TF<JPN>:TW023656		x		
LC678986.1	*Camellia japonica* voucher TF<JPN>:TW024624		x		
LC679132.1	*Camellia japonica* voucher TF<JPN>:TW024751		x		
LC678314.1	*Camellia japonica* voucher TF<JPN>:TW025361		x		
LC689760.1	*Camellia japonica* voucher TF<JPN>:TW026076			x	
LC677835.1	*Camellia japonica* voucher TF<JPN>:YK0056		x		
NC_079935.1	*Camellia jinshajiangica*	x			
NC_035648.1	*Polyspora dalglieshiana* voucher BROWP 501		x	x	x
NC_035689.1	*Polyspora longicarpa* voucher YangSX 4779		x	x	x
NC_054364.1	*Camellia perpetua*	x			x
NC_057957.1	*Camellia chrysanthoides*	x			x
NC_053889.1	*Polyspora tiantangensis* voucher Ma 15701		x	x	x
KX216414.1	*Camellia longipedicellata*		x		
NC_065391.1	*Camellia longipedicellata*				x

Table 3. Continued

NC_061051.1	*Camellia pingguoensis*	x	x	x	
NC_067609.1	*Camellia longissima* voucher S. X. Yang5079			x	
NC_061599.1	*Camellia limonia*	x	x		x
NC_061600.1	*Camellia petelotii* var. microcarpa	x	x	x	
MN579509.2	*Camellia lungzhouensis*	x			
NC_060777.1	*Camellia polyodonta*		x	x	x
NC_061610.1	*Camellia amplexifolia*	x	x	x	x
KU669092.2	*Camellia luteoflora* voucher Y.Q.Liufu1534				x
NC_062050.1	*Camellia aurea*	x	x	x	
NC_035688.1	*Camellia mairei* voucher YangSX 5054	x			
NC_065391.1	*Camellia longipedicellata* voucher YangSX 5001	x	x	x	x
MT956593.1	*Camellia meiocarpa*				x
NC_067082.1	*Camellia saluenensis*	x			x
NC_067088.1	*Camellia pingguoensis* var. terminalis	x	x	x	
NC_058881.1	*Camellia meiocarpa* clone 3				x
NC_067085.1	*Camellia bambusifolia*	x			x
NC_067090.1	*Camellia ptilosperma*	x	x	x	
KU669078.1	*Camellia micrantha* voucher Q.Q.Ye 1314				x
NC_065198.1	*Camellia formosensis*			x	x
NC_039645.1	*Camellia nitidissima*	x			
MT157617.1	*Camellia nitidissima* var. nitidissima	x			
NC_063576.1	*Camellia suaveolens*		x	x	
NC_067086.1	*Camellia lienshanensis*	x			x
NC_067081.1	*Camellia edithae*		x	x	x
NC_067087.1	*Camellia subintegra*		x	x	x
NC_067091.1	*Camellia indochinensis* var. tunghinensis	x	x	x	
NC_067089.1	*Camellia parvipetala*		x	x	x
NC_067092.1	*Camellia wumingensis*	x	x	x	
NC_067734.1	*Polyspora tonkinensis* voucher FZF20220217	x	x	x	x
NC_068780.1	*Camellia flavida*	x	x	x	
OP953554.1	*Camellia oleifera*		x	x	
NC_067613.1	*Camellia lipingensis*	x		x	x
NC_068781.1	*Camellia longzhouensis*	x	x	x	
NC_067764.1	*Camellia lanceoleosa*	x		x	x
NC_080228.1	*Camellia oligophlebia*	x			
NC_069227.1	*Camellia tamdaoensis*	x	x	x	
NC_069309.1	*Camellia insularis*	x	x	x	x
KU669079.1	*Camellia parvipetala* voucher Q.Q.Ye 1316				x
MT157621.1	*Camellia perpetua*	x			
NC_069310.1	*Camellia minima*	x		x	x
KU669085.1	*Camellia perpetua* voucher Y.Q.Liufu 1531				x
MT157619.1	*Camellia petelotii* var. microcarpa	x			
NC_070214.1	*Camellia pyxidiacea*	x	x	x	
NC_068785.1	*Camellia obtusifolia*		x	x	x
NC_080274.1	*Camellia uraku*	x	x	x	x
NC_072608.1	*Camellia lungshenensis*	x			x
ON208849.1	*Camellia pingguoensis*	x		x	
OK046127.1	*Camellia leyensis*	x	x	x	
OK149109.1	*Camellia pingguoensis* var. terminalis	x			
OK235334.1	*Camellia petelotii* var. microcarpa	x	x	x	
AB207877.1	*Camellia pitardii*	x			
OP709388.1	*Camellia pitardii* var. alba	x			
NC_079666.1	*Camellia brevipetiolata*	x			x
NC_080233.1	*Camellia omeiensis*	x			x
NC_022462.1	*Camellia pitardii* voucher HKAS:S.X.Yang3148				x
OK546696.1	*Camellia luteocalpandria*	x	x	x	
NC_080884.1	*Camellia bailinshanica*	x			x
NC_072174.1	*Camellia hongkongensis*		x	x	x
NC_081063.1	*Camellia magniflora*	x			x
OL450398.1	*Camellia pingguoensis* var. terminalis	x	x		
KU669091.1	*Camellia ptilosperma* voucher Q.Q.Ye 1315				x
KU669075.1	*Camellia ptilosperma* voucher Y.Q.Liufu 1530				x
MW543444.1	*Camellia pubipetala*	x			
NC_054365.1	*Camellia pubipetala*	x			
OK181904.1	*Camellia trichosperma*		x	x	
MW629114.1	*Camellia pyxidiacea* var. rubituberculata	x			
MZ424202.1	*Camellia pyxidiacea* var. rubituberculata	x			

Table 3. Continued

MZ766253.1	*Camellia pyxidiacea* var. rubituberculata	x		x	
NC_041672.1	*Camellia renshanxiangiae*	x			
OK377261.1	*Camellia polyodonta*	x			x
OL685018.1	*Camellia sp. 'longruiensis'*	x	x	x	
NC_050389.1	*Camellia rhytidophylla*	x			
OM935753.1	*Camellia chrysanthoides*	x	x		
NC_041473.1	*Camellia sasanqua*				x
OL689015.1	*Camellia gauchowensis*			x	x
MZ403753.1	*Camellia semiserrata*				x
NC_058880.1	*Camellia semiserrata*				x
ON208846.1	*Camellia indochinensis* var. tunghinensis	x	x	x	
ON208847.1	*Camellia petelotii* var. petelotii	x	x	x	
OL689023.1	*Camellia semiserrata* var_GN1				x
MZ359672.1	*Camellia semiserrata* var. magnocarpa	x			
OP404083.1	*Camellia sinensis*	x			
OL840044.1	*Camellia sinensis* var. assamica			x	
OM677554.1	*Camellia sinensis* var. assamica voucher_TCM172K-		x		
OM677553.1	*Camellia sinensis* var. assamica voucher TCM170K-		x		
OL944068.1	*Camellia sinensis* var. assamica voucher TCM193K-		x		
OM677563.1	*Camellia sinensis* var. assamica voucher TCM196K-		x		
OM677583.1	*Camellia sinensis* var. assamica voucher TCM238K-		x		
ON208848.1	*Camellia ptilosperma*	x	x	x	x
OQ281601.1	*Camellia sinensis* var. sinensis	x			
ON208850.1	*Camellia wumingensis*	x	x	x	
MZ128138.1	*Camellia sp.* XJ-2021		x		
OL689014.1	*Camellia chekiangoleosa*		x	x	
OL689016.1	*Camellia meiocarpa* cultivar X3			x	x
OL689018.1	*Camellia oleifera*		x	x	x
OL689019.1	*Camellia oleifera*		x	x	x
OL689020.1	*Camellia oleifera*		x	x	
OL689021.1	*Camellia oleifera*		x	x	x
NC_035651.1	*Camellia szechuanensis* voucher YangSX 5066				x
AF380052.1	*Camellia taliensis*			x	
AF380095.1	*Camellia taliensis*		x		
OL689024.1	*Camellia vietnamensis*		x	x	x
OK405020.1	*Camellia tetracocca*			x	
AB207882.1	*Camellia tsaii*	x			
OL742653.1	*Camellia polyodonta*		x	x	x
ON755226.1	*Polyspora hainanensis* voucher FZF20220110	x	x	x	x
MN078084.1	*Camellia vietnamensis*			x	
ON755227.1	*Polyspora speciosa* voucher FZF20220121	x	x	x	x
NC_060778.1	*Camellia vietnamensis*			x	
ON755229.1	*Polyspora speciosa* voucher FZF20220310	x	x	x	x
OL689022.1	*Camellia sasanqua*			x	x
MK820035.1	*Camellia weiningensis*	x			
ON755230.1	*Polyspora axillaris* voucher FZF20220406	x	x	x	x
ON367462.1	*Camellia semiserrata*		x	x	
KU669080.1	*Camellia xiashiensis* voucher Y.Q.Liufu 1528				x
ON072481.1	*Camellia yokdonensis* cultivar Dung & Hakoda		x		
OL689025.1	*Camellia yuhsienensis*			x	x
NC_058253.1	*Camellia zhaiana* voucher YangSX 6023	x			
MH332607.1	*Gordonia sp.* gp-413		x		
AF089716.1	*Polyspora axillaris*			x	
AF380047.1	*Polyspora axillaris*			x	
AF380090.1	*Polyspora axillaris*		x		
AF421092.1	*Polyspora axillaris*			x	
KJ440031.1	*Polyspora axillaris*			x	
KJ510931.1	*Polyspora axillaris*		x		
OM262114.1	*Camellia sinensis* var. pubilimba		x		x
OL537881.1	*Polyspora axillaris* voucher BRIT:Gostel593		x		
ON418964.1	*Camellia meiocarpa*		x	x	x
AF380091.1	*Polyspora chrysandra*		x		
ON418963.1	*Camellia suaveolens*			x	x
ON418965.1	*Camellia sp.* XJ-2021		x	x	x
AF380092.1	*Polyspora hainanensis*		x		
KJ197938.1	*Polyspora hainanensis*		x		
ON755225.1	*Polyspora longicarpa* voucher FZF20211220		x	x	x

Table 3. Continued

OP580978.1	*Camellia euryoides*	x	x	x	x
OP936137.1	*Camellia sp.* XJ-2021	x	x	x	x
AF380051.1	*Polyspora longicarpa*			x	
AF380094.1	*Polyspora longicarpa*		x		
KJ197937.1	*Polyspora longicarpa*		x		
OP723864.1	*Camellia pitardii* var. cryptoneura	x			x
OQ630970.1	*Camellia cordifolia*	x	x	x	
OP953553.1	*Camellia semiserrata*	x			x
AF380093.1	*Polyspora speciosa*		x		
OP036120.1	*Camellia oleifera*			x	x
OP953555.1	*Camellia vietnamensis*	x		x	x
OP953554.1	*Camellia oleifera*		x	x	
OQ707217.1	*Camellia borealiyunnanica*	x	x		
NC_067734.1	*Polyspora tonkinensis*			x	
OQ538305.1	*Camellia pitardii* var. compressa	x			x

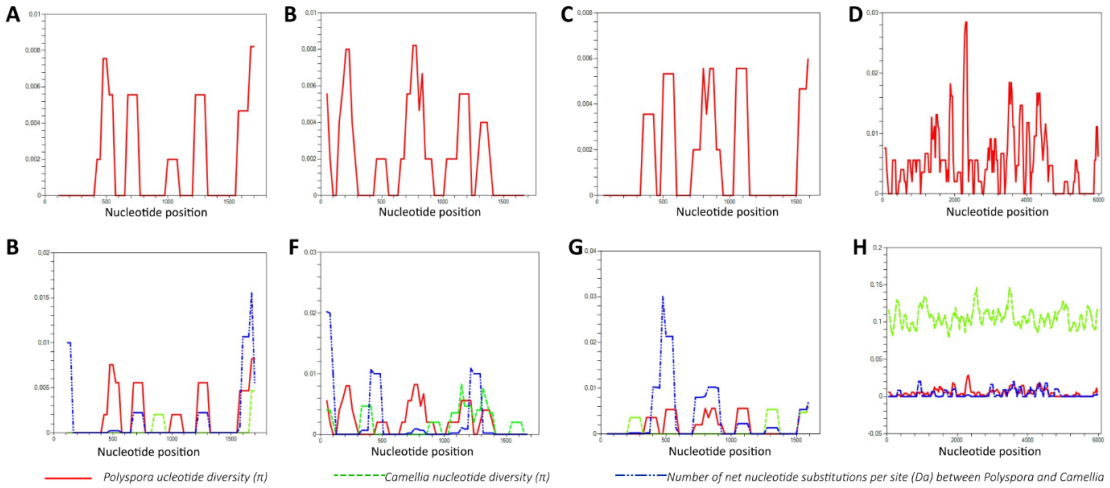

Figure 1. The DNA polymorphism of the *Polyspora* and *Camellia* sequences. The nucleotide diversity of *accD* (A), *matK* (B), *rbcL* (C), and *ycf1* (D) genes with peripheral 100 nucleotides supplement of the genus of *Polyspora*. The DNA polymorphism of of *accD* (E), *matK* (F), *rbcL* (G), and *ycf1* (H) genes comparing between *Polyspora* and *Camellia* genera.

Table 4. Genetic parameters of evaluated *Polyspora* and *Camellia* populations.

Values	Populations		Sequences			
	Polyspora	***Camellia***	***accD***	***matK***	***rbcL***	***ycf1***
Nucleotide diversity (π)	x		0.0020	0.00232	0.00164	0.00531
		x	0.0004	0.00168	0.00083	0.10650
	x	x	0.00217	0.00334	0.00314	0.05768
Average number of nucleotide differences (k)	x		3.356	3.933	2.667	29.222
		x	0.667	2.844	1.356	585.622
	x	x	3.637	5.658	5.111	317.163
The average number of nucleotide differences between populations	x	x	5.100	7.700	7.900	325.930
The average number of nucleotide substitutions per site between populations (Dxy)	x	x	0.00304	0.00455	0.00485	0.05927
The number of net nucleotide substitutions per site between populations	x	x	0.00184	0.00254	0.00362	0.00337

model (T92), and the one for *rbcL* was the Jukes–Cantor model (JC). The phylogenic trees were constructed with maximum likelihood in 1000 bootstraps (Figure 3). The *rbcL* and *ycf1* sequences clearly separated the *Polyspora* species population (Figure 3).

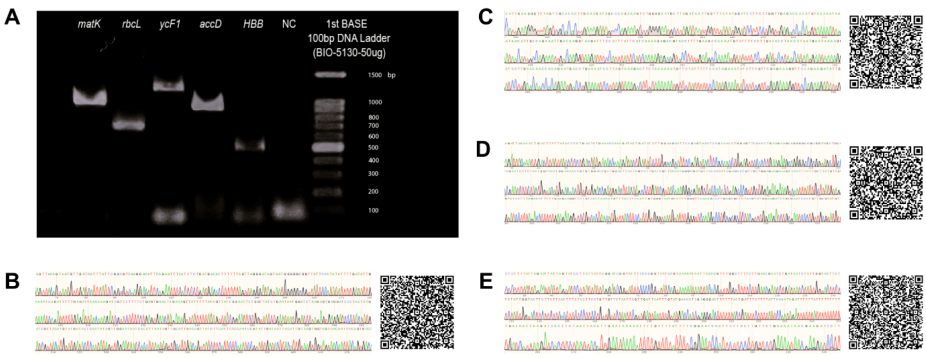

Figure 2. The amplicons of the evaluated marker genes. The amplicons were tested for quality by gel electrophoresis with the positive markers (A). The nucleotide signals by Sanger sequencing and the DDQR codes of the sequences of *accD* (B), *matK* (C), *rbcL* (D), and *ycf1* (E).

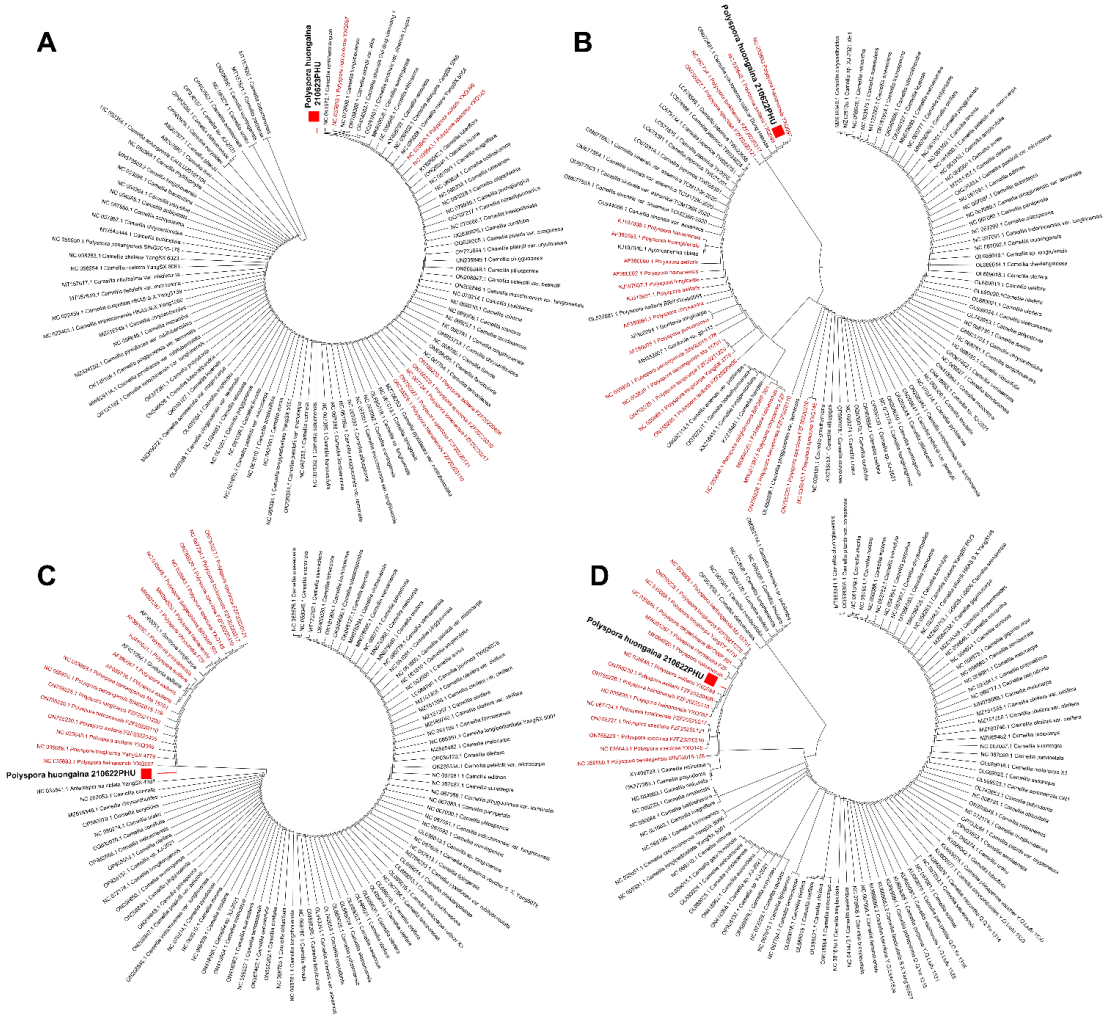

Figure 3. The topology trees built from single-sequence. The topology trees of *accD* (A), *matK* (B), *rbcL* (C), and *ycf1* (D) were conducted from the 100 most related sequences by using Maximum Likelihood algorithm with 1000 bootstraps and a fixed model. *Polyspora* species were in red.

A Phylogenetic Tree Built from Multiple Sequences

Sequence combinations were performed using Snapgene V5.3.2 and were coded into DDQR codes (Figure 4). The combination sequences were coalesced by two, three, or four markers, further contributing to phylogenetic tree conduction (Figures 5-7). The combination demonstrated

the ability to distinguish genera better than the single factor mentioned above.

Two-sequence coherence revealed the authentic separation of the *Polyspora* out of the *Camellia*. The nucleotide diversities between populations of *Polyspora* and *Camellia* were respectively recorded as 0.00582, 0.00121, 0.05682, 0.00969, 0.0376, and 0.04222 for the fusions of *accD + matK, accD + rbcL, accD + ycf1, matK + rbcL, matK + ycf1,* and *rbcL + ycf1*. Evolutionary analysis with maximum likelihood and the T92 model were performed on the fused sequences, with the exception of *rbcL + ycf1*.[26] The T92 evolutionarily invariable (+I, 40,03% sites) model was chosen for the *rbcL + ycf1* evolutionary analyses.[26,27] The topology of *accD + matK* showed that *P. huongiana* had the most related sequence to *Camellia pingguoensis* (OL450398.1), while the rest of the analysis reflexed *P. axillaris* and *P. hainanensis*.[28] *AcD + matK + rbcL, matK + rbcL + ycf1, rbcL + ycf1 + accD*, and *ycf1 + accD + matK* were obtained by assembling sequences from *P. huongiana* and the most-sequence-related collection. The nucleotide diversity were 0.00105 ± 0.00019, 0.00170 ± 0.01135, 0.0442 ± 0.02269, and 0.02712 ± 0.01989 for the fusions in the order. The pairwise distances from *P. huongiana* to *Camellia* (from 0.0005998 ± 0.0005010 to 0.005519 ± 0.002343) and that within *Polyspora* (from 0.001701 ± 0.0001957 to 0.008859 ± 0.001912) indicated a significant difference, p-value < 0.0001 (Figure 7). The *accD + matK + rbcL, matK + rbcL + ycf1, and rbcL + ycf1 + accD* algorithms were built evolutionary using the T92 model and Maximum likelihood applying Neighbour-joining and BioNJ algorithms.[26] The *ycf1 + accD + matK* were analysed by the T92 with a discrete Gamma distribution (+G, parameter = 0.3937) model featuring evolutionary rate differences among sites (Figure 6).

The combination of the four sequence regions allowed the distinction of two genera at large genetic distances (Figure 8). There was a significant difference between the pairwise distances from *P. huongiana* within *Polyspora* (0.003725 ± 0.002167) and to *Camellia* (0.007074 ± 0.0003353), p-value = 0.0013; the analysis was based on the T92+G model with 1000 bootstrap replication.[26,29] The nucleotide diversity of the populations was 0.02234 ± 0.01563 in the *Polyspora* and Camellia combination and was 0.00344 ± 0.00037 only in the *Polyspora* genus. The phylogenic tree was obtained using the T92+G model. *P. huongiana* was separated from the most related species *P. axillaris and P. hainanensis* with a frequency of 94% bootstraps.

DISCUSSION

matK and *rbcL* are two commonly used plant classification genes suggested by CBOL.[11] The sequence of *matK* has been recorded to have the fastest evolution in chloroplasts, so it is often used in analysing the evolution and phylogeny of plant species.[30,31] However, the analysis of closely related plant families requires a strong method for identification, as the two sequence regions are not strong enough to distinguish between *Camellia* and *Polyspora*. Nucleotide variation at loci >0.035 is considered highly polymorphic, and it was recorded through the nucleotide density values.[32,33] Based on the analysis of this study, the *matK* and *rbcL* markers did not meet the value. Therefore, two other proposed sequences were added to improve species identity, namely *accD* and *ycf1*.[15] In particular, the *accD* sequence was determined to have low genetic variability, thereby helping to ensure the specific identification of species within the genus. On the other hand, the *ycf1* sequence exhibited high polymorphism in both *Polyspora* and *Camellia* genera, enhancing the ability to separate species between genera.[16] DNA polymorphism in *accD* sequences was higher among *Polyspora* species than within *Camellia species*, which was also detected in *rbcL* and *matK* genes. The *ycf1* sequence exhibited high genetic variability and was evenly distributed throughout the gene region. the DNA variability of *ycf1* was significantly higher in *Camellia* than in *Polyspora*. Most of the nucleotide differences between *Polyspora* and *Camellia* were located in the first half of the *matK* and *rbcL* genes but in the two ends of the *accD* gene. The length-appropriate sequences were selected based on nucleotide diversity distributed according to the nucleotide position to perform Sanger sequencing. The sequences and fusions of *P. huongiana* were translated into DDQR for easy retrieval.

The results showed that *Camellia weiningensis (accD* sequence), *P. hainanensis (rbcL* and *matK fusion*), and *P. axillaris (ycf1* sequence) had the most similar genetic sequences. These two genera of plants are often confused with each other due to common morphological characteristics; for example, *Polyspora (Gordonia) yunnanensis* Hu (wfo-0001219828) and *Camellia taliensis* (wfo-0000582705) have been still considered two names for the same species. All 100 most compatible sequences observed in this study belonged to the genera *Camellia* and *Polyspora*, indicating a deep genetic connection. Using a single sequence to distinguish these two genera faced many obstacles. Hence, sequence combination is necessary for differentiated augments. The combination of two or more sequences allowed for better separation of *Polyposra* from *Camellia*. The two-gene markers proved effective; these results were also analysed for other plant species,

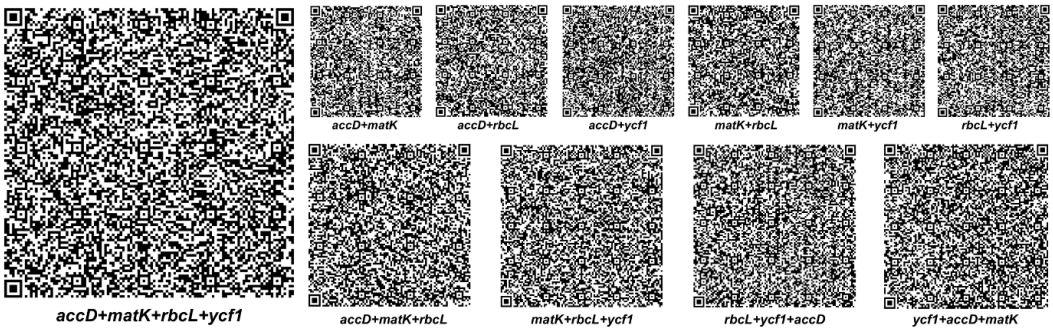

Figure 4. The DDQR codes for the sequence combinations of *P. huongiana.*

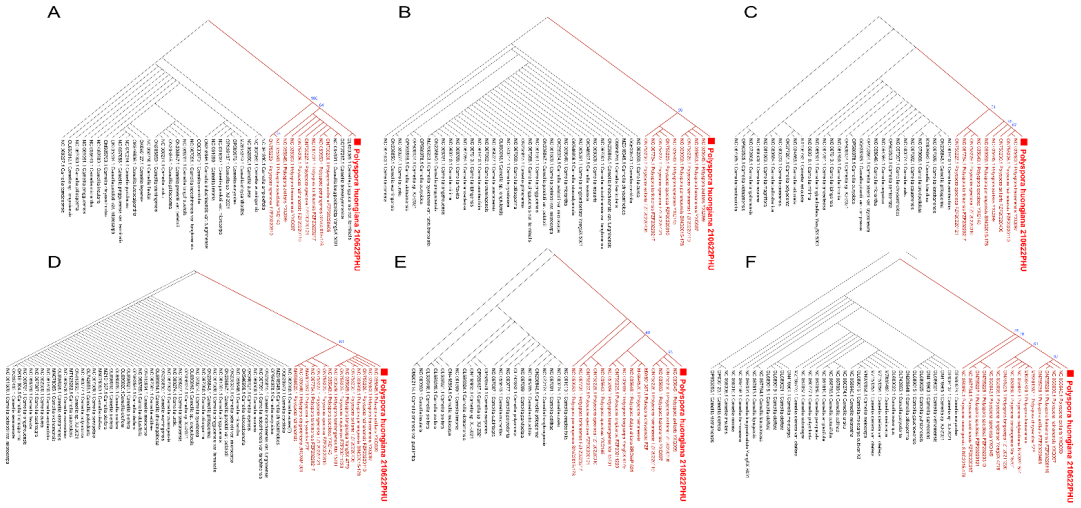

Figure 5. The topology trees of the two-region combinations. Topology trees of the combinations of *accD+matK* (A), *accD+rbcL* (B), *accD+ycf1* (C), *matK+rbcL* (D), *matK+ycf1*(E), and *rbcL+ycf1* (F) were built up by using Maximum Likelihood algorithm with 1000 bootstraps and a fixed model. *Polyspora* species were in red.

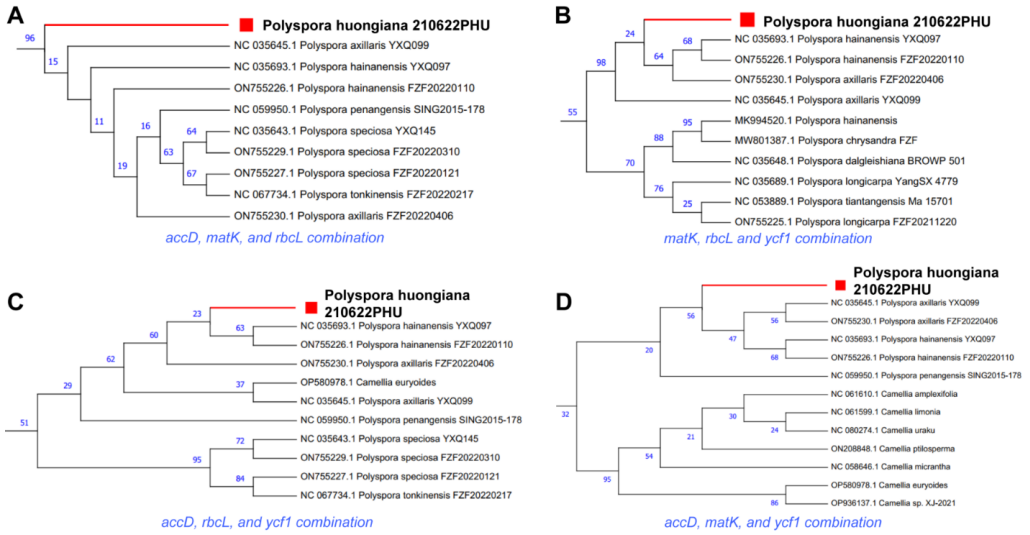

Figure 6. The topology trees of the tri-sequence fusions. Topology trees of the combinations of *accD+matK+rbcL* (A), *matK+rbcL+ycf1* (B), *rbcL+ycf1+accD* (C), and *ycf1+accD+matK* (D) were conducted by using Maximum Likelihood algorithm with 1000 bootstraps and a fixed model.

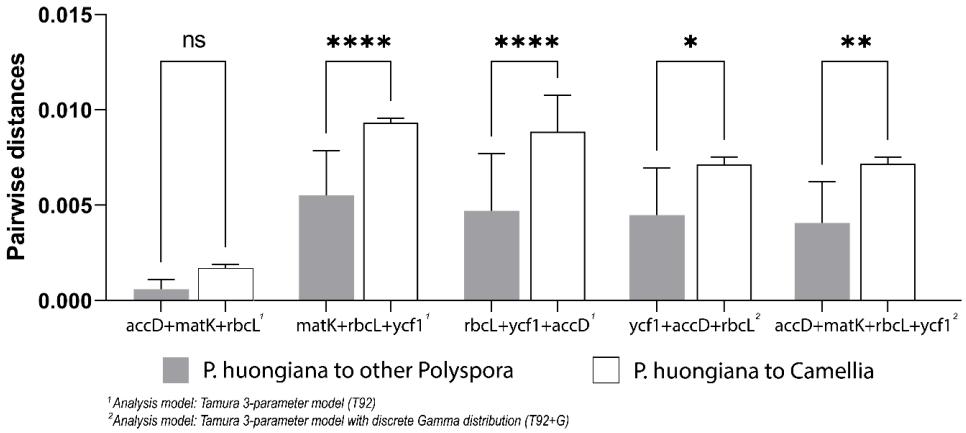

Figure 7. The genetic distances were measured from *P. huongiana* to the other evaluated. The genetic distances of *P. huongiana* to the others were determined by using the best-fixed model conducted by Mega 11 software. The P-values expressed as "ns" for not statistically significant, "*", "**", and "****" for the values less than 0.03, 0.002, and 0.0001, respectively.

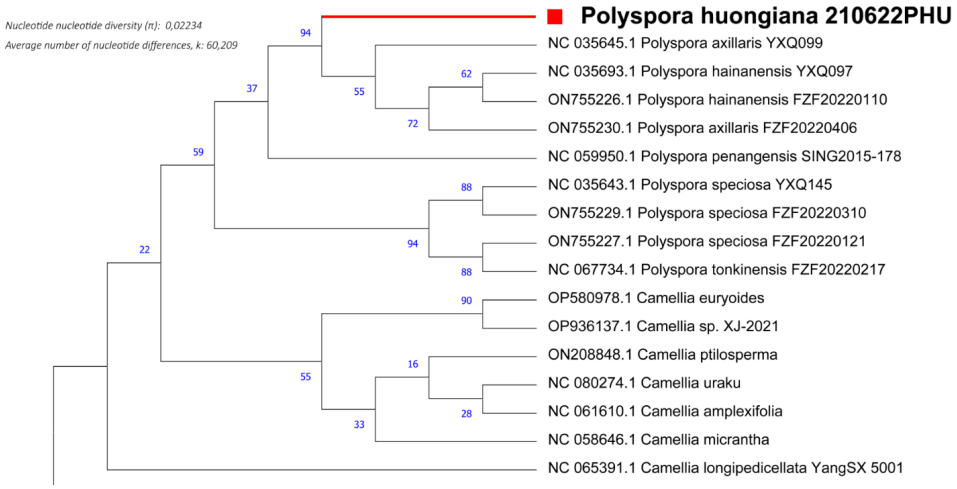

Figure 8. The topology tree of the quart-sequence fusion of *accD+matK+rbcL+ycf1*. The *accD+matK+rbcL+ycf1* topology tree was conducted by using the Maximum Likelihood algorithm with 1000 bootstraps and the T92+G model.

such as jewel orchids, in previous research.[20,34] The combination of the three sequences provided good species discrimination within the genus *Polyspora*, and the most effective combination was *rbcL + ycf1 + accD*. Similarly, combining all four sequence regions gave a large genetic distance and separation between the *Camellia* and *Polyspora* populations, besides, it illustrated good species discrimination within the genus.

The genetic distances between individuals can be used as an element to identify individuals with species, genera, families, and orders.[29,35,36] In this study, the genetic distance between *P. huongiana* and the closest neighbour ranged from 0.0016 to 0.008 in tri- or quant-sequence fusion analysis. *P. axillaris* and *P. hainanensis* were the most connected species published in the NCBI database to *P. huongiana*, based on nucleotide analysis of four investigated regions. Thus far, the plants were judged to share common characteristics and have a deep relationship with the species native to Vietnam, namely *P. bidoupensis, P. gigantiflora, P. intricata, P. balansae, P. tonkinensis, P. axillaris*.[3,14] The close relative relationship between *P. huongiana* and P. axillaris was genetically confirmed in this study. However, the lack of genetic data on endemic species in Vietnam, especially native tea species, has prevented an in-depth analysis. Research on the genetic diversity of plant species has required attention and implementation plans to address the issue of the lack of genetic information; it helps determine the relationships among phytosociological factors, thereby helping to define the interaction between species.[37] As a result, the most effective exploitation, use, and conservation strategies for plant species in general and area-highly specific plant species, in particular, will be deployed.

CONCLUSION

The combination of two or more of the sequences *accD, matK, rbcL*, and *ycf1* can be considered as a strategy to recognise the *Polyspora* or *Camellia* genera. It is suggested that the tri-sequence or quat-sequence fusion of the mentioned regions can be used to identify *Polyspora* species, including *P. huongiana*, which have a close genetic relationship with *Polyspora axillaris* and *Polyspora hainanensis*. The combination of genetic markers in accurately identifying *P. huongiana* species will contribute positively to the rapid identification and conservation of this rare tea species.

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Peer Review: Externally peer-reviewed.

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