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Comprehensive Genomic Analysis for Accurate Identification of *Cytospora* Species: Insights from *ITS*, *TUB*, *RPB2*, and *ACT* Gene Regions

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Abstract:

The classification of Cytospora species, has historically presented challenges due to the limitations of conventional methodologies. Understanding the genetic characteristics of these species has become a vital necessity for their precise identification and classification. In this comprehensive study, we conducted an in-depth analysis of the genetic composition of various Cytospora species, focusing on four distinct gene regions: ITS, ACT, TUB, and RPB2. Our primary objective was to elucidate the evolutionary relationships among these species and their capability to differentiate from other taxonomic groups. Within the ITS region, our analysis revealed the presence of 68 clades. However, this region was able to effectively separate only 43% of the studied species. Notably, a basal clade featuring C. valsoide was observed, with relatively modest bootstrap values for species containing multiple sequences. Turning our attention to the ACT region, we identified 45 clades, achieving a 53% success rate in species separation. Importantly, this region exhibited higher bootstrap values for certain species, including C. populinopsis, C. japonica, and C. leucostoma sequences. The TUB region emerged as particularly successful, presenting 34 clades and achieving an impressive 76% success rate in species separation. High bootstrap values were observed for species such as C. chrysosperma, C. davidiana, and C. brevispora. In the RPB2 region, we identified 63 clades, effectively separating 80% of the studied species. Species with multiple sequences formed cohesive clusters with high bootstrap values. The combination of gene regions yielded robust results. In the combined phylogenetic analysis of gene regions, ITS+ACT, ITS+RPB2, ITS+TUB, ITS+ACT+TUB and ITS+TUB+RPB2+ACT were able to separate 97, 97, 100, 116 and 125 species among 137, respectively. This research underscores the necessity of analyzing multiple gene regions for precise Cytospora species identification. It notably highlights the significance of the RPB2 and TUB genes, particularly when resource constraints are a concern. These findings offer valuable insights into the genetic diversity and relationships within the Cytospora genus, significantly advancing the accuracy of taxonomic classification and enhancing our understanding of these fungal species.

Keywords: Phylogenetic analysis, Single-Gene, Multigene phylogeny, Species identification.

Introduction

*Cytospora, introduced by Ehrenberg in 1818, is a significant pathogenic genus responsible for causing canker disease on branches of various tree and shrub species. This disease often results in extensive dieback in a wide range of plants, including forest trees, fruit trees, and ornamental species (*Adams et al., 2004*) (Extension Utah State University, 2025*). *Cytospora* represents the asexual form of *Valsa*, which serves as the type genus of Valsaceae within the order Diaporthales Nannf. Additionally, the family Valsaceae includes other genera such as *Amphicytostroma*, *Chadefaudiomyces*, *Cryptascoma*, *Ditopellina*, *Durispora*, *Harpostroma*, *Hypospilina*, *Kapooria, Leptosillia, Maculatipalma, Pachytrype*, and *Paravalsa* (Yang et al., 2015).

In addition to its association with the genus *Valsa*, *Cytospora* has also been recognized as the asexual form of other genera, including *Leucocytospora*, *Leucostoma*, *Valsella*, and *Valseutypella*. These genera share a morphological connection with *Cytospora*, indicating the presence of different stages or forms within the life cycle of these fungal organisms (Adams et al., 2002).

Consequently, Adams et al., in 2006 unified all of these sexual genera under the umbrella of *Valsa*, merging them either as subgenera or species. This taxonomic consolidation aimed to establish a more comprehensive classification system and facilitate a better understanding of the interrelationships among these fungal genera (X. Fan et al., 2015; X. L. Fan et al., 2015; Wikee et al., 2011).

Consequently, the genus *Valsa* (1849) was considered synonymous with *Cytospora* (1818), with the latter being recognized as the oldest and most extensively employed name. This decision was made to maintain consistency and avoid confusion in the taxonomy of these organisms (Fan et al., 2014; Fotouhifar et al., 2010).

The majority of the names formerly attributed to *Valsa* already possess an earlier epithet within *Cytospora* according to the SMML Fungal Database (http://nt.arsgrin.gov/fungaldatabases/). Additionally, Rossman et al., (2015) introduced certain new names for the widespread species of *Cytospora* that were previously classified under *Valsa (*Rossman et al., 2015*)*. This update aimed to enhance the accuracy and consistency of nomenclature within the field of mycology.

Cytospora species usually produce asexual fruiting bodies and contain a single or labyrinthine of locules, filamentous conidiophores or asci, and allantoid hyaline conidia or allantoid hyaline ascospores (X. Fan et al., 2015; X. L. Fan et al., 2015). In moist conditions, the conidia will emerge from the fruiting bodies forming yellow, orange to red gelatinous tendrils ((Adams et al., 2006).

Cytospora species are typically identified based on their association with specific host plants, as morphological descriptions often lack distinctive differences. As a result, it is possible for a single *Cytospora* species to be found on multiple unrelated host plants, or for a single host plant to host multiple *Cytospora* species (Wang et al., 2015). In recent years, phylogenetic approaches have become essential tools for the accurate identification of *Cytospora* species and related fungi. Traditional morphological identification alone often leads to misclassification because of high phenotypic plasticity and overlapping host affiliations (Pan et al., 2020) (Wang, Jiang, & Ma, 2024). Therefore, molecular phylogenetic analyses based on multiple gene regions provide a more reliable framework for distinguishing closely related taxa (Reeb et al., 2004; Tang et al., 2007). Among the available markers, the internal transcribed spacer (ITS) region has been widely used as a universal fungal DNA barcode due to its relatively high interspecific variability, although it sometimes lacks sufficient resolution among closely related species (Pan et al., 2020). The β-tubulin (TUB) and RNA polymerase II second largest subunit (RPB2) genes offer higher phylogenetic resolution at species and genus levels (Reeb et al., 2004; Tang et al., 2007), while the actin (ACT) gene contributes additional support for clade differentiation (Guan et al., 2020).

Multi-locus phylogenetic analysis provides a more robust and reliable framework for species delimitation in *Cytospora*, overcoming the limitations of both traditional morphological identification and single-gene approaches (Pan et al., 2020; Reeb et al., 2004; Tang et al., 2007).

In Index Fungorum (2017), there are 585 epithets assigned to *Cytospora*, with an estimated count of 110 species according to Kirk et al., (2013) (Fungorum, 2017; Kirk et al., 2013). However, ex-type sequence data is only accessible for 23 species in GenBank (Benson et al., 2018). Consequently, identifying species solely based on a phylogenetic perspective poses difficulties (Hyde et al., 2014).

The objective of this study was to identify *Cytospora* species within a specific region through phylogenetic analysis. Additionally, a multi-locus phylogeny was constructed using ITS, LSU, RPB2, and ACT sequence data to enhance the resolution of *Cytospora* species. Furthermore, the distribution of *Cytospora* species on different hosts is also discussed in this research. The comprehensive analysis of *Cytospora* species in this study not only addresses long-standing taxonomic challenges but also provides valuable insights into their ecological associations, distribution, and potential implications for forest health management and biodiversity conservation.

Materials and methods

Information Retrieval

A comprehensive search was carried out on renowned academic databases, including Google Scholar, Scopus, and PubMed, utilizing the keywords "*Cytospora*" and "*Cytospora* sp. no." The purpose of this search was to compile a selection of reliable articles related to valid species of the genus *Cytospora*. These articles were carefully reviewed to identify newly reported species and access phylogenetic data. Subsequently, a definitive list of valid species was prepared.

Dataset Compilation

Table 1 presents the sequences of valid species sourced from gene repositories, which were essential for conducting phylogenetic analyses of *Cytospora* species. Within this dataset, strains marked with 'T' represent the type species, while 'NA' indicates caseswhere the desired isolate lacked a corresponding sequence in the gene pools.

Table 1: Authentic species sequences obtained from gene bank for use in phylogenetic study of the genus *Cytospora* species (strains marked with T are types and NA indicates the absence of the sequence in gene bank)

Species	Strain	ITS	*ACT1*	*RPB2*	*TUB2*
C. ailanthicola	CFCC 89970^T	MH933618	MH933526	MH933592	MH933565
C. abyssinica	CMW 10181^T	AY347353	NA	NA	NA
C. abyssinica	CMW 10178	AY347354	NA	NA	NA
C. acaciae	CBS 468.69	DQ243804	NA	NA	NA
C. ampulliformis	MFLUCC 16-0583^T	KY417726	KY417692	KY417794	NA
C. ampulliformis	MFLUCC 16-0629	KY417727	KY417693	KY417795	NA
C. amygdali	CBS 144233^T	MG971853	MG972002	NA	MG971718
C. atrocirrhata	CFCC 89615	KR045618	KF498673	KU710946	KR045659
C. atrocirrhata	CFCC 89616	KR045619	KF498674	KU710947	KR045660
C. atrocirrhata	CFCC 89625	EF447305	NA	NA	NA
C. austromontana	CMW 6735^T	AY347361	NA	NA	NA
C. beilinensis	CFCC 50493^T	MH933619	MH933527	NA	MH933561
C. beilinensis	CFCC 50494	MH933620	MH933528	NA	MH933562
C. berberidis	CFCC 89927^T	KR045620	KU710990	KU710948	KR045661
C. berberidis	CFCC 89933	KR045621	KU710991	KU710949	KR045662
C. berkeleyi	StanfordT3^T	AY347350	NA	NA	NA
C. berkeleyi	UCBTwig3	AY347349	NA	NA	NA
C. brevispora	CBS 116829	AF192321	NA	NA	NA
C. brevispora	CBS 116811^T	AF192315	NA	NA	NA
C. bungeana	CFCC 50495^T	MH933621	MH933529	MH933593	MH933563
C. bungeana	CFCC 50496	MH933622	MH933530	MH933594	MH933564
C. californica	CBS 144234^T	MG971935	MG972083	NA	NA
C. castanae	DBT 183^T	KC963921	NA	NA	NA
C. carbonacea	CFCC 89947	KR045622	KP310842	KU710950	KP310825
C. carpobroti	CMW 48981^T	MH382812	NA	NA	MH411207
C. cedri	CBS 196.50	AF192311	NA	NA	NA
C. celtidicola	CFCC 50497^T	MH933623	MH933531	MH933595	MH933566
C. celtidicola	CFCC 50498	MH933624	MH933532	MH933596	MH933567
C. centrivillosa	MFLUCC 16-1206^T	MF190122	NA	MF377600	NA
C. centrivillosa	MFLUCC 17-1660	MF190123	NA	MF377601	NA

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Species	Strain	ITS	*ACT1*	*RPB2*	*TUB2*
C. ceratosperma	CFCC 89624*	KR045645	NA	KU710976	KR045686
C. ceratosperma	CFCC 89625*	KR045646	NA	KU710977	KR045687
C. ceratospermopsis	CFCC 89626^{T}	KR045647	KU711011	KU710978	KR045688
C. ceratospermopsis	CFCC 89627	KR045648	KU711012	KU710979	KR045689
C. chrysosperma	CFCC 89629	KF765673	NA	KF765705	NA
C. chrysosperma	CFCC 89981	MH933625	MH933533	MH933597	MH933568
C. chrysosperma	CFCC 89982	KP281261	KP310835	NA	KP310818
C. cinerostroma	CMW 5700^{T}	AY347377	NA	NA	NA
C. cinnamomea	CFCC 53178*^{T}	MK673054	MK673024	NA	MK672970
C. coryli	CFCC 53162^{T}	MN854450	NA	MN850751	MN861120
C. cotoneastricola	CF 20197027*	MK673072	MK673042	MK673012	MK672988
C. cotoneastricola	CF 20197031*^{T}	MK673075	MK673045	MK673015	MK672991
C. cotini	MFLUCC 14-1050^{T}	KX430142	NA	KX430144	NA
C. curvata	MFLUCC 15-0865^{T}	KY417728	KY417694	KY417796	NA
C. davidiana	CXY 1350^{T}	KM034870	NA	NA	NA
C. davidiana	CXY 1374	KM034869	NA	NA	NA
C. diatrypelloidea	CMW 8549^{T}	AY347368	NA	NA	NA
C. diopuiensis	MFLUCC 18-1419^{T}	MK912137	MN685819	NA	NA
C. disciformis	CMW 6509^{T}	AY347374	NA	NA	NA
C. disciformis	CMW 6750	AY347359	NA	NA	NA
C. elaeagni	CFCC 89632	KR045626	KU710995	KU710955	KR045667
C. elaeagni	CFCC 89633	KF765677	KU710996	KU710956	KR045668
C. elaeagnicola	CFCC 52882^{T}	MK732341	MK732344	MK732347	NA
C. eriobotryae	IMI 136523^{T}	AY347327	NA	NA	NA
C. erumpens	CFCC 50022*	MH933627	MH933534	NA	MH933569
C. erumpens	CFCC 53163*	MK673059	MK673029	MK673000	MK672975
C. eucalypti	LSEQ	AY347340	NA	NA	NA
C. eucalypti	CBS 144241	MG971907	MG972056	NA	MG971772
C. eucalypticola	ATCC 96150^{T}	AY347358	NA	NA	NA
C. eucalypticola	CMW 5309	AF260266	NA	NA	NA
C. eucalyptina	CMW 5882	AY347375	NA	NA	NA
C. eugeniae	CMW 7029	AY347364	NA	NA	NA
C. eugeniae	CMW 8648	AY347344	NA	NA	NA
C. euonymicola	CFCC 50499^{T}	MH933628	MH933535	MH933598	MH933570
C. euonymicola	CFCC 50500	MH933629	MH933536	MH933599	MH933571
C. euonymina	CFCC 89993^{T}	MH933630	MH933537	MH933600	MH933590
C. euonymina	CFCC 89999	MH933631	MH933538	MH933601	MH933591
C. fraxinigena	MFLU 17–0880	MF190134	NA	NA	NA
C. fraxinigena	MFLUCC 14-0868^{T}	MF190133	NA	NA	NA
C. friesii	CBS 194.42	AY347328	NA	NA	NA
C. fugax	CXY 1371	KM034852	NA	NA	KM034891
C. fugax	CXY 1381	KM034853	NA	NA	KM034890
C. fugax	CBS 203.42	AY347323	NA	NA	NA
C. galegicola	MFLUCC 18-1199^{T}	MK912128	MN685810	MN685820	NA
C. germanica	CXY 1322	JQ086563	NA	NA	NA
C. gigalocus	CFCC 89620^{T}	KR045628	KU710997	KU710957	KR045669
C. gigalocus	CFCC 89621	KR045629	KU710998	KU710958	KR045670
C. gigaspora	CFCC 50014	KR045630	KU710999.	KU710959	KR045671
C. gigaspora	CFCC 89634^{T}	KF765671	KU711000	KU710960	KR045672
C. granati	CBS 144237^{T}	MG971799	MG971949	NA	MG971664
C. hippophaës	CFCC 89639	KR045632	KU711001	KU710961	KR045673
C. hippophaës	CFCC 89640	KF765682	KF765730	KU710962	KR045674
C. japonica	CBS 375.29*	AF191185	NA	NA	NA
C. japonica	CFCC 89956*	KR045624	KU710993	KU710953	KR045665
C. japonica	CFCC 89960*	KR045625	KU710994	KU710954	KR045666
C. joaquinensis	CBS 144235^{T}	MG971895	MG972044	NA	MG971761
C. junipericola	BBH 42444	MF190126	NA	NA	NA
C. junipericola	MFLU 17-0882^{T}	MF190125	NA	NA	NA
C. juniperina	CFCC 50501^{T}	MH933632	MH933539	MH933602	NA
C. juniperina	CFCC 50502	MH933633	MH933540	MH933603	MH933572
C. juniperina	CFCC 50503	MH933634	MH933541	MH933604	NA
C. kantschavelii	CXY 1383	KM034867	NA	NA	NA
C. kantschavelii	CXY 1386	KM034867	NA	NA	NA
C. kuanchengensis	CFCC 52464^{T}	MK432616	MK442940	MK578076	NA
C. kuanchengensis	CFCC 52465	MK432617	MK442941	MK578077	NA
C. kunzei	CBS 118556	DQ243791	NA	NA	NA
C. longiostiolata	MFLUCC 16-0628^{T}	KY417734	KY417700	KY417802	NA
C. longispora	CBS 144236^{T}	MG971905	MG972054	NA	MG971764
C. leucosperma	CFCC 89622*	KR045616	KU710988	KU710944	KR045657
C. leucosperma	CFCC 89894*	KR045617	KU710989	KU710945	KR045658

Table 1 Continuation: Authentic species sequences obtained from gene bank for use in phylogenetic study of the genus *Cytospora* species (strains marked with T are types and NA indicates the absence of the sequence in gene bank)

Species	Strain	ITS	*ACT1*	*RPB2*	*TUB2*
C. leucostoma	CFCC 50016*	MH820400	MH820408	NA	MH820389
C. leucostoma	CFCC 50017*	MH933635	MH933542	NA	MH933573
C. leucostoma	CFCC 50018*	MH933636	MH933543	NA	MH933574
C. leucostoma	CFCC 50019*	MH933637	MH933544	NA	NA
C. leucostoma	CFCC 50020*	MH933638	MH933545	NA	NA
C. leucostoma	CFCC 50021*	MH933639	MH933546	NA	MH933575
C. leucostoma	CFCC 50023*	KR045635	KU711003	KU710964	KR045676
C. leucostoma	CFCC 50024*	MH933640	MH933547	MH933605	MH933576
C. leucostoma	CFCC 53156*	MN854447	MN850762	MN850748	MN861117
C. leucostoma	MFLUCC 16-0574*	KY417731	KY417696	KY417798	NA
C. leucostoma	MFLUCC 16-0589*	KY417732	KY417698	KY417800	NA
C. leucostoma	CFCC 53167*	MK673056	MK673026	MK672998	MK672972
C. lumnitzericola	MFLUCC 17-0508^{T}	MG975778	MH253457	MH253453	NA
C. mali	CFCC 50028*	MH933641	MH933548	MH933606	MH933577
C. mali	CFCC 50029*	MH933642	MH933549	MH933607	MH933578
C. mali-spectabilis	CFCC 53181*^{T}	MK673066	MK673036	MK673006	MK672982
C. melnikii	CFCC 89984	MH933644	MH933551	MH933609	MH933580
C. melnikii	MFLUCC 15-0851^{T}	KY417735	KY417701	KY417803	NA
C. melnikii	MFLUCC 16-0635	KY417736	KY417702	KY417804	NA
C. mougeotii	ATCC 44994	AY347329	NA	NA	NA
C. multicollis	CBS 105.89^{T}	DQ243803	NA	NA	NA
C. myrtagena	CFCC 52454	MK432614	MK442938	MK578074	NA
C. myrtagena	CFCC 52455	MK432615	MK442939	MK578075	NA
C. myrtagena	CBS 116843^{T}	AY347363	NA	NA	NA
C. nitschkii	CMW 10180^{T}	AY347356	NA	NA	NA
C. nitschkii	CMW 10184	AY347355	NA	NA	NA
C. nivea	MFLUCC 15-0860	KY417737	KY417703	KY417805	NA
C. nivea	CFCC 89641	KF765683	KU711006	KU710967	KR045679
C. nivea	CFCC 89643	KF765685	NA	KU710968	KP310829
C. notastroma	NE_TFR5	JX438632	NA	NA	NA
C. notastroma	NE_TFR8	JX438633	NA	NA	NA
C. ochracea	CFCC 53164*^{T}	MK673060	MK673030	MK673001	MK672976
C. oleicola	CBS 144248^{T}	MG971944	MG972098	NA	MG971752
C. olivacea	CFCC 53174*	MK673058	MK673028	MK672999	MK672974
C. olivacea	CFCC 53175*	MK673062	MK673032	MK673003	MK672978
C. olivacea	CFCC 53176*^{T}	MK673068	MK673038	MK673008	MK672984
C. olivacea	CFCC 53177*	MK673071	MK673041	MK673011	MK672987
C. palm	CXY 1276	JN402990	NA	NA	NA
C. palm	CXY 1280^{T}	JN411939	NA	NA	NA
C. parakantschavelii	MFLUCC 15-0857^{T}	KY417738	KY417704	KY417806	NA
C. parakantschavelii	MFLUCC 16-0575	KY417739	KY417705	KY417807	NA
C. parapersoonii	T28.1^{T}	AF191181	NA	NA	NA
C. parapistaciae	CBS 144506^{T}	MG971804	MG971954	NA	MG971669
C. parasitica	MFLUCC 15-0507*^{T}	KY417740	KY417706	KY417808	NA
C. parasitica	CFCC 53172*	MK673069	MK673039	MK673009	MK672985
C. parasitica	CFCC 53173*	MK673070	MK673040	MK673010	MK672986
C. paratranslucens	MFLUCC 15-0506^{T}	KY417741	KY417707	KY417809	NA
C. paratranslucens	MFLUCC 16-0627	KY417742	KY417708	KY417810	NA
C. piceae	CFCC 52841^{T}	MH820398	MH820406	MH820395	MH820387
C. piceae	CFCC 52842	MH820399	MH820407	MH820396	MH820388
C. pingbianensis	MFLUCC 18-1204^{T}	MK912135	MN685817	MN685826	NA
C. pini	CBS 197.42	AY347332	NA	NA	NA
C. pini	CBS 224.52^{T}	AY347316	NA	NA	NA
C. pistaciae	CBS 144238^{T}	MG971802	MG971952	NA	MG971667
C. platanicola	MFLU 17-0327	MH253451	MH253449	MH253450	NA
C. platyclada	CFCC 50504^{T}	MH933645	MH933552	MH933610	MH933581
C. platyclada	CFCC 50505	MH933646	MH933553	MH933611	MH933582
C. platyclada	CFCC 50506	MH933647	MH933554	MH933612	MH933583
C. platycladicola	CFCC 50038^{T}	KT222840	MH933555	MH933613	MH933584
C. platycladicola	CFCC 50039	KR045642	KU711008	KU710973	KR045683
C. plurivora	CBS 144239^{T}	MG971861	MG972010	NA	MG971726
C. populicola	CBS 144240^{T}	MG971891	MG972040	NA	MG971757
C. populina	CFCC 89644^{T}	KF765686	KU711007	KU710969	KR045681
C. populinopsis	CFCC 50032*^{T}	MH933648	MH933556	MH933614	MH933585
C. populinopsis	CFCC 50033*	MH933649	MH933557	MH933615	MH933586
C. predappioensis	MFLUCC 17-2458^{T}	MG873484	NA	NA	NA
C. prunicola	MFLU 17-0995^{T}	MG742350	MG742353	MG742352	NA

Table 1 Continuation: Authentic species sequences obtained from gene bank for use in phylogenetic study of the genus *Cytospora* species (strains marked with T are types and NA indicates the absence of the sequence in gene bank)

Species	Strain	ITS	*ACT1*	*RPB2*	*TUB2*
C. pruni-mume	CFCC 53179*	MK673057	MK673027	NA	MK672973
C. pruni-mume	CFCC 53180*^{T}	MK673067	MK673037	MK673007	MK672983
C. pruinopsis	CFCC 50034^{T}	KP281259	KP310836	KU710970	KP310819
C. pruinopsis	CFCC 50035	KP281260	KP310837	KU710971	KP310820
C. pruinopsis	CFCC 53153	MN854451	MN850763	MN850752	MN861121
C. pruinosa	CBS 201.42^{T}	DQ243801	NA	NA	NA
C. pruinosa	CFCC 50036	KP310800	KP310832	NA	KP310815
C. pruinosa	CFCC 50037	MH933650	MH933558	NA	MH933589
C. pubescentis	MFLUCC 18-1201^{T}	MK912130	MN685812	MN685821	NA
C. punicae	CBS 144244	MG971943	MG972091	NA	MG971798
C. quercicola	MFLU 17–0881	MF190128	NA	NA	NA
C. quercicola	MFLUCC 14-0867^{T}	MF190129	NA	NA	NA
C. rhizophorae	MUCC302	EU301057	NA	NA	NA
C. ribis	CFCC 50026	KP281267	KP310843	KU710972	KP310826
C. ribis	CFCC 50027	KP281268	KP310844	NA	KP310827
C. rosae	MFLU 17-0885	MF190131	NA	NA	NA
C. rosicola	CF 20197024*^{T}	MK673079	MK673049	MK673019	MK672995
C. rostrate	CFCC 89909^{T}	KR045643	KU711009	KU710974	KR045684
C. rostrate	CFCC 89910	KR045644	KU711010	KU710975	NA
C. rusanovii	MFLUCC 15-0853	KY417743	KY417709	KY417811	NA
C. rusanovii	MFLUCC 15-0854^{T}	KY417744	KY417710	KY417812	NA
C. salicacearum	MFLUCC 16-0576	KY417741	KY417707	KY417809	NA
C. salicacearum	MFLUCC 16-0587	KY417742	KY417708	KY417810	NA
C. salicicola	MFLUCC 15-0866	KY417749	KY417715	KY417817	NA
C. salicicola	MFLUCC 14-1052^{T}	KU982636	KU982637	NA	NA
C. salicina	MFLUCC 15-0862^{T}	KY417750	KY417716	KY417818	NA
C. salicina	MFLUCC 16-0637	KY417751	KY417717	KY417819	NA
C. schulzeri	CFCC 50040*	KR045649	KU711013	KU710980	KR045690
C. schulzeri	CFCC 50042*	KR045650	KU711014	KU710981	KR045691
C. sibiraeae	CFCC 50045*^{T}	KR045651	KU711015	KU710982	KR045692
C. sibiraeae	CFCC 50046*	KR045652	KU711015	KU710983	KR045693
C. sophorae	CFCC 50047	KR045653	KU711017	KU710984	KR045694
C. sophoricola	CFCC 89596	KR045656	KU711020	KU710987	KR045697
C. sophoricola	CFCC 89595^{T}	KR045655	KU711019	KU710986	KR045696
C. sophoriopsis	CFCC 89600^{T}	KR045623	KU710992	KU710951	KP310817
C. sorbi	MFLUCC 16-0631^{T}	KY417752	KY417718	KY417820	NA
C. sorbicola	MFLUCC 16-0584^{T}	KY417755	KY417721	KY417823	NA
C. sorbicola	MFLUCC 16-0633	KY417758	KY417724	KY417826	NA
C. sorbina	CF 20197660*^{T}	MK673052	MK673022	NA	MK672968
C. spiraeae	CFCC 50049*^{T}	MG707859	MG708196	MG708199	NA
C. spiraeae	CFCC 50050*	MG707860	MG708197	MG708200	NA
C. spiraeicola	CFCC 53138^{T}	MN854448	NA	MN850749	MN861118
C. spiraeicola	CFCC 53139	MN854449	NA	MN850750	MN861119
C. tamaricicola	CFCC 50507*	MH933651	MH933559	MH933616	MH933587
C. tamaricicola	CFCC 50508*^{T}	MH933652	MH933560	MH933617	MH933588
C. tanaitica	MFLUCC 14-1057^{T}	KT459411	KT459413	NA	NA
C. thailandica	MFLUCC 17-0262^{T}	MG975776	MH253459	MH253455	NA
C. thailandica	MFLUCC 17-0263^{T}	MG975777	MH253460	MH253456	NA
C. tibetensis	CF 20197026*	MK673076	MK673046	MK673016	MK672992
C. tibetensis	CF 20197029*	MK673077	MK673047	MK673017	MK672993
C. tibetensis	CF 20197032*^{T}	MK673078	MK673048	MK673018	MK672994
C. tibouchinae	CPC 26333^{T}	KX228284	NA	NA	NA
C. translucens	CXY 1351	KM034874	NA	NA	KM034895
C. ulmi	MFLUCC 15-0863^{T}	KY417759	NA	NA	NA
C. valsoidea	CMW 4309^{T}	AF192312	NA	NA	NA
C. valsoidea	CMW 4310	AF192312	NA	NA	NA
C. variostromatica	CMW 6766^{T}	AY347366	NA	NA	NA
C. variostromatica	CMW 1240	AF260263	NA	NA	NA
C. vinacea	CBS 141585^{T}	KX256256	NA	NA	KX256235
C. viridistroma	CBS 202.36^{T}	MN172408	NA	NA	NA
C. viticola	Cyt2	KX256238	NA	NA	KX256217
C. viticola	CBS 141586^{T}	KX256239	NA	NA	KX256218
C. xinjiangensis	CFCC 53182*	MK673064	MK673034	MK673004	MK672980
C. xinjiangensis	CFCC 53183*^{T}	MK673065	MK673035	MK673005	MK672981
C xinglongensis	CFCC 52458	MK432622	MK442946	MK578082	NA
C. xylocarpi	MFLUCC 17-0251^{T}	MG975775	MH253458	MH253454	NA

Data Analysis

Sequence Preparation and Alignment

The core objective of phylogenetic analysis is to infer the evolutionary relationships and history of genes or gene fragments by comparing their homologous positions. To do this, alignment of homologous sequences in the study is essential.. To facilitate this alignment, we employed BioEdit version 4.0.6.2 (Hall, 1999), incorporating the Clustal W algorithm for multiple sequence alignment. In cases where sequences were short and exhibited significant nucleotide variations in specific regions, additional data were introduced to prevent unwanted deletion of these regions during subsequent phylogenetic analyses

.**Phylogenetic Analysis for Species Discrimination and Evaluation of Gene Regions**

For species with multiple sequence alignments accessible in the gene repositories for the four gene regions, two or more sequences were selected for analysis. Additionally, two outgroup taxa, *Diaporthe vaccinii*, and *Diaporthe eres*, were chosen to root the phylogenetic trees. The phylogenetic analysis was conducted utilizing the maximum likelihood algorithm and validated with 1000 bootstrap replicates through the utilization of MEGA 11 software (Tamura et al., 2021). The best-fitting model for maximum likelihood analysis, as determined by the minimum Akaike Information Criterion (AIC) score, was employed. Furthermore, a bootstrap analysis with 1000 pseudoreplicates was performed to assess the support values of clades, branches, and tree topology.

Results

Separation Based on Individual Gene Regions

ITS Region

The phylogenetic tree based on the ITS region (Figure 1) revealed a total of 68 clades. However, this region exhibited a separation success rate of only 43% concerning the studied species in comparison to other taxa (Table 2). Notably, the clade representing the species *C. valsoide* emerged as the most basal clade at the terminus of the phylogenetic tree. It is important to mention that bootstrap values for species with multiple sequences used in the phylogenetic analysis were relatively low. Among these, the clade comprising *C. atrocinrhata* and *C. valsoidea* species displayed bootstrap values of 92 and 98, respectively.

Table 2: Separated species using ITS region

C. joaquinensis	*C. xinjiangensis*
C. galegicola	*C. multicollis*
C. ulmi	*C. notastroma*
C. friesii	*C. bungeanae*
C. davidiana	*C. kunze*
C. Rusanovii	*C. pini*
C. Rusanovii	*C. cinereostroma*
C. Viticola	*C. diatrypelloidea*
C. Elaeagnicola	*C. berkeleyi*
C. Oleicola	*C. parapistaciae*
C. pruinosa	*C. pistaciae*
C. sibiraeae	*C. parapersoonii*
C. cotoneastricola	*C. variostromatica*
C. rosicola	*C. variostromatica*
C. tibetensis	*C. eucalypti*
C. japonica	*C. californica*
C. sorbina	*C. vinacea*
C. atrocirrhata	*C. abyssinica*
C. sorbi	*C. acacia*
C. olivacea	*C. abyssinica*
C. amygdal	*C. palm*
C. sorbicola	*C. platyclada*
C. erumpens	*C. pingbianensis*
C. leucostoma	*C. brevispora*
C. pruinopsis	*C. rhizophorae*
C. salicicola	*C. viridistroma*
C. castanae	*C. eugeniae*
C. punicae	*C. myrtagena*
C. valsoidea	*C. tibouchinae*
C. gigalocus	

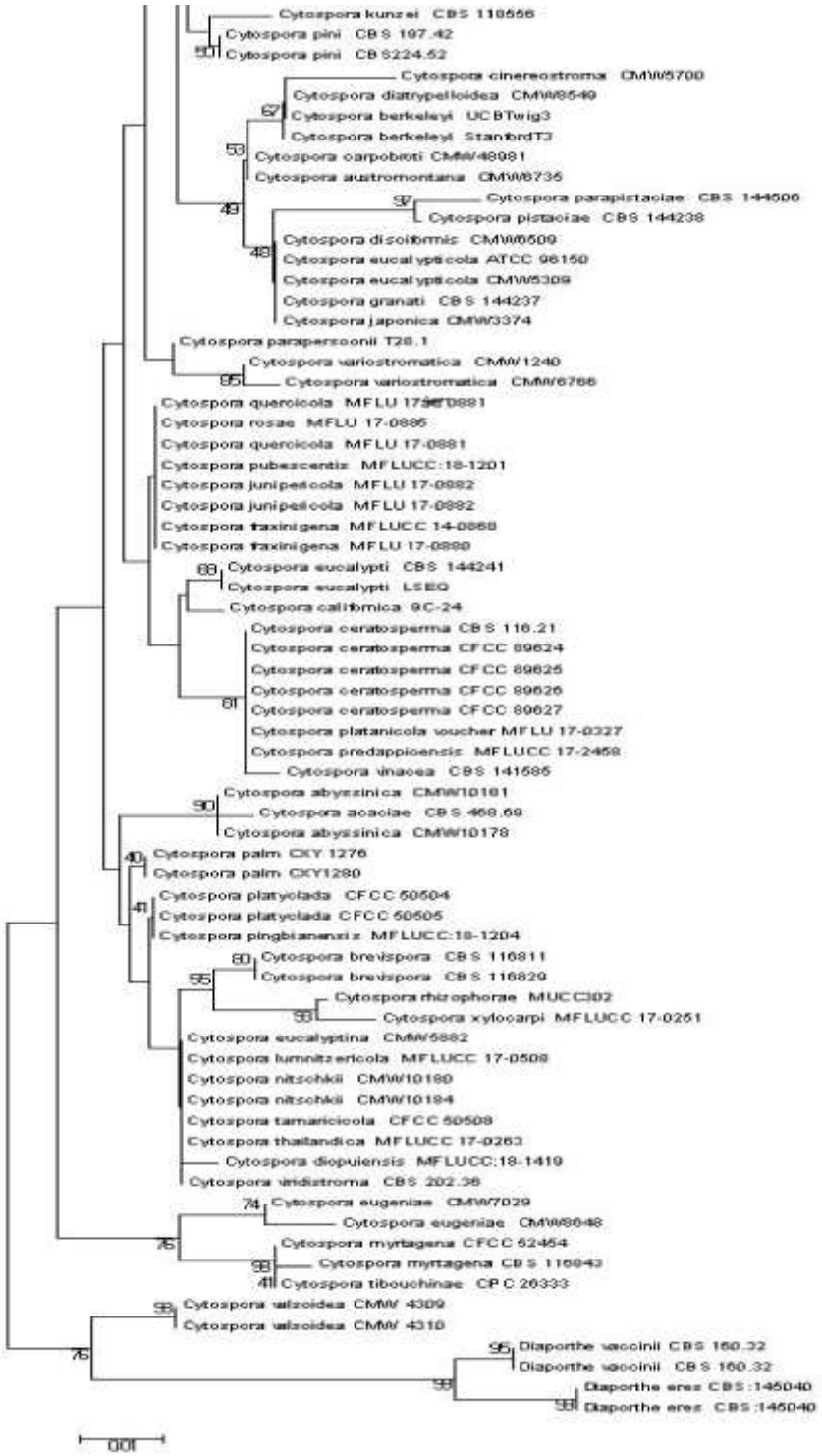
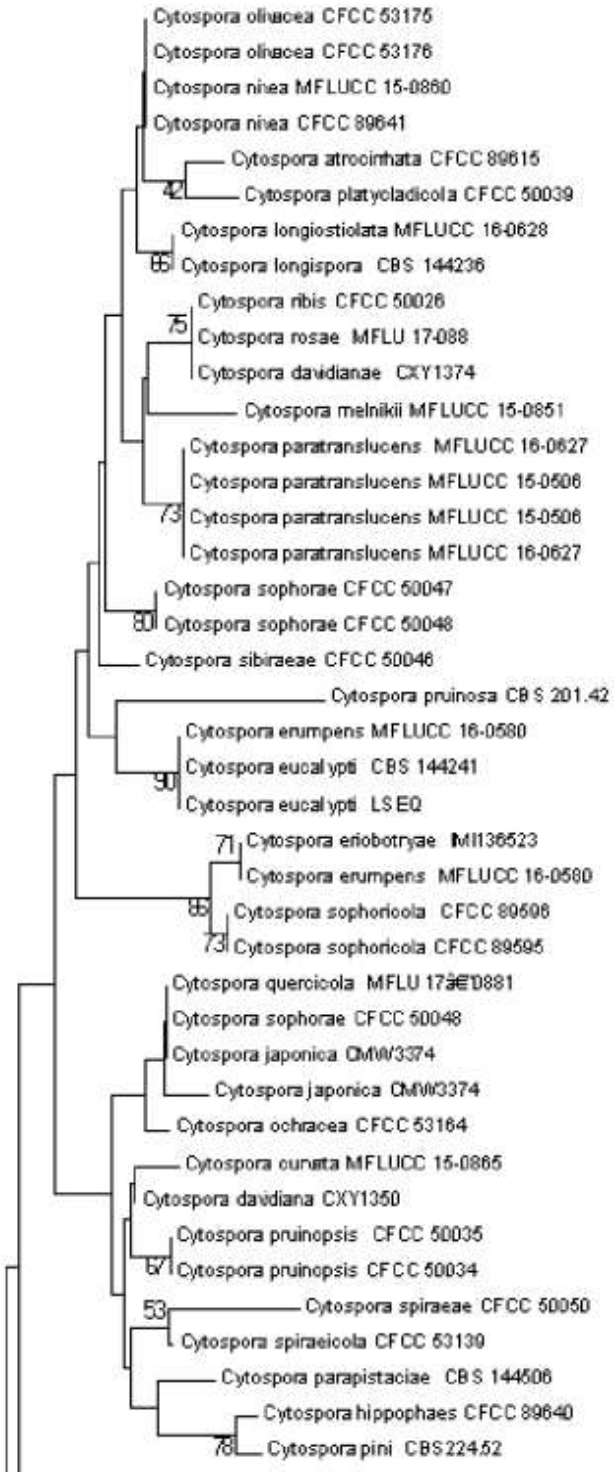


Figure 1: Phylogenetic tree based on ITS region using the model TN93+G+I in maximum likelihood analysis

ACT Region

Within the phylogenetic tree of the ACT region (Figure 2), 45 clades were identified. Impressively, this region succeeded in effectively separating 53% of the studied species (equivalent to 36% of the total species) from other taxa (Table 3). In the analysis, species with multiple sequences showed notably high bootstrap values. The most supported clade within this region included sequences of *C. populinopsis, C. japonica,* and *C. leucostoma*, featuring bootstrap values of 99, 99, and 95, respectively.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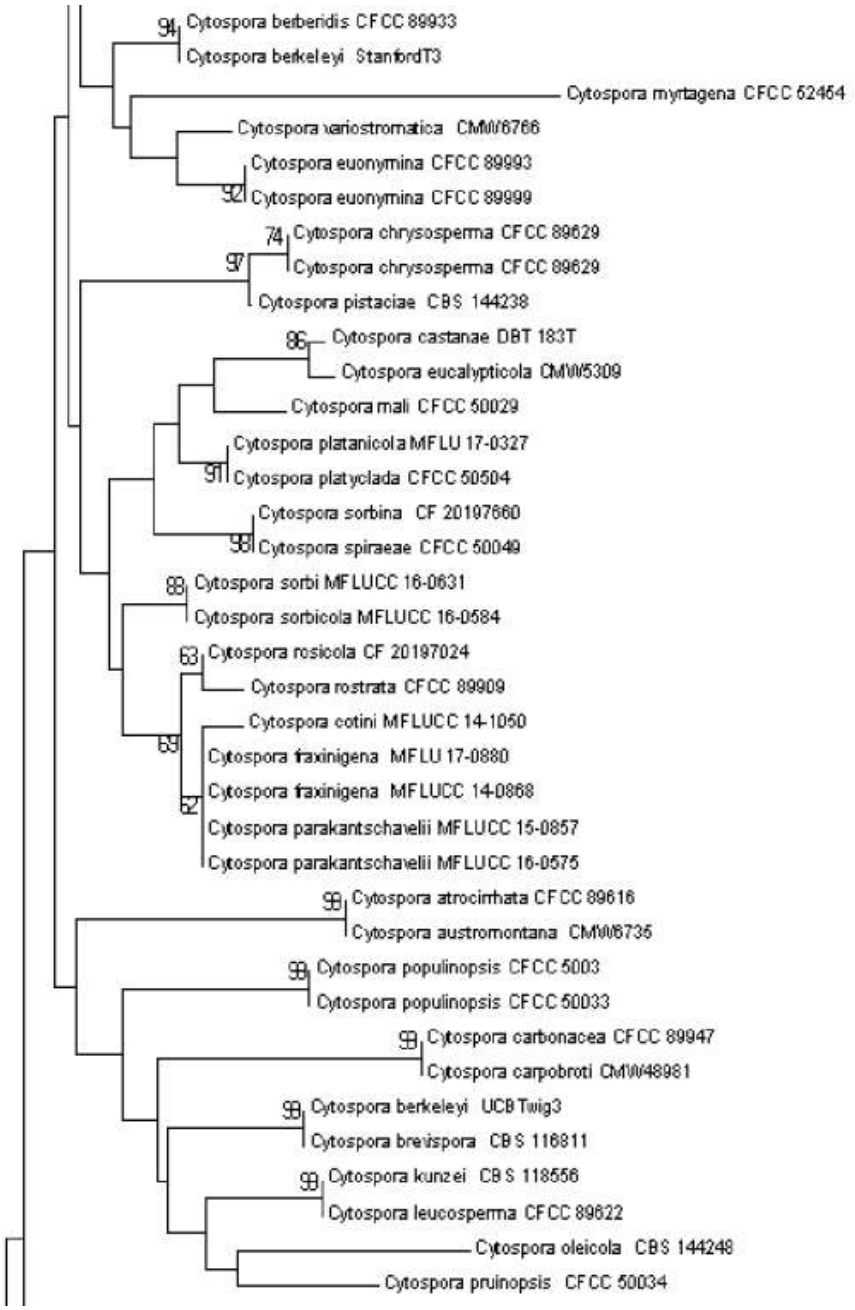
Cytospora oliviacea CFCC 53176
Cytospora nivea MFLUCC 15-0860
Cytospora nivea CFCC 89641
Cytospora atrocinhata CFCC 89615
42
Cytospora platycladicola CFCC 50039
Cytospora longiostiolata MFLUCC 16-0628
85
Cytospora longispora CBS 144236
Cytospora ribis CFCC 50026
75
Cytospora rosae MFLU 17-088
Cytospora davidianae CXY1374
Cytospora melnikii MFLUCC 15-0851
Cytospora paratranslucens MFLUCC 16-0627
Cytospora paratranslucens MFLUCC 15-0506
73
Cytospora paratranslucens MFLUCC 15-0506
Cytospora paratranslucens MFLUCC 16-0627
Cytospora sophorae CFCC 50047
80
Cytospora sophorae CFCC 50048
Cytospora sibiraeae CFCC 50046
Cytospora pruinosa CBS 201.42
Cytospora erumpens MFLUCC 16-0580
Cytospora eucalypti CBS 144241
90
Cytospora eucalypti LSEQ
71
Cytospora eriobotryae MI136523
Cytospora erumpens MFLUCC 16-0580
86
Cytospora sophoricola CFCC 89596
73
Cytospora sophoricola CFCC 89595
Cytospora quercicola MFLU 17â€“0881
Cytospora sophorae CFCC 50048
Cytospora japonica CMW3374
Cytospora japonica CMW3374
Cytospora ochracea CFCC 53164
Cytospora cuneata MFLUCC 15-0865
Cytospora davidiana CXY1350
Cytospora pruinopsis CFCC 50035
67
Cytospora pruinopsis CFCC 50034
53
Cytospora spiraeae CFCC 50050
Cytospora spiraeicola CFCC 53139
Cytospora parapistaciae CBS 144506
Cytospora hippophaes CFCC 89640
78
Cytospora pini CBS224.52


94
Cytospora berberidis CFCC 89933
Cytospora berkeleyi StanfordT3
Cytospora myrtagena CFCC 52464
Cytospora variostromatica CMW6766
Cytospora euonymina CFCC 89993
92
Cytospora euonymina CFCC 89999
74
Cytospora chrysosperma CFCC 89629
97
Cytospora chrysosperma CFCC 89629
Cytospora pistaciae CBS 144238
86
Cytospora castanae DBT 183T
Cytospora eucalypticola CMW5309
Cytospora mali CFCC 50029
Cytospora platanicola MFLU 17-0327
91
Cytospora platyclada CFCC 50504
Cytospora sorbina CF 20197660
98
Cytospora spiraeae CFCC 50049
88
Cytospora sorbi MFLUCC 16-0631
Cytospora sorbicola MFLUCC 16-0584
63
Cytospora rosicola CF 20197024
Cytospora rostrata CFCC 89909
Cytospora cotini MFLUCC 14-1050
69
Cytospora fraxinigena MFLU 17-0880
82
Cytospora fraxinigena MFLUCC 14-0868
Cytospora parakantschavelii MFLUCC 15-0857
Cytospora parakantschavelii MFLUCC 16-0575
98
Cytospora atrocirrhata CFCC 89616
Cytospora austromontana CMW6735
99
Cytospora populinopsis CFCC 50003
Cytospora populinopsis CFCC 50033
99
Cytospora carbonacea CFCC 89947
Cytospora carpobroti CMW48981
99
Cytospora berkeleyi UCB Twig3
Cytospora brevispora CBS 118811
99
Cytospora kunzei CBS 118556
Cytospora leucosperma CFCC 89622
Cytospora oleicola CBS 144248
Cytospora pruinopsis CFCC 50034

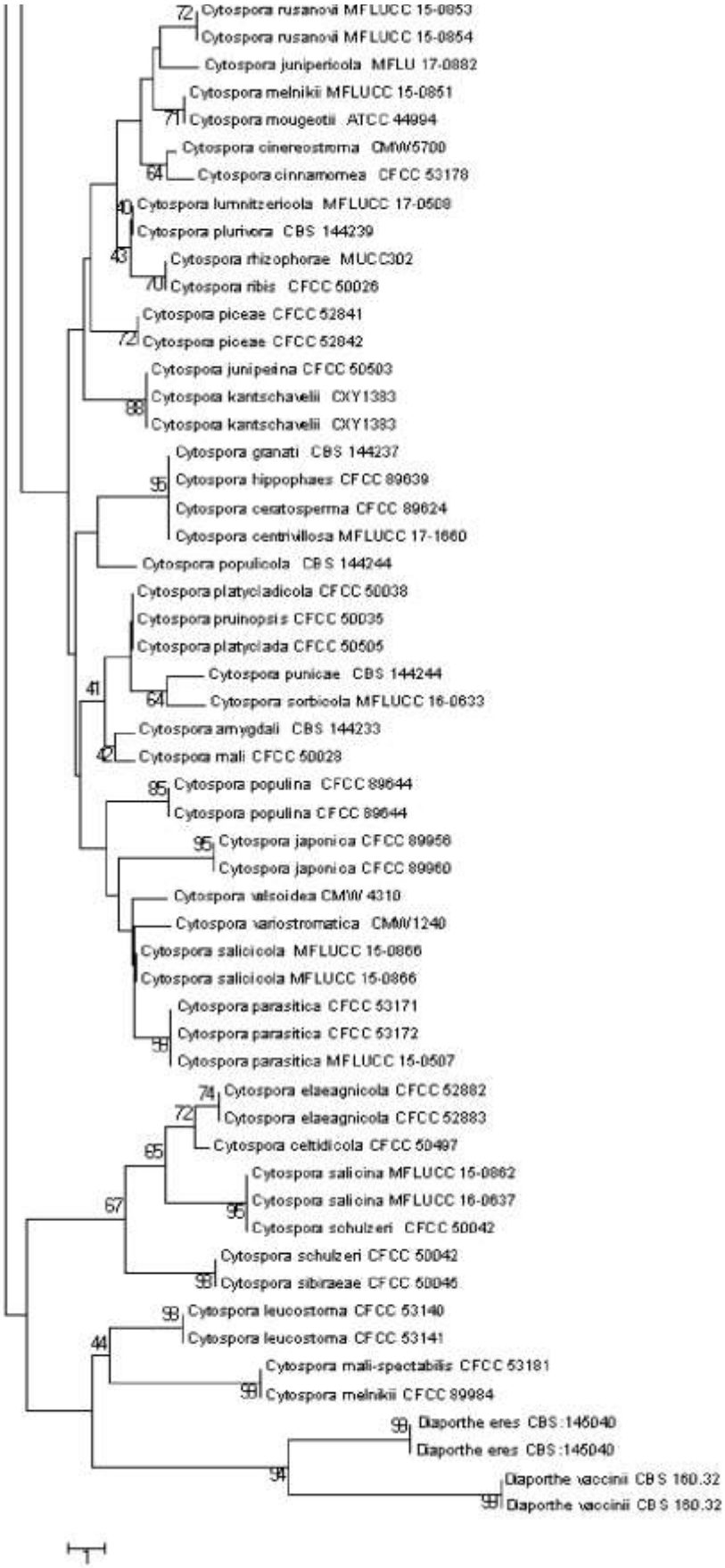


Figure 2: Phylogenetic tree based on ACT region using the model TN93+G+I in maximum likelihood analysis

Table 3: Separated species using ACT region

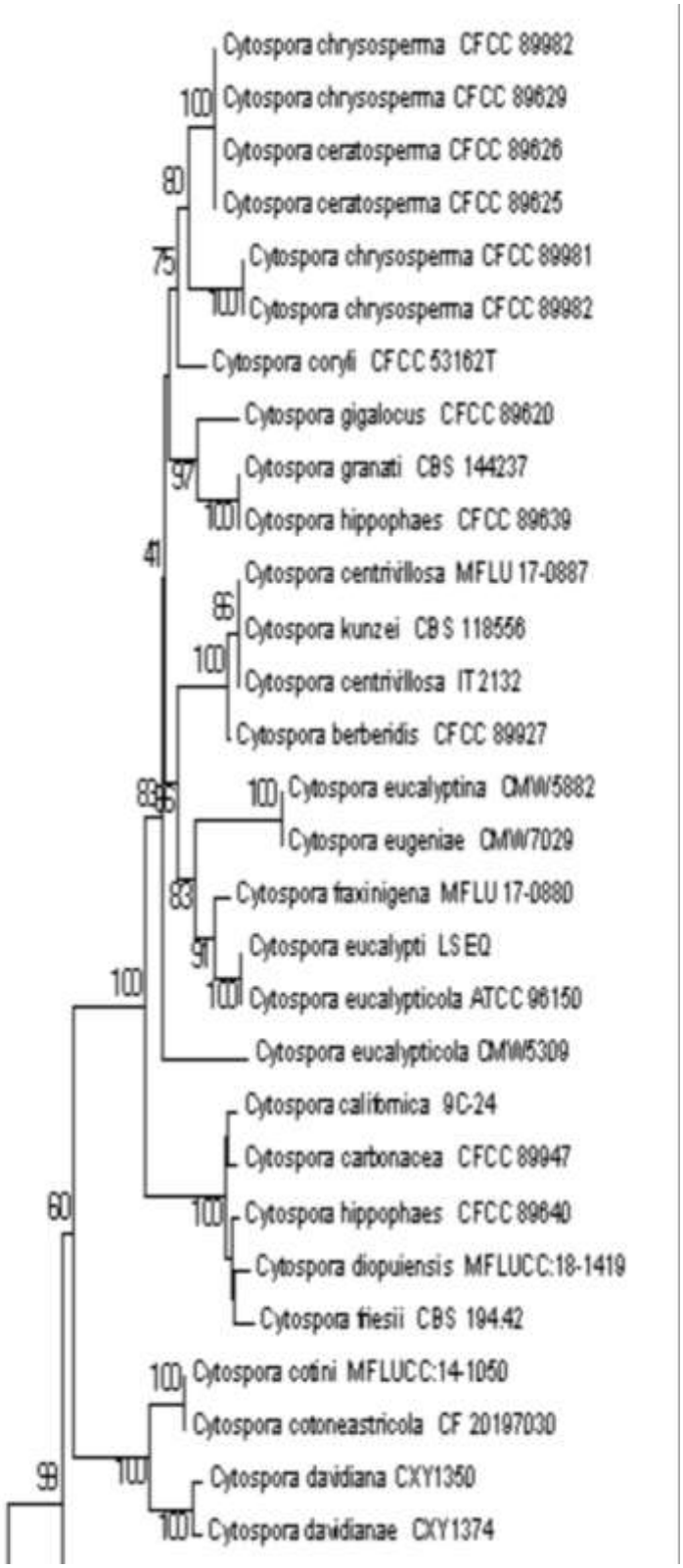
C. atrocirrhata	*C. eucalypticola*
C. platycladicola	*C. mali*
C. melnikii	*C. rosicola*
C. paratranslucens	*C. rostrate*
C. sophorae	*C. cotini*
C. sibiraeae	*C. populinopsis*
C. pruinosa	*C. oleicola*
C. sophoricola	*C. pruinopsis*
C. japonica	*C. junipericola*
C. ochracea	*C. cinereostroma*
C. curvata	*C. cinnamomea*
C. davidiana	*C. piceae*
C. pruinopsis	*C. populicola*
C. spiraeae	*C. punicae*
C. spiraeicola	*C. sorbicola*
C. parapistaciae	*C. amygdali*
C. hippophaes	*C. mal*
C. pini	*C. castanae*
C. berberidis	*C. eucalypticola*
C. berkeleyi	*C. mali*
C. myrtagena	*C. rosicola*
C. variostromatica	*C. rostrate*
C. euonymina	*C. cotini*
C. chrysosperma	*C. populinopsis*
C. pistaciae	*C. oleicola*
C. castanae	

TUB Region

The TUB region-based phylogenetic tree (Figure 3) exhibited a total of 34 clades. Remarkably, this region achieved a substantial separation success rate, successfully segregating 76% of the studied species, which accounts for 23% of all species, from other taxa (Table 4). Species with multiple sequences in the phylogenetic analysis displayed robust bootstrap values and were clustered together. The most well-supported clade was constituted by the sequences of *C. chrysosperma, C. davidiana*, and *C. brevispora*, exhibiting an impressive bootstrap value of 100.

Table 4: Separated species using TUB region

C. chrysosperma	*C. brevispora*
C. coryli	*C. davidiana*
C. gigalocus	*C. erumpens*
C. granati	*C. eucalypti*
C. berberidis	*C. japonica*
C. eucalyptina	*C. joaquinensis*
C. eugeniae	*C. cinereostroma*
C. fraxinigena	*C. amygdali*
C. eucalypti	*C. atrocirrhata*
C. eucalypticola	*C. beilinensis*
C. eucalypticola	*C. kuanchengensis*
C. californica	*C. ampulliformis*
C. carbonacea	*C. eugeniae*
C. hippophaes	*C. euonymicola*
C. diopuiensis	*C. berkeleyi*
C. friesi	*C. atrocirrhata*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Cytospora chrysosperma CFCC 89629
Cytospora ceratosperma CFCC 89626
Cytospora ceratosperma CFCC 89625
Cytospora chrysosperma CFCC 89981
Cytospora chrysosperma CFCC 89982
Cytospora coryli CFCC 53162T
Cytospora gigalocus CFCC 89620
Cytospora granati CBS 144237
Cytospora hippophaes CFCC 89639
Cytospora centrivillosa MFLU 17-0887
Cytospora kunzei CBS 118556
Cytospora centrivillosa IT 2132
Cytospora berberidis CFCC 89927
Cytospora eucalyptina CMW5882
Cytospora eugeniae CMW7029
Cytospora taxinigena MFLU 17-0880
Cytospora eucalypti LSEQ
Cytospora eucalypticola ATCC 96150
Cytospora eucalypticola CMW5309
Cytospora californica 9C-24
Cytospora carbonacea CFCC 89947
Cytospora hippophaes CFCC 89640
Cytospora diopuiensis MFLUCC:18-1419
Cytospora friesii CBS 194.42
Cytospora cotini MFLUCC:14-1050
Cytospora cotoneastricola CF 20197030
Cytospora davidiana CXY1350
Cytospora davidianae CXY1374

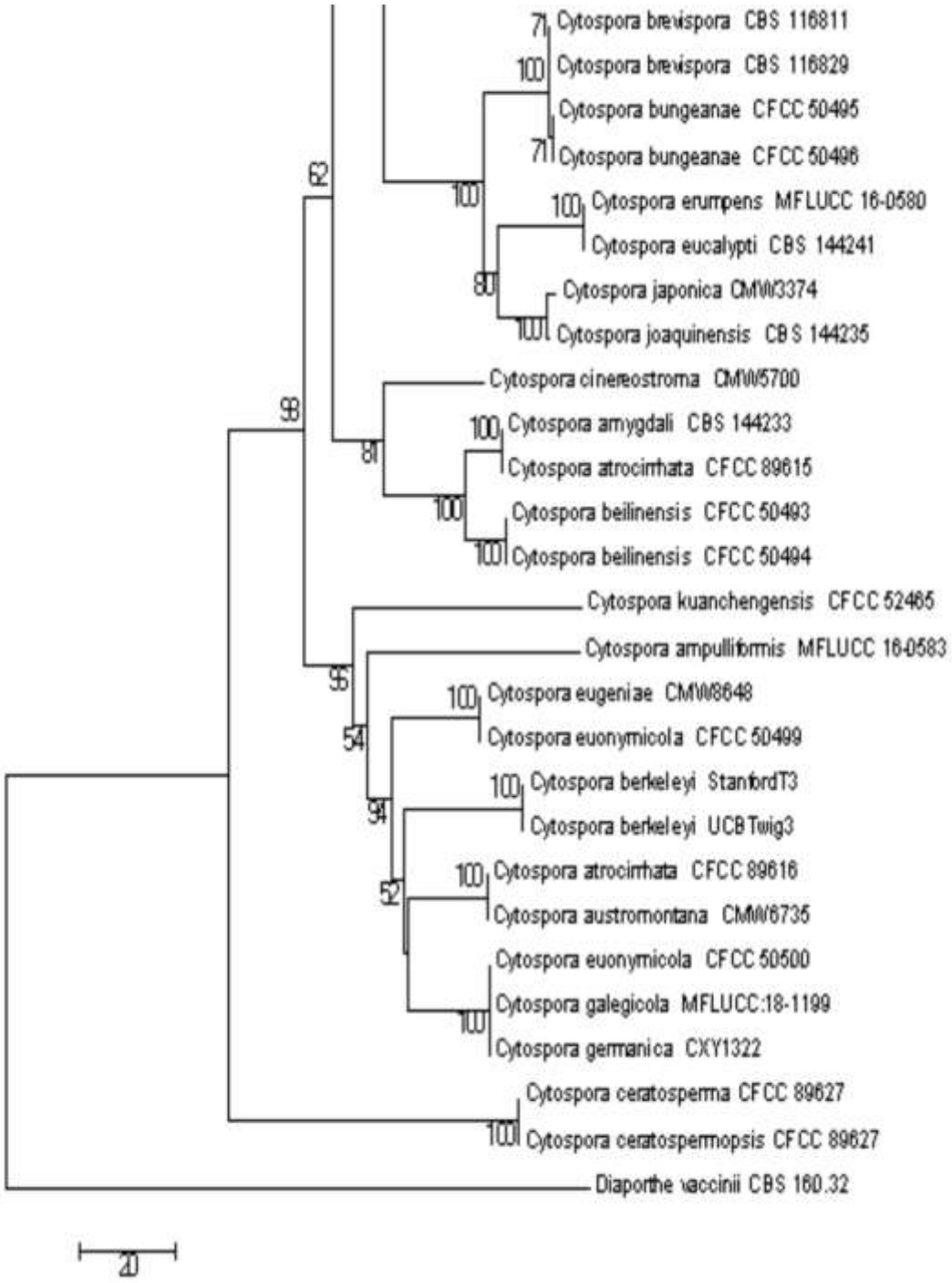
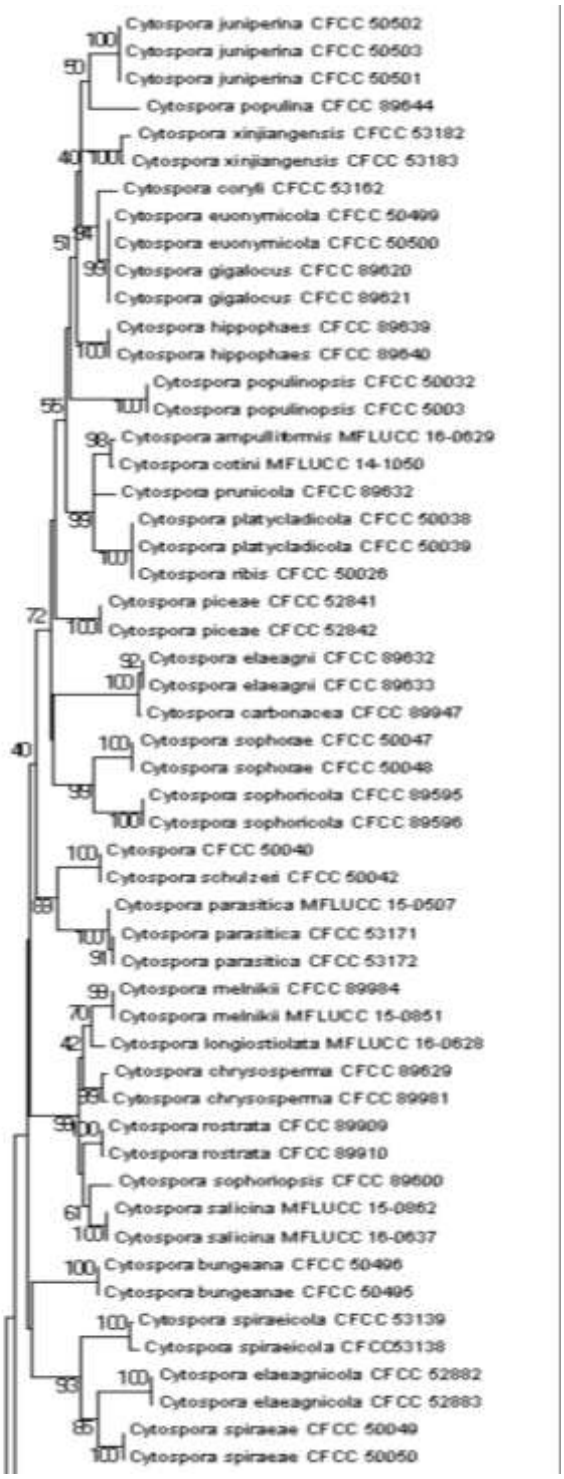


Figure3: Phylogenetic tree based on TUB region using the model TN93+G+I in maximum likelihood analysis

RPB2 Region

In the phylogenetic tree based on the RPB2 region (Figure 4), a total of 63 clades were depicted. This region demonstrated its efficacy by successfully separating 80% of the studied species, which corresponds to 47% of all species, from other taxa (refer to Table 5). Notably, species with multiple sequences included in the phylogenetic analysis showcased high bootstrap values and were consolidated within a single clade.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Cytospora populina CFCC 89644
Cytospora xinjiangensis CFCC 53182
Cytospora xinjiangensis CFCC 53183
Cytospora coryli CFCC 53162
Cytospora euonymicola CFCC 50499
Cytospora euonymicola CFCC 50500
Cytospora gigalocus CFCC 89620
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Cytospora hippophaes CFCC 89639
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Cytospora populinopsis CFCC 50032
Cytospora populinopsis CFCC 5003
Cytospora ampulliformis MFLUCC 16-0629
Cytospora cotini MFLUCC 14-1050
Cytospora prunicola CFCC 89632
Cytospora platycladicola CFCC 50038
Cytospora platycladicola CFCC 50039
Cytospora ribis CFCC 50026
Cytospora piceae CFCC 52841
Cytospora piceae CFCC 52842
Cytospora elaeagni CFCC 89632
Cytospora elaeagni CFCC 89633
Cytospora carbonacea CFCC 89947
Cytospora sophorae CFCC 50047
Cytospora sophorae CFCC 50048
Cytospora sophoricola CFCC 89595
Cytospora sophoricola CFCC 89596
Cytospora CFCC 50040
Cytospora schulzeri CFCC 50042
Cytospora parasitica MFLUCC 15-0507
Cytospora parasitica CFCC 53171
Cytospora parasitica CFCC 53172
Cytospora melnikii CFCC 89984
Cytospora melnikii MFLUCC 15-0851
Cytospora longiostiolata MFLUCC 16-0628
Cytospora chrysosperma CFCC 89629
Cytospora chrysosperma CFCC 89981
Cytospora rostrata CFCC 89909
Cytospora rostrata CFCC 89910
Cytospora sophoriopsis CFCC 89600
Cytospora salicina MFLUCC 15-0862
Cytospora salicina MFLUCC 16-0637
Cytospora bungeana CFCC 50496
Cytospora bungeanae CFCC 50495
Cytospora spiraeicola CFCC 53139
Cytospora spiraeicola CFCC53138
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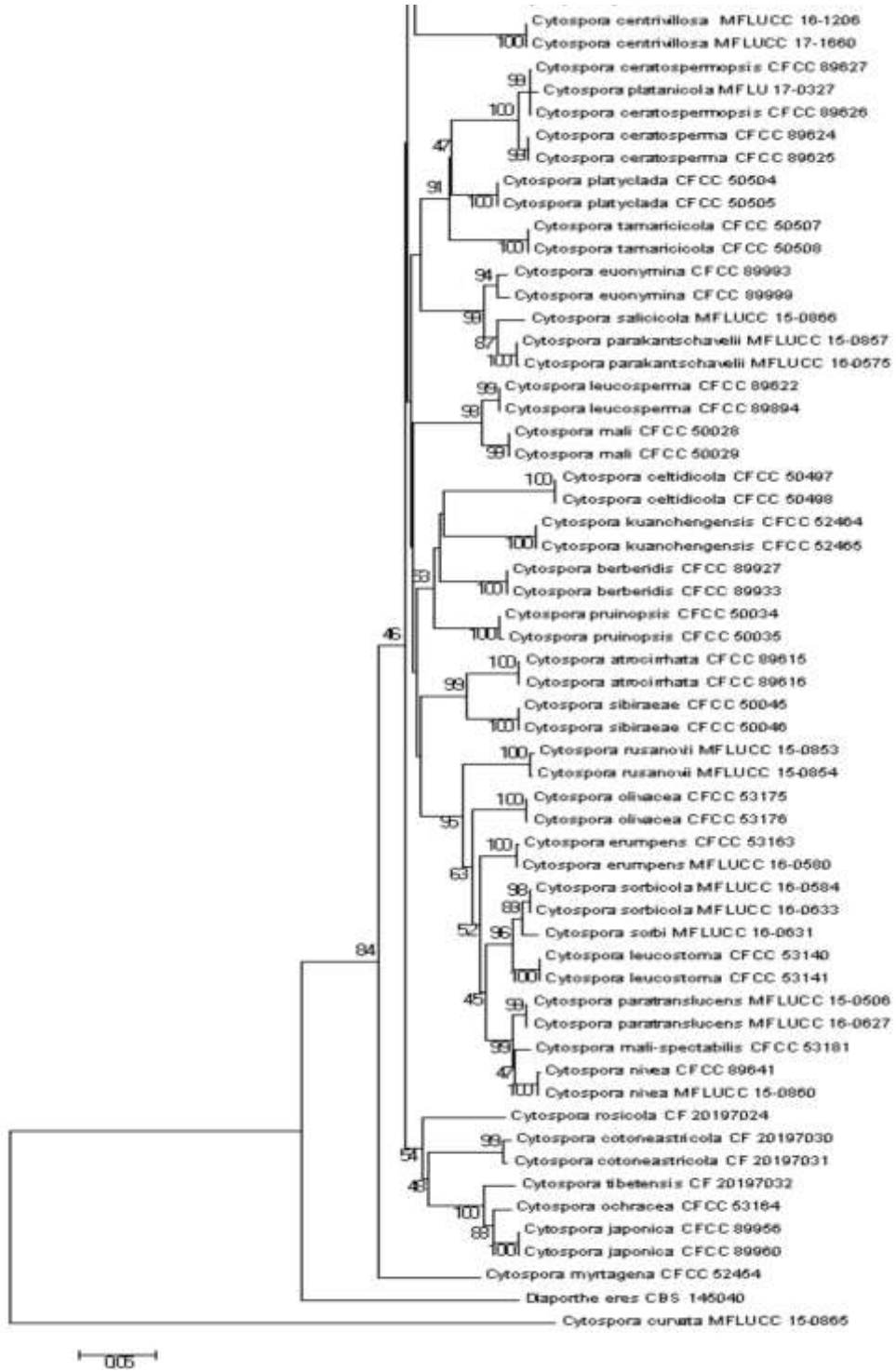

Figure 4: Phylogenetic tree based on RPB2 region using the model TN93+G+I in maximum likelihood analysis

Table 5: Separated species using RPB2 region

C. juniperina	*C. parakantschavelii*
C. populina	*C. leucosperma*
C. xinjiangensis	*C. mali*
C. coryli	*C. celtidicola*
C. hippophaes	*C. kuanchengensis*
C. populinopsis	*C. berberidis*
C. prunicola	*C. pruinopsis*
C. piceae	*C. atrocirrhata*
C. elaeagn	*C. sibiraeae*
C. carbonacea	*C. rusanovii*
C. sophorae	*C. olivacea*

C. sophoricola	*C. erumpens*
C. schulzeri	*C. erumpens*
C. parasitica	*C. atrocirrhata*
C. melnik	*C. atrocirrhata*
C. longiostiolata	*C. sibiraeae*
C. chrysosperma	*C. rusanovii*
C. rostrate	*C. olivacea*
C. sophoriopsis	*C. erumpens*
C. salicina	*C. sorbicola*
C. spiraeicola	*C. sorbi*
C. bungeanae	*C. leucostoma*
C. spiraeicola	*C. paratranslucens*
C. elaeagnicola	*C. mali-spectabilis*
C. spiraeae	*C. nivea*
C. centrivillosa	*C. rosicola*
C. ceratospermopsis	*C. cotoneastricola*
C. platanicola	*C. tibetensis*
C. ceratospermopsis	*C. japonica*
C. ceratosperma	*C. myrtagena*
C. platyclada	*C. curvata*
C. tamaricicola	*C. salicicola*
C. euonymina	*C. parakantschavelii*
C. salicicola	

Combined ITS + ACT Regions

The phylogenetic tree resulting from the combined ITS + ACT regions (Figure 5) unveiled the presence of 86 clades. These clades were effective in distinguishing 71% of the studied species from other taxa, as elaborated in Table 6. It's noteworthy that species with multiple sequences employed in the phylogenetic analysis exhibited elevated bootstrap values, forming coherent clusters within distinct clades.

Table 6: Separated species using ITS+ACT regions

C. atrocirrhata	*C. notastroma*	*C. juniperina*
C. brevispora	*C. oleicola*	*C. kantschavelii*
C. brevispora	*C. olivacea*	*C. kunzei*
C. bungeana	*C. palm*	*C. leucosperma*
C. carbonacea	*C. parakantschaveli*	*C. leucostoma*
C. carpobroti	*C. parapersooni*	*C. lumnitzericola*
C. castanae	*C. parapistaciae*	*C. mali*
C. celtidicola	*C. parasitica*	*C. mali-spectabilis*
C. ceratosperma	*C. paratranslucens*	*C. melnikii*
C. ceratospermopsis	*C. pingbianensis*	*C. mougeotii*
C. chrysosperma	*C. pini*	*C. multicollis*
C. cinnamomea	*C. pistaciae*	*C. myrtagena*
C. coryli	*C. platyclada*	*C. juniperina*
C. cotini	*C. plurivora*	*C. kantschavelii*
C. davidiana	*C. populicola*	*C. sibiraeae*
C. diopuiensis	*C. populinopsis*	*C. sophorae*
C. disciformis	*C. pruinopsis*	*C. sophoricola*
C. elaeagni	*C. pruinosa*	*C. sophoriopsis*
C. eriobotryae	*C. pruinosa*	*C. sorbicola*
C. erumpens	*C. kuanchengensis*	*C. sorbina*
C. eucalypti	*C. prunicola*	*C. spiraeicola*
C. eucalypticola	*C. pruni-mume*	*C. tamaricicola*
C. eucalyptina	*C. pubescentis*	*C. tibetensis*
C. eugeniae	*C. punicae*	*C. translucens*
C. euonymina	*C. quercicola*	*C. valsoidea*
C. friesii	*C. rhizophorae*	
C. galegicola	*C. ribis*	
C. germanica	*C. rosae*	
C. gigalocus	*C. rostrate*	
C. granati	*C. rusanovii*	
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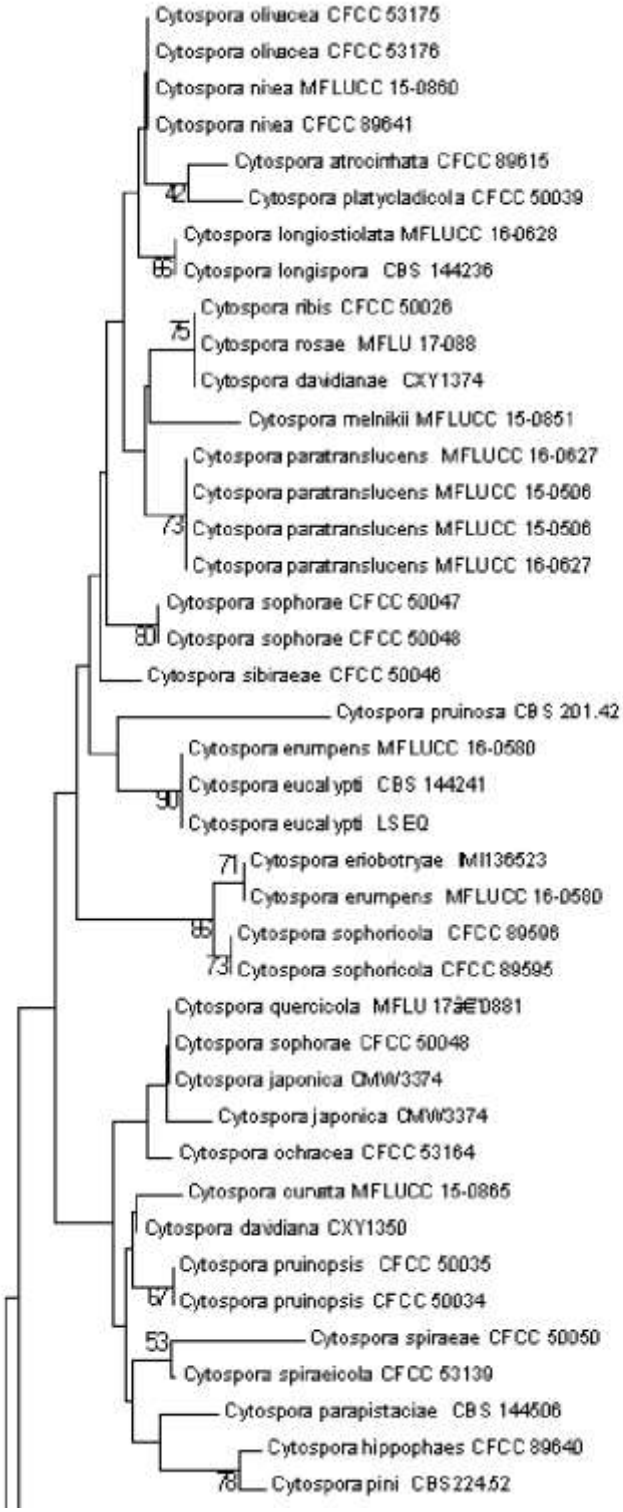
C. joaquinensis	*C. schulzeri*	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Cytospora davidiana CXY1350
Cytospora pruinopsis CFCC 50035
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Cytospora pruinopsis CFCC 50034
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Cytospora spiraeae CFCC 50050
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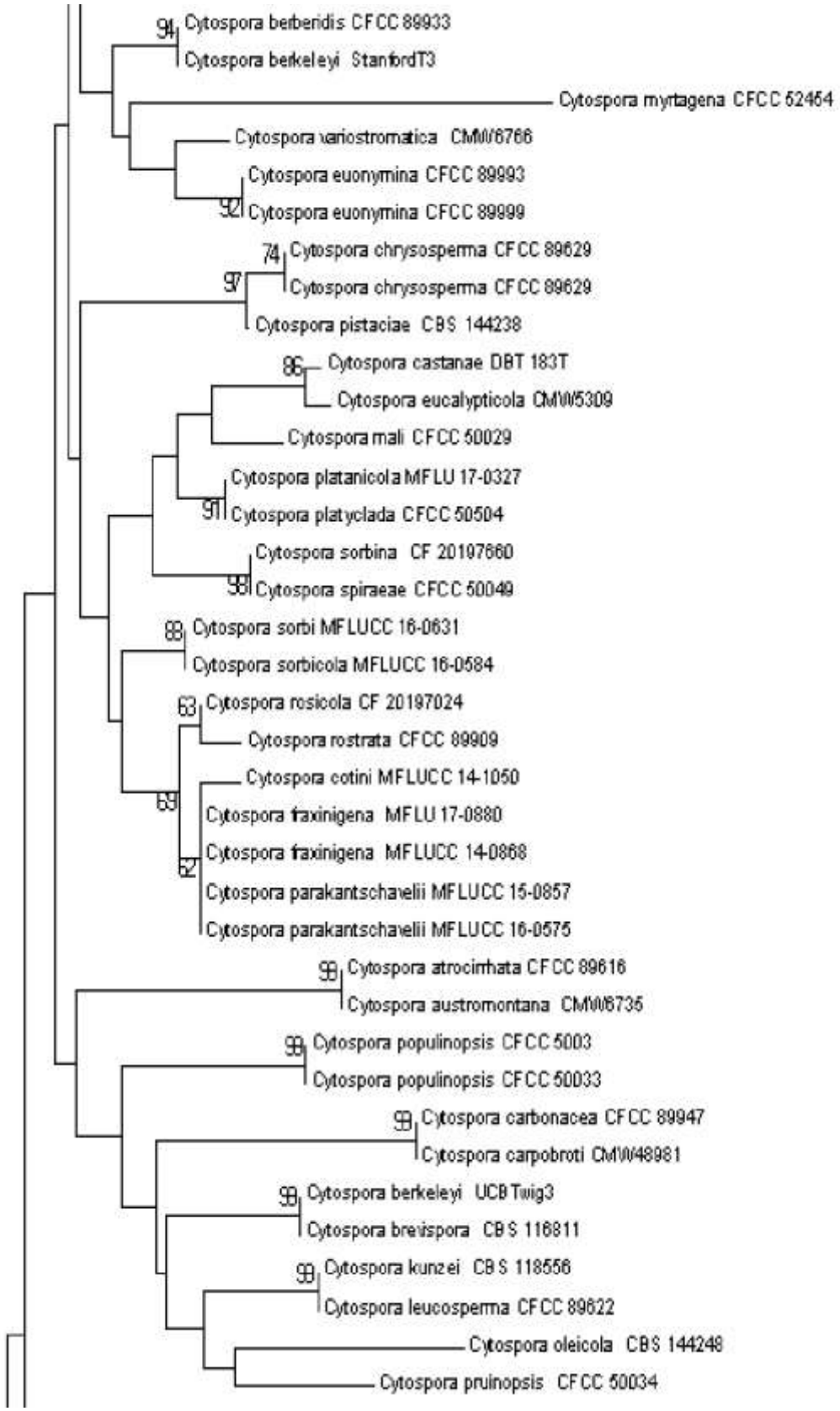
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Cytospora platyclada CFCC 50504
Cytospora sorbina CF 20197660
Cytospora spiraeae CFCC 50049
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Cytospora sorbicola MFLUCC 16-0584
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Cytospora fraxinigena MFLUCC 14-0868
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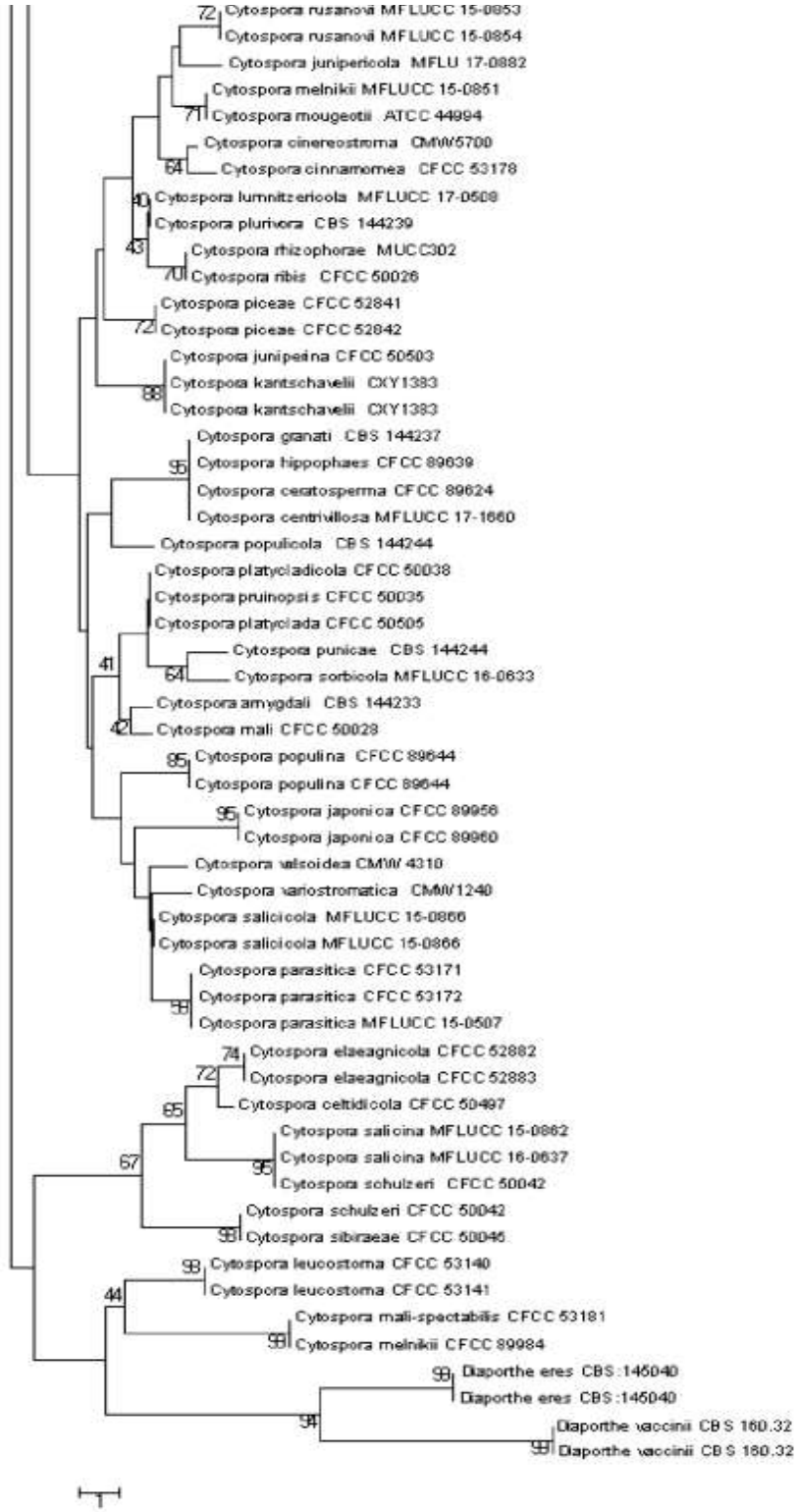
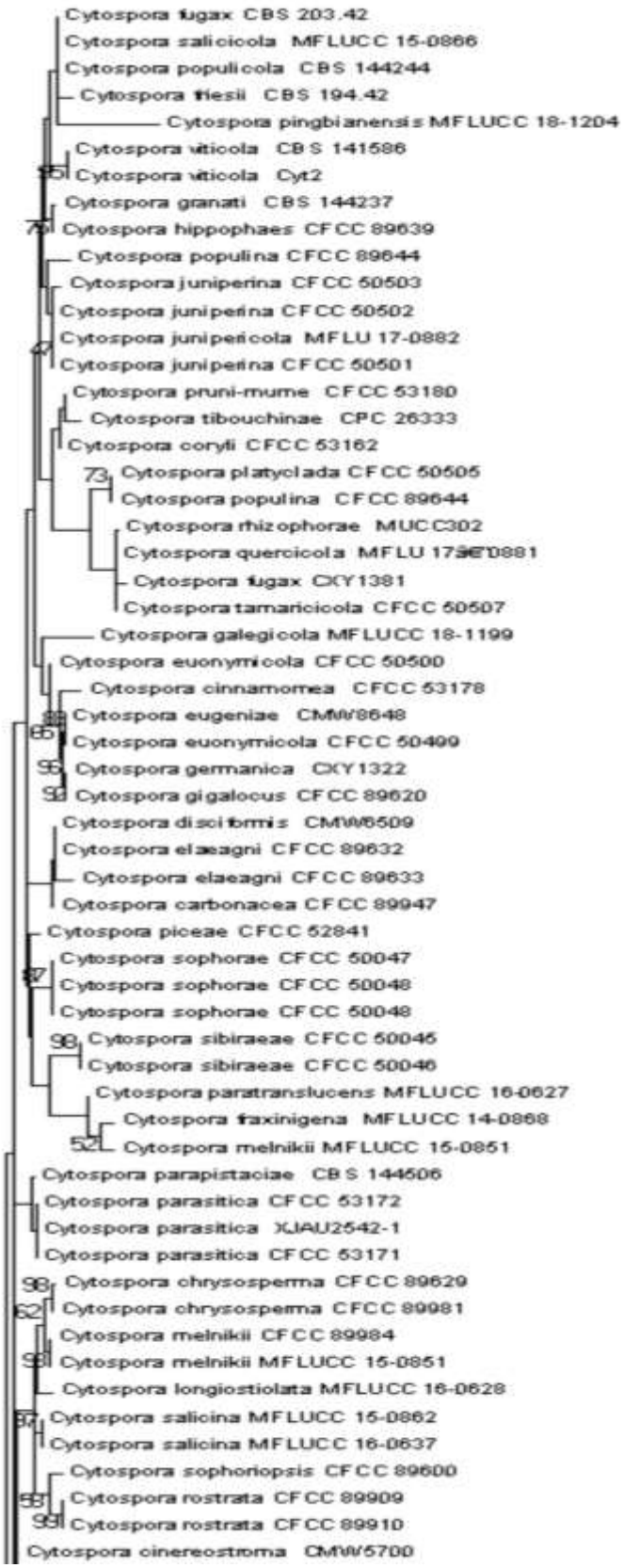


Figure 5: Phylogenetic tree based on ITS+ACT regions using the model TN93+G+I in maximum likelihood analysis

Combined ITS + RPB2 Regions

In the phylogenetic tree derived from the combination of ITS + RPB2 regions (Figure 6), a total of 86 clades was identified. This combination succeeded in effectively separating 71% of the studied species from other taxa, a fact elucidated in Table 7. Species with multiple sequences used in the analysis showcased high bootstrap values within a unified clade.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Cytospora sibiraeae CFCC 50046
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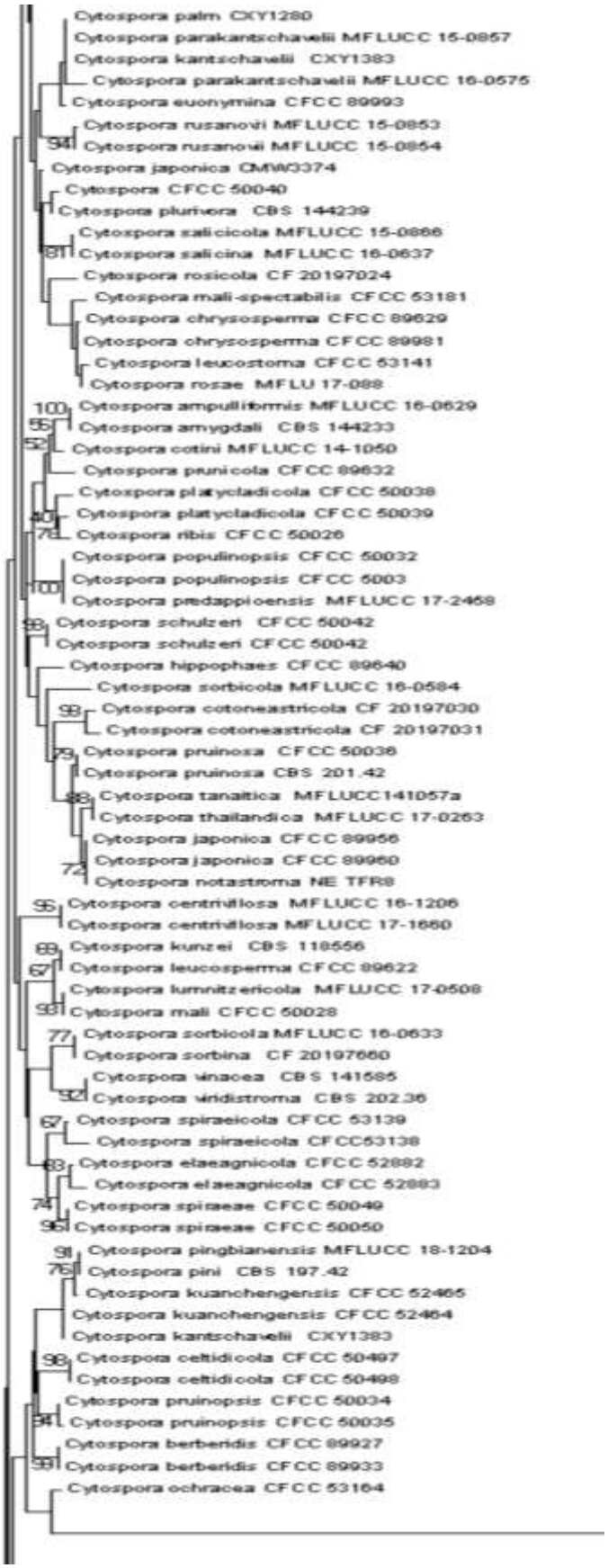
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Cytospora spiraeae CFCC 50049
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91
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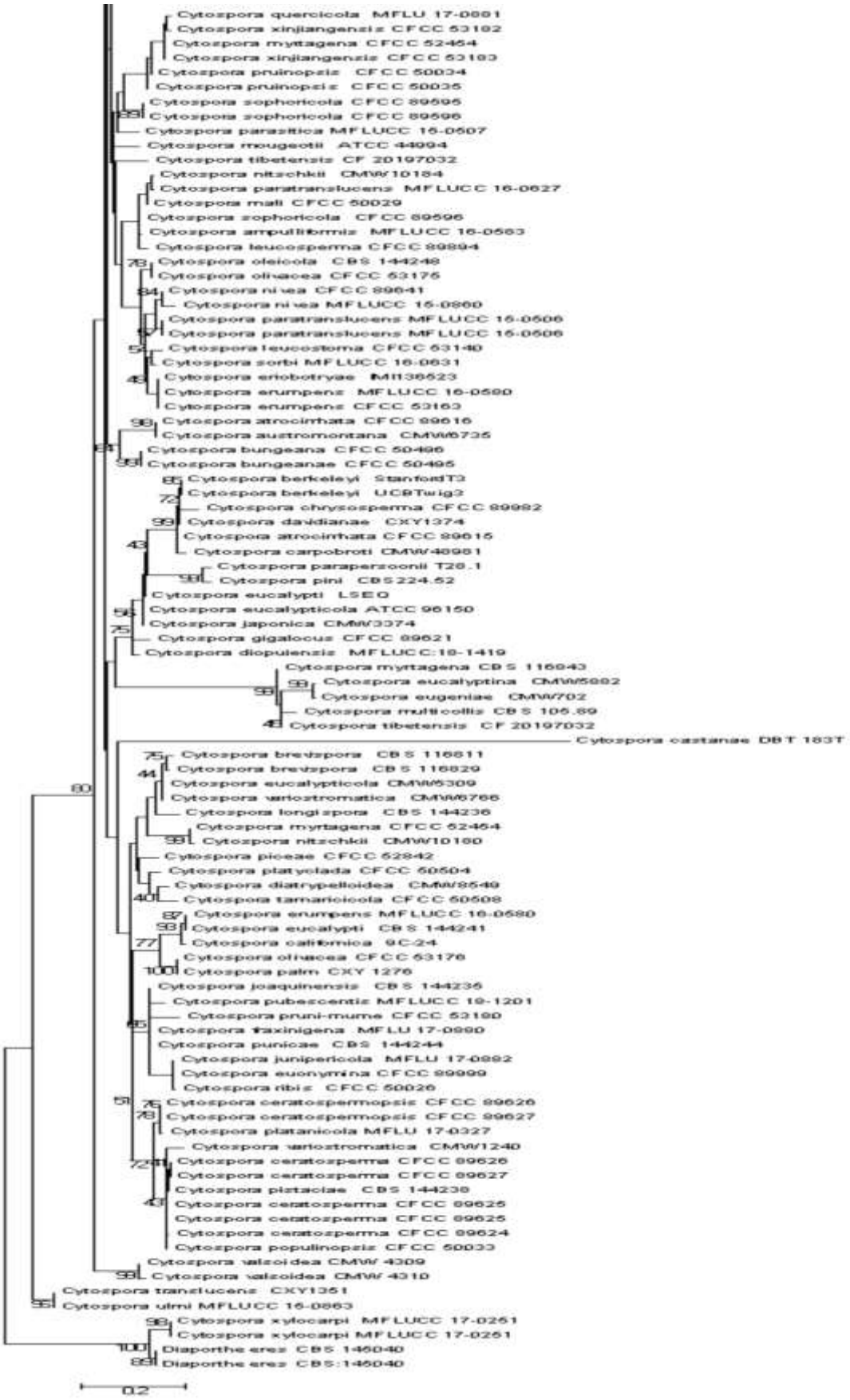
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Diaporthe eres CBS:145040
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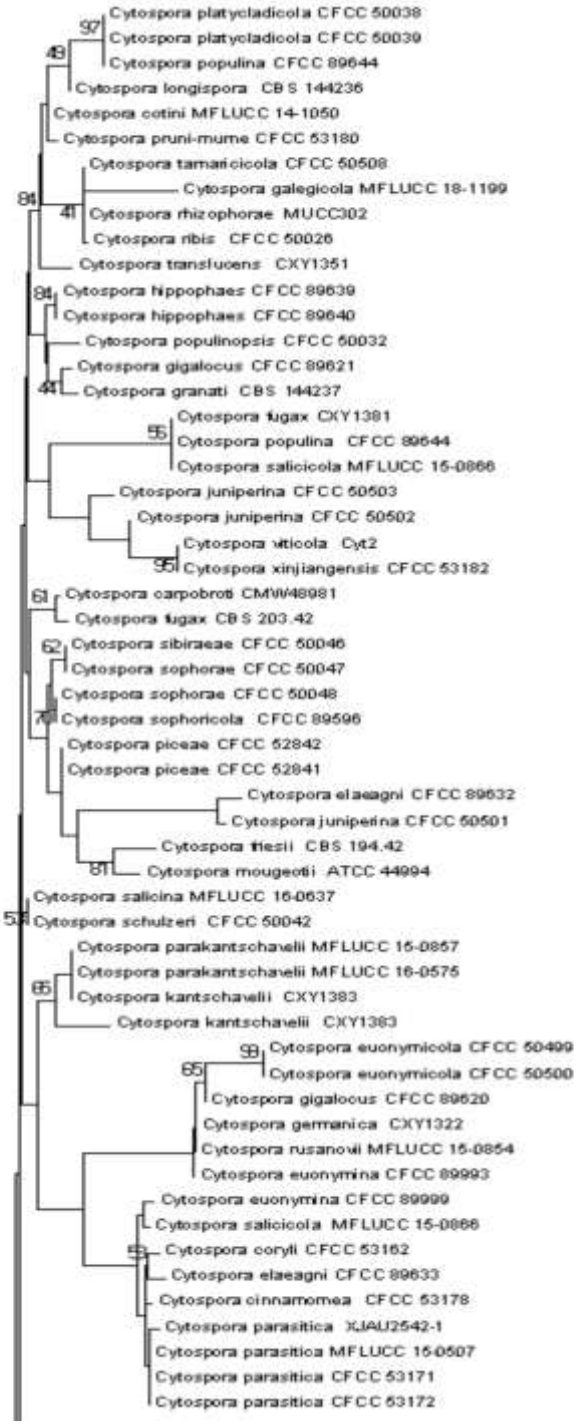
Figure 6: Phylogenetic tree based on ITS+RPB2 regions using the model TN93+G+I in maximum likelihood analysis

Table 7: Separated species using ITS+RPB2 regions

C. ampulliformis	*C. nitschkii*	*C. kuanchengensis*
C. amygdal	*C. nivea*	*C. leucosperma*
C. berberidis	*C. notastroma*	*C. leucostoma*
C. berkeleyi	*C. ochracea*	*C. longiostiolata*
C. brevispora	*C. oleicola*	*C. longispora*
C. bungeana	*C. palm*	*C. lumnitzericola*
C. californica	*C. parakantschaveli*	*C. mali*
C. carbonacea	*C. parapersoonii*	*C. mali-spectabilis*
C. carpobroti	*C. parapistaciae*	*C. melnikii*
C. castanae	*C. parasitica*	*C. mougeotii*
C. celtidicola	*C. paratranslucens*	*C. multicollis*
C. centrivillosa	*C. piceae*	*C. myrtagena*
C. ceratosperma	*C. pini*	*C. sorbicola*
C. ceratospermopsis	*C. pistaciae*	*C. spiraeae*
C. chrysosperma	*C. platanicola*	*C. spiraeicola*
C. cinnamomea	*C. platyclada*	*C. tamaricicola*
C. coryli	*C. platycladicola*	*C. tanaitica*
C. cotini	*C. plurivora*	*C. tibetensis*
C. cotoneastricola	*C. populicola*	*C. tibouchinae*
C. davidianae	*C. populina*	*C. variostromatica*
C. diopuiensis	*C. pruinopsis*	*C. viticola*
C. elaeagnicol	*C. pruinosa*	*C. xinjiangensis*
C. erumpens	*C. prunicol*	*C. xylocarp*
C. eucalypti	*C. pruni-mume*	*C. nitschkii*
C. eucalypticola	*C. quercicola*	
C. eugeniae	*C. ribis*	
C. euonymicola	*C. rosae*	
C. euonymina	*C. rosicola*	
C. fraxinigena	*C. rostrate*	
C. galegicola	*C. rusanovi*	
C. germanica	*C. salicina*	
C. gigalocus	*C. schulzeri*	
C. hippophaes	*C. sibiraeae*	
C. japonica	*C. sophorae*	
C. joaquinensis	*C. sophoricola*	
C. juniperina	*C. sophoriopsis*	
C. kantschaveli	*C. sorbi*	

Combined ITS + TUB Regions

In the phylogenetic tree derived from the combination of ITS + TUB regions (Figure 7), a total of 90 clades was identified. This combination succeeded in effectively separating 73% of the studied species from other taxa, a fact elucidated in Table 8. Species with multiple sequences used in the analysis showcased high bootstrap values within a unified clade.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Cytospora rhizophorae MUCC302
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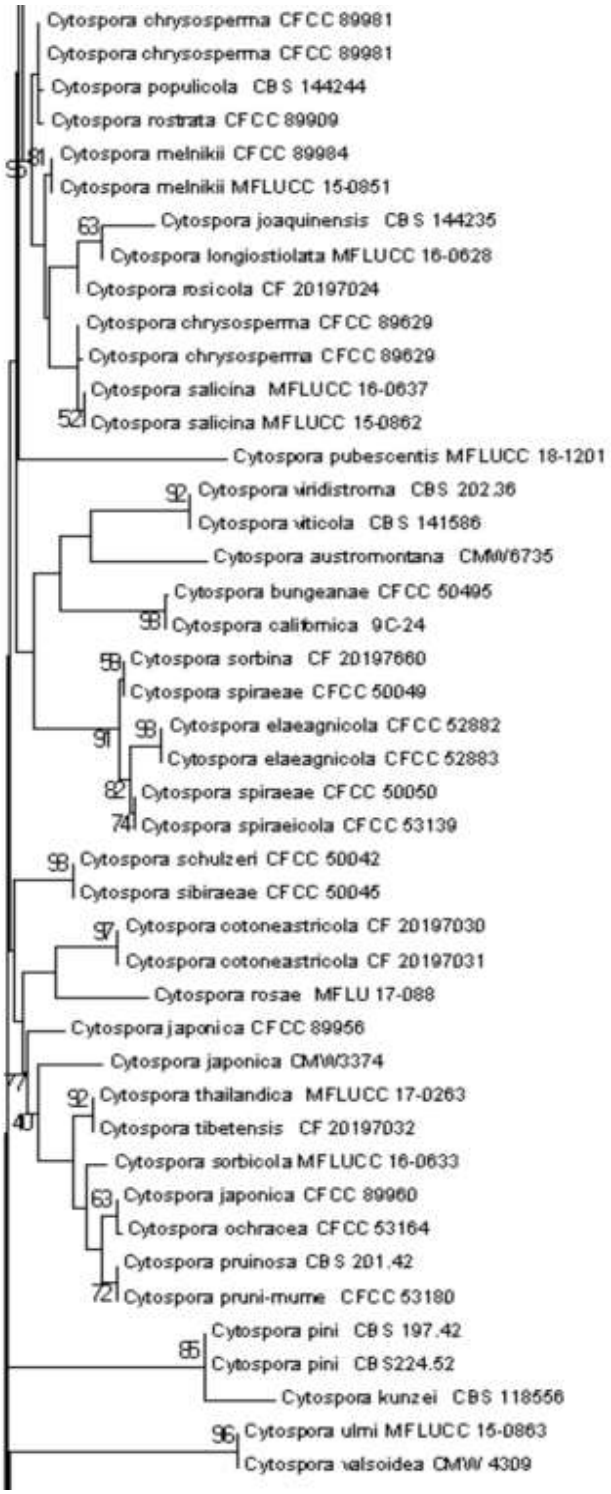
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Cytospora bungeanae CFCC 50495
Cytospora californica 9C-24
Cytospora sorbina CF 20197660
Cytospora spiraeae CFCC 50049
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Cytospora spiraeae CFCC 50050
Cytospora spiraeicola CFCC 53139
Cytospora schulzeri CFCC 50042
Cytospora sibiraeae CFCC 50046
Cytospora cotoneastricola CF 20197030
Cytospora cotoneastricola CF 20197031
Cytospora rosae MFLU 17-088
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Cytospora japonica CMW3374
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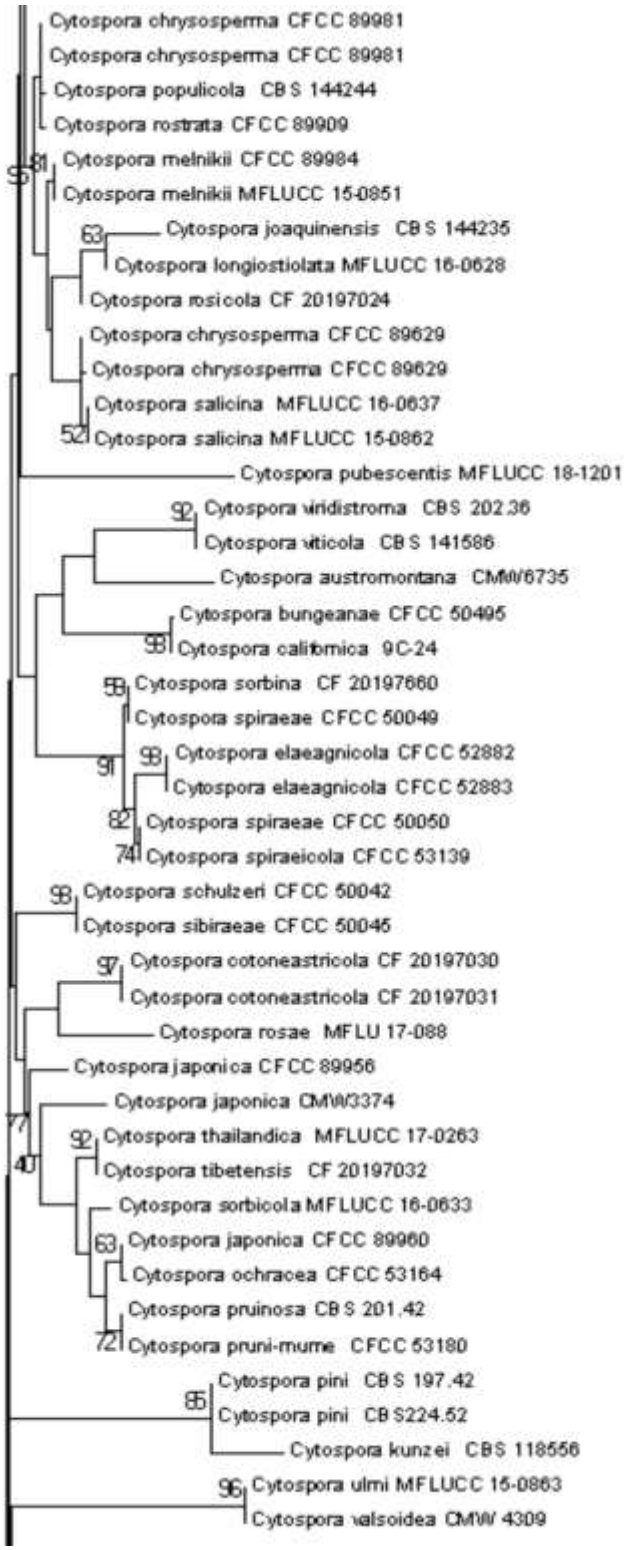
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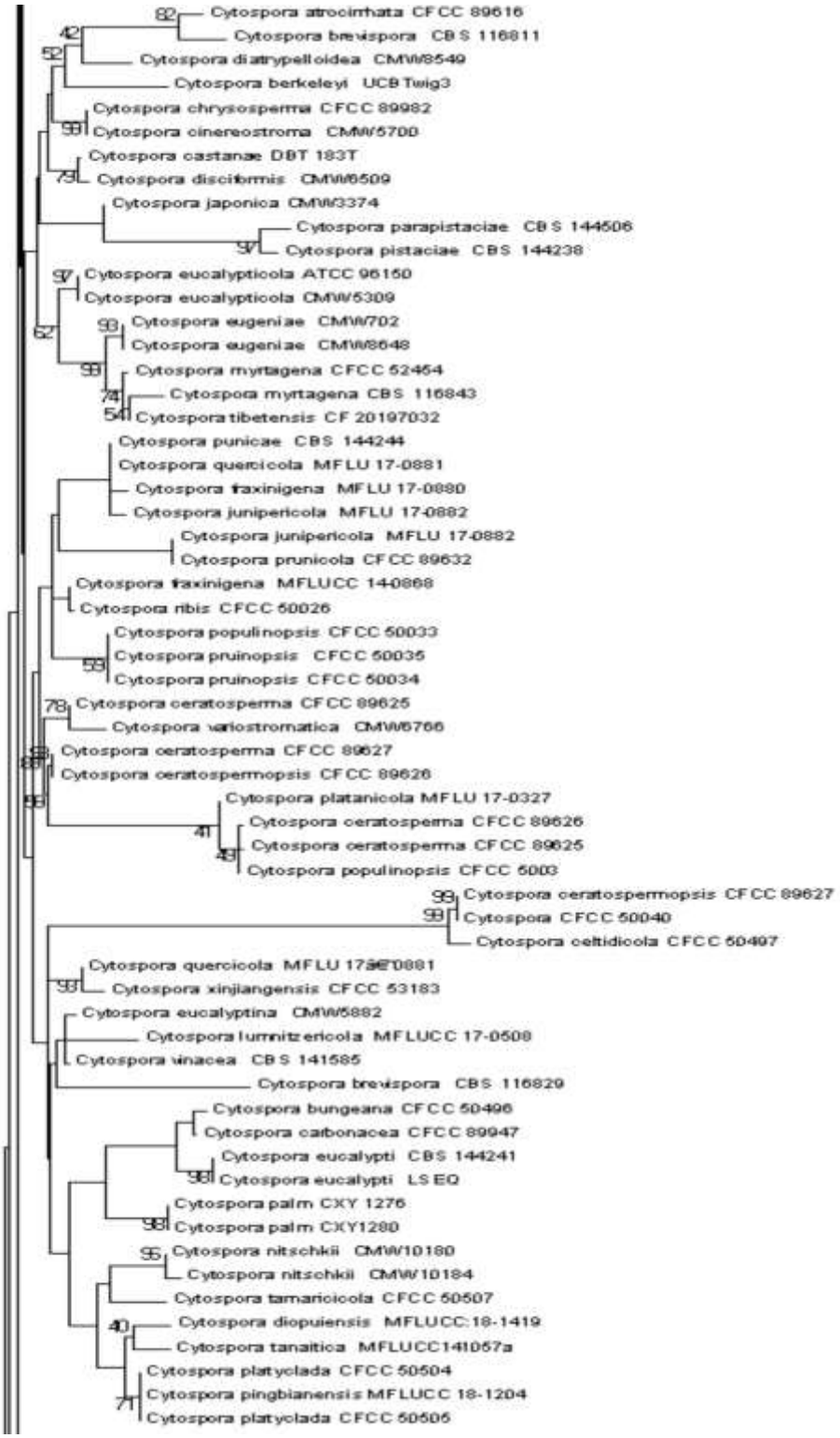
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Cytospora lumnitzericola MFLUCC 17-0508
Cytospora vinacea CBS 141585
Cytospora brevispora CBS 116829
Cytospora bungeana CFCC 50496
Cytospora carbonacea CFCC 89947
Cytospora eucalypti CBS 144241
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Figure 7: Phylogenetic tree based on ITS+TUB regions using the model TN93+G+I in maximum likelihood analysis

Table 8: Separated species using ITS+TUB regions

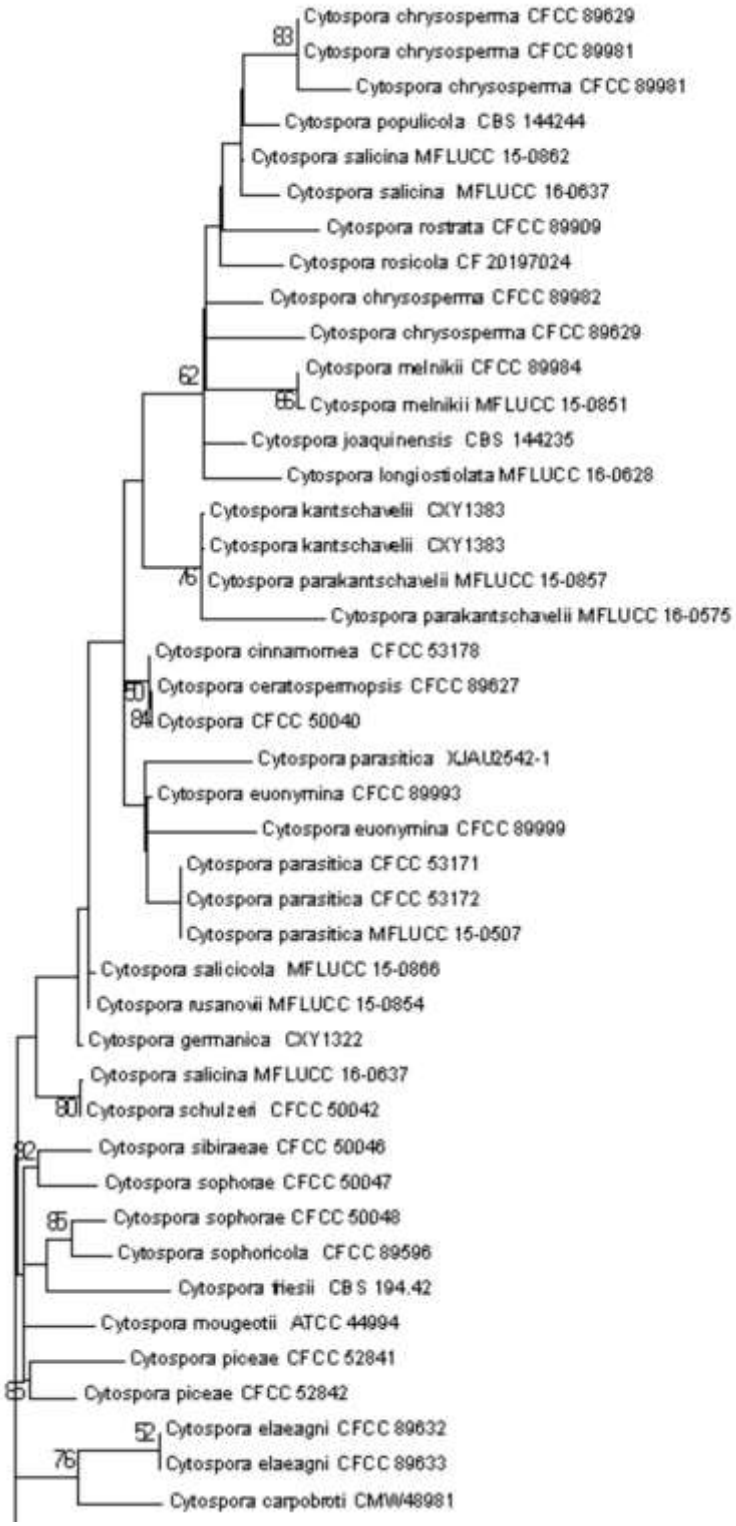
C. ampulliformis	*C. kantschavelii*	*C. eucalypti*
C. atrocirrhata	*C. kuanchengensis*	*C. eucalypticola*
C. austromontana	*C. kunzei*	*C. eucalyptina*
C. berberidis	*C. leucostoma*	*C. eugeniae*
C. berkeleyi	*C. longiostiolata*	*C. euonymicola*
C. berkeleyi	*C. longispora*	*C. euonymina*
C. brevispora	*C. lumnitzericola*	*C. fraxinigena*
C. brevispora	*C. mali*	*C. friesi*
C. bungeana	*C. mali-spectabilis*	*C. fugax*
C. bungeanae	*C. melnikii*	*C. germanica*
C. californica	*C. mougeotii*	*C. gigalocus*
C. carbonacea	*C. multicollis*	*C. granati*
C. carpobroti	*C. myrtagena*	*C. hippophaes*
C. castanae	*C. nitschkii*	*C. japonica*
C. celtidicola	*C. notastroma*	*C. joaquinensis*
C. celtidicola	*C. ochracea*	*C. juniperina*
C. centrivillosa	*C. oleicola*	*C. pubescentis*
C. ceratosperma	*C. olivacea*	*C. quercicola*
C. ceratosperma	*C. palm*	*C. ribis*
C. ceratospermopsis	*C. parapersoonii*	*C. rosae*
C. chrysosperma	*C. parapistaciae*	*C. rosicola*
C. chrysosperma	*C. parasitica*	*C. rostrate*
C. chrysosperma	*C. paratranslucens*	*C. rusanovii*
C. cinnamomea	*C. piceae*	*C. salicicola*
C. coryli	*C. pingbianensis*	*C. salicina*
C. cotini	*C. pini*	*C. sorbicola*
C. cotoneastricola	*C. pistaciae*	*C. spiraeicola*
C. davidianae	*C. platanicola*	*C. tamaricicola*
C. diatrypelloidea	*C. plurivora*	*C. tanaitica*
C. diopuiensis	*C. populinopsis*	*C. tibetensis*
C. disciformis	*C. predappioensis*	*C. translucens*
C. elaeagni	*C. pruinopsis*	
C. elaeagnicola	*C. pruinosa*	
C. eriobotryae	*C. pruinosa*	
C. erumpens	*C. pruni-mume*	

Combined ITS +ACT+ RPB2 Regions

In the phylogenetic tree derived from the combination of ITS + ACT+ RPB2 regions (Figure 8), a total of 95 clades was identified. This combination succeeded in effectively separating 73% of the studied species from other taxa, a fact elucidated in Table 9. Species with multiple sequences used in the analysis showcased high bootstrap values within a unified clade.

Table 9: Non separated species using ITS+ACT+RPB2 regions

C. austromontana	*C. fugax*
C. berberidis	*C. joaquinensis*
C. berkeleyi	*C. populina*
C. bungeanae	*C. xinjiangensis*
C. californica	*C. ribis*
C. centrivillosa	*C. pruni-mume*
C. predappioensis	*C. tamaricicola*
C. pruinopsis	*C. platycladicola*
C. rostrate	*C. ceratosperma*
C. rusanovii	*C. rhizophorae*
C. sorbi	*C. longiostiolata*
C. tibouchinae	
C. viridistroma	
C. viticola	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Cytospora ceratospermopsis CFCC 89627
Cytospora CFCC 50040
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Cytospora euonymina CFCC 89993
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Cytospora parasitica CFCC 53171
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Cytospora germanica CXY1322
Cytospora salicina MFLUCC 16-0637
Cytospora schulzeri CFCC 50042
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Cytospora sophorae CFCC 50047
Cytospora sophorae CFCC 50048
Cytospora sophoricola CFCC 89596
Cytospora thesii CBS 194.42
Cytospora mougeotii ATCC 44994
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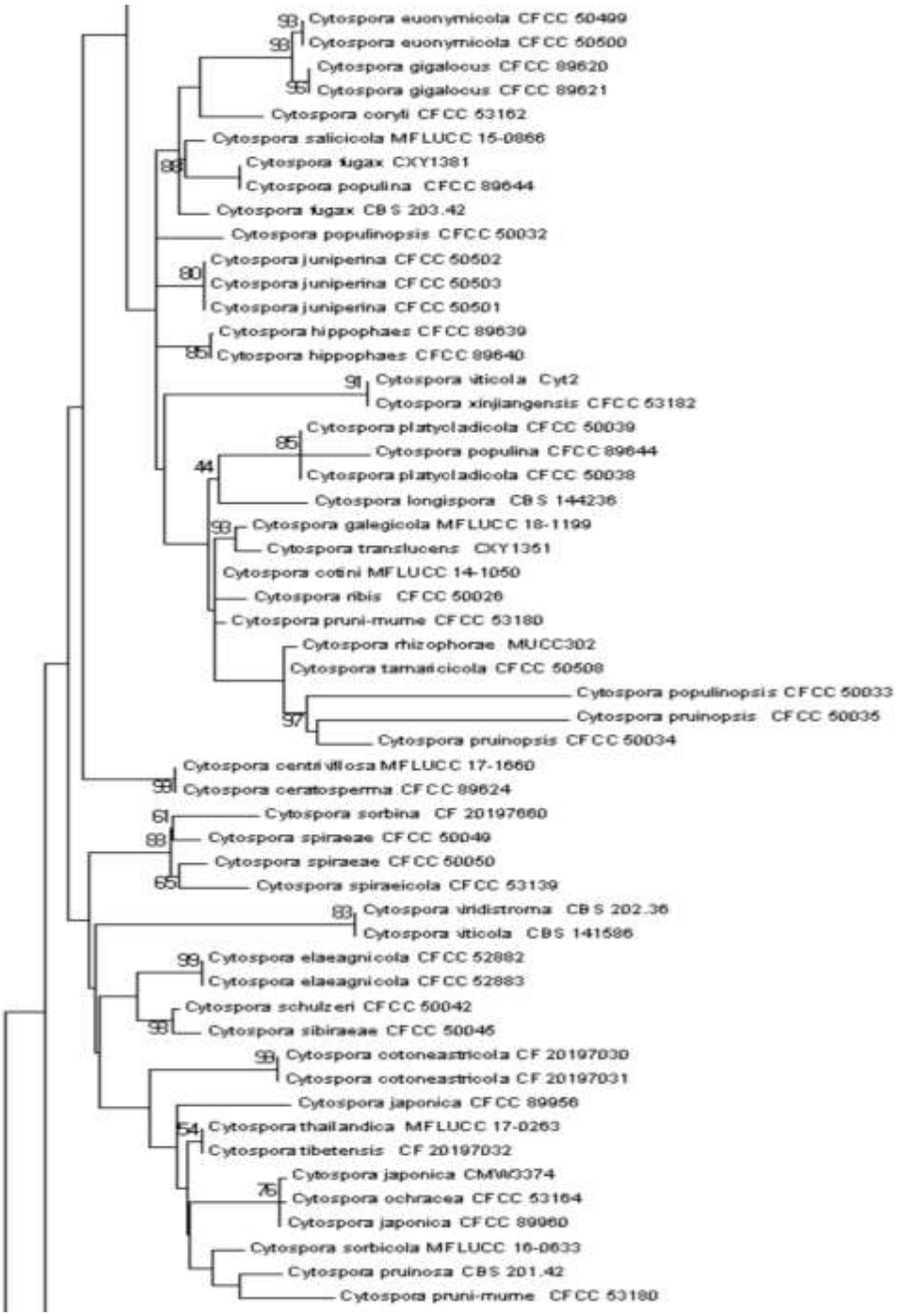
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Cytospora spiraeicola CFCC 53139
Cytospora viridistroma CBS 202.36
Cytospora viticola CBS 141586
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Cytospora schulzeri CFCC 50042
Cytospora sibiraeae CFCC 50045
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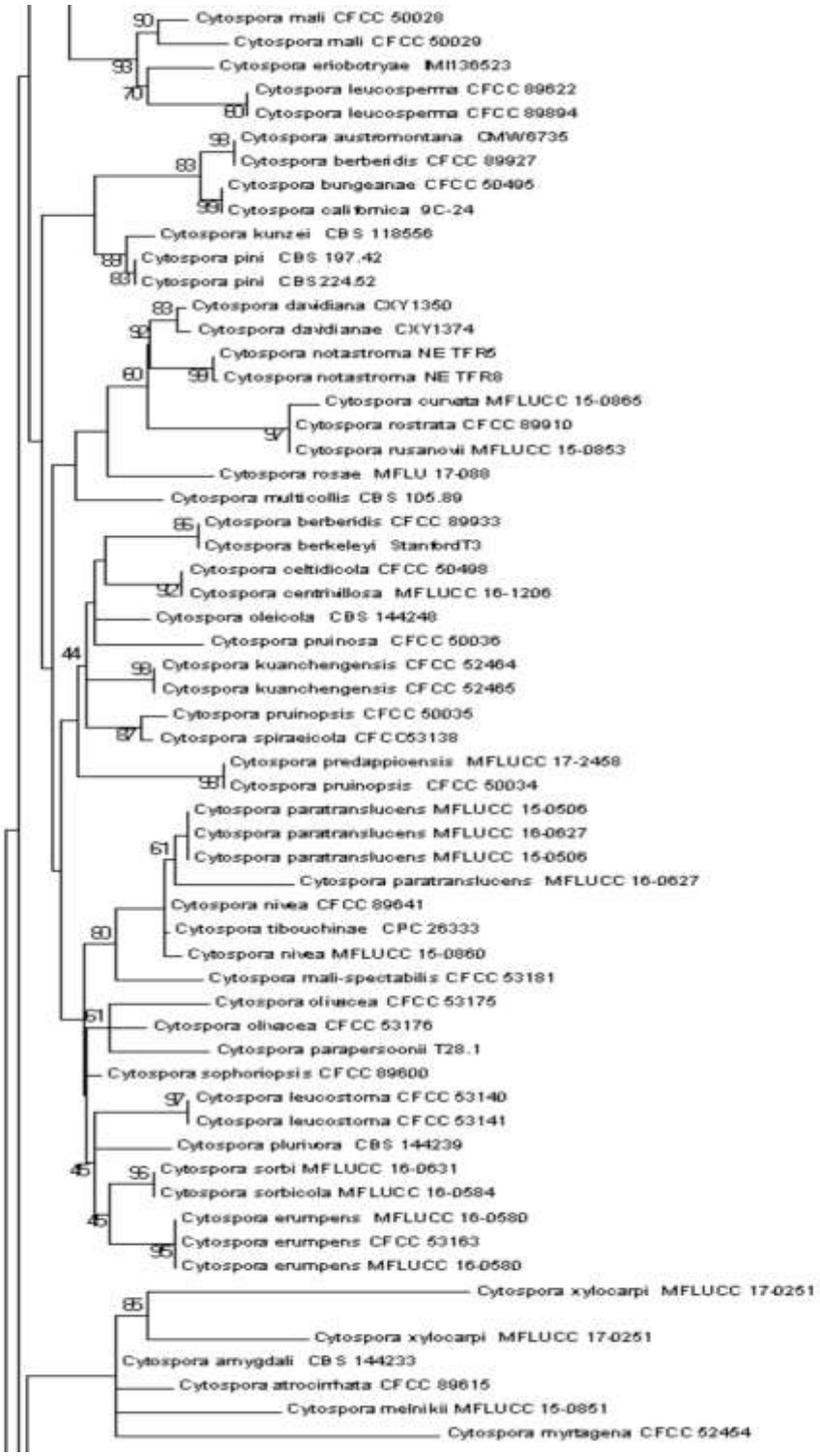
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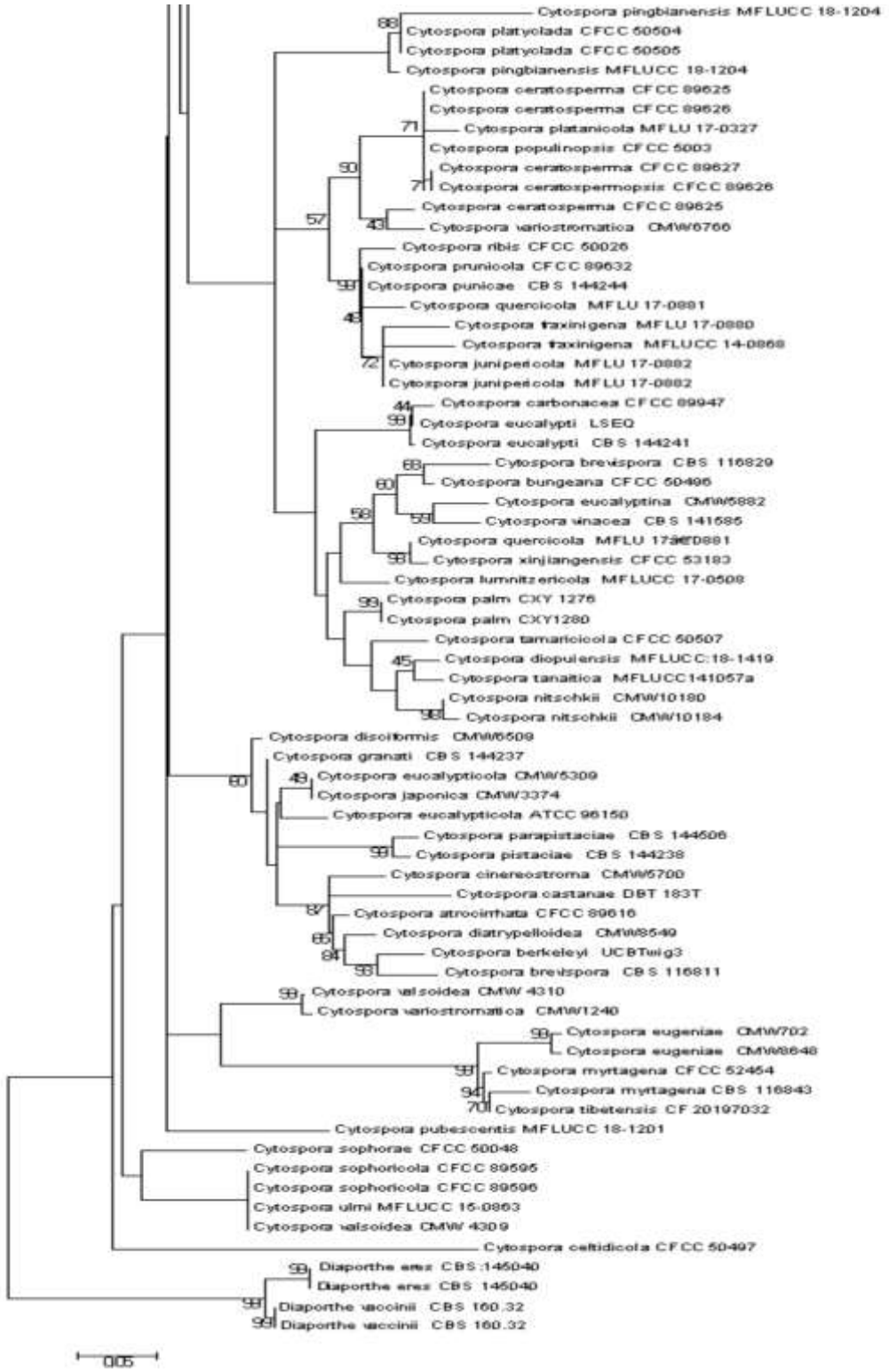
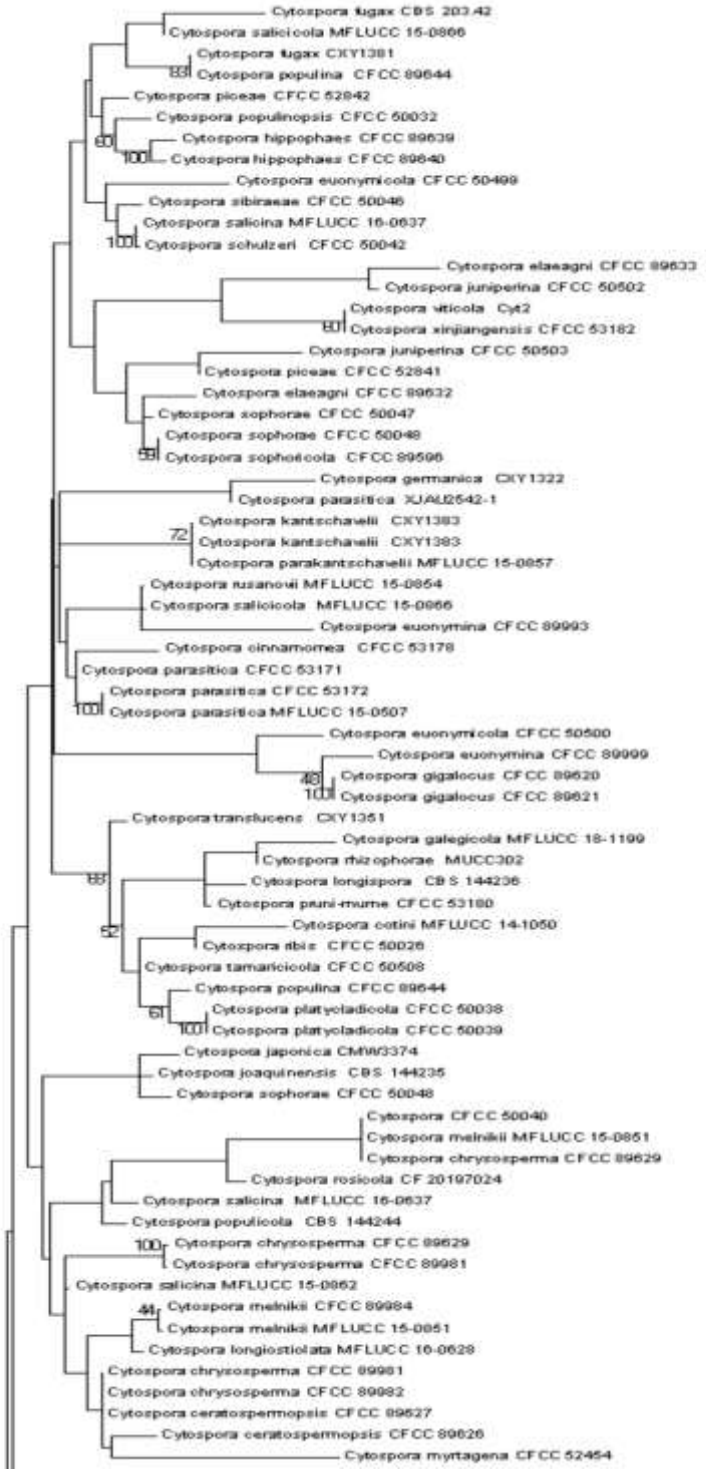


Figure 8: Phylogenetic tree based on ITS+ACT+RPB2 regions using the model TN93+G+I in maximum likelihood analysis

Combined ITS + ACT+TUB Regions

In the phylogenetic tree derived from the combination of ITS + ACT+ RPB2 regions (Figure 9), a total of 117 clades was identified. This combination succeeded in effectively separating 85% of the studied species from other taxa, a fact elucidated in Table 10. Species with multiple sequences used in the analysis showcased high bootstrap values within a unified clade.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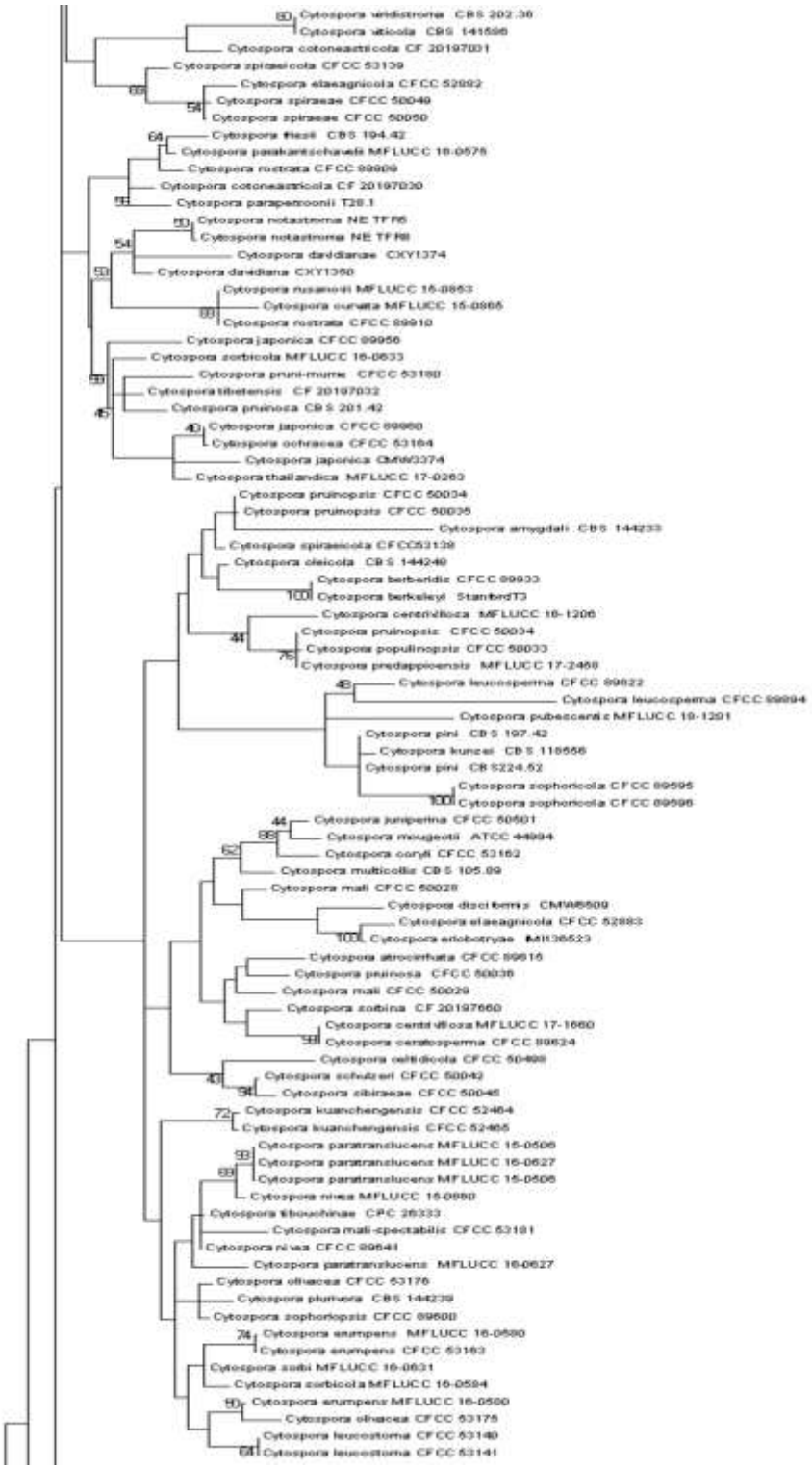
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Cytospora celtidicola CFCC 50488
Cytospora schulzeri CFCC 50042
Cytospora sibiraeae CFCC 50045
Cytospora kuanchengensis CFCC 52464
Cytospora kuanchengensis CFCC 52465
Cytospora paratranslucens MFLUCC 15-0506
Cytospora paratranslucens MFLUCC 16-0627
Cytospora paratranslucens MFLUCC 15-0506
Cytospora nivea MFLUCC 15-0980
Cytospora tibouchinae CPC 26333
Cytospora mali-spectabilis CFCC 53191
Cytospora nivea CFCC 89641
Cytospora paratranslucens MFLUCC 16-0627
Cytospora ochracea CFCC 53176
Cytospora plurivora CBS 144239
Cytospora sophoriopsis CFCC 89600
Cytospora enumpens MFLUCC 16-0580
Cytospora enumpens CFCC 53163
Cytospora sorbi MFLUCC 16-0631
Cytospora sorbicola MFLUCC 16-0594
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Cytospora ochracea CFCC 53175
Cytospora leucostoma CFCC 53140
Cytospora leucostoma CFCC 53141

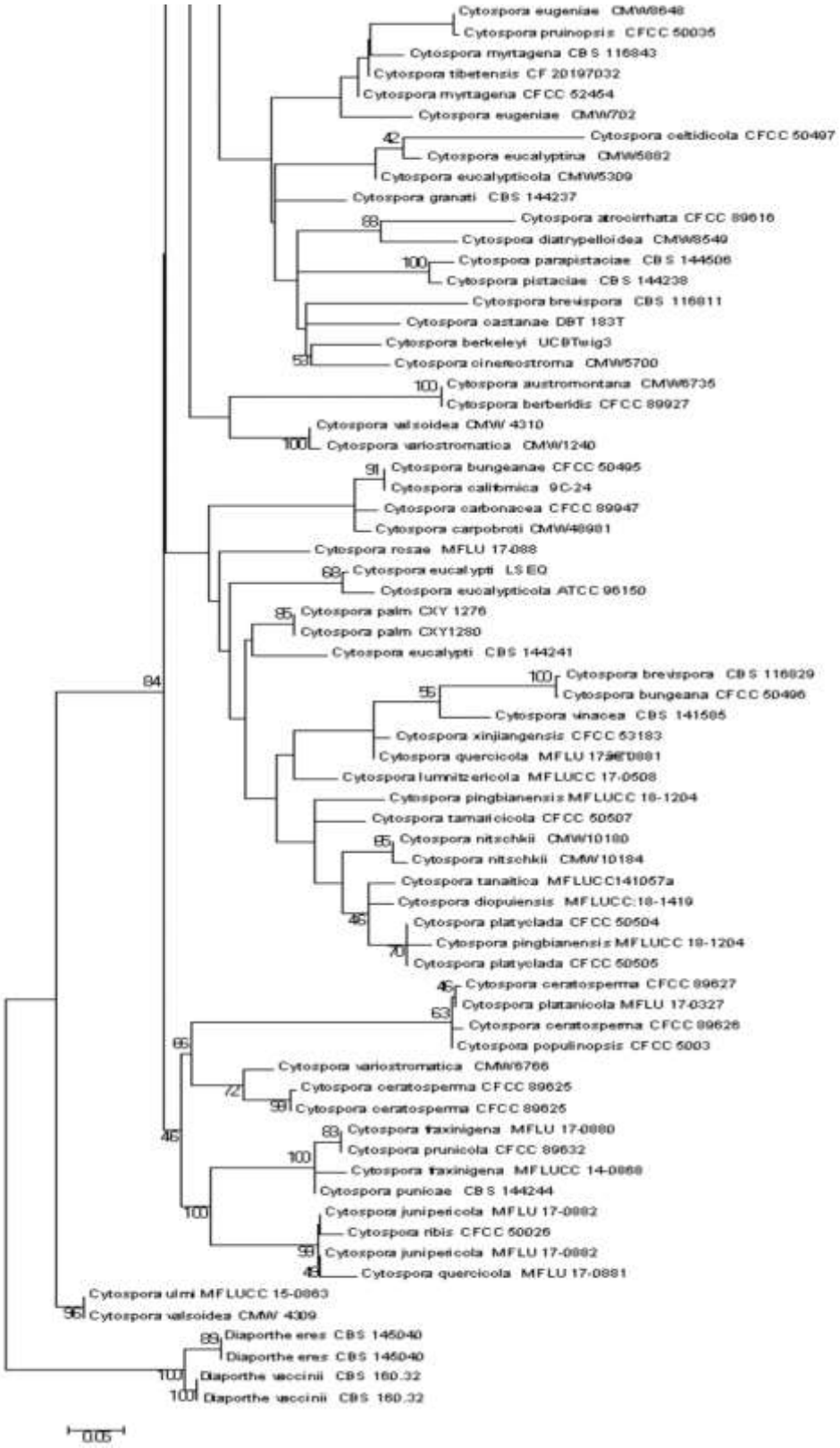


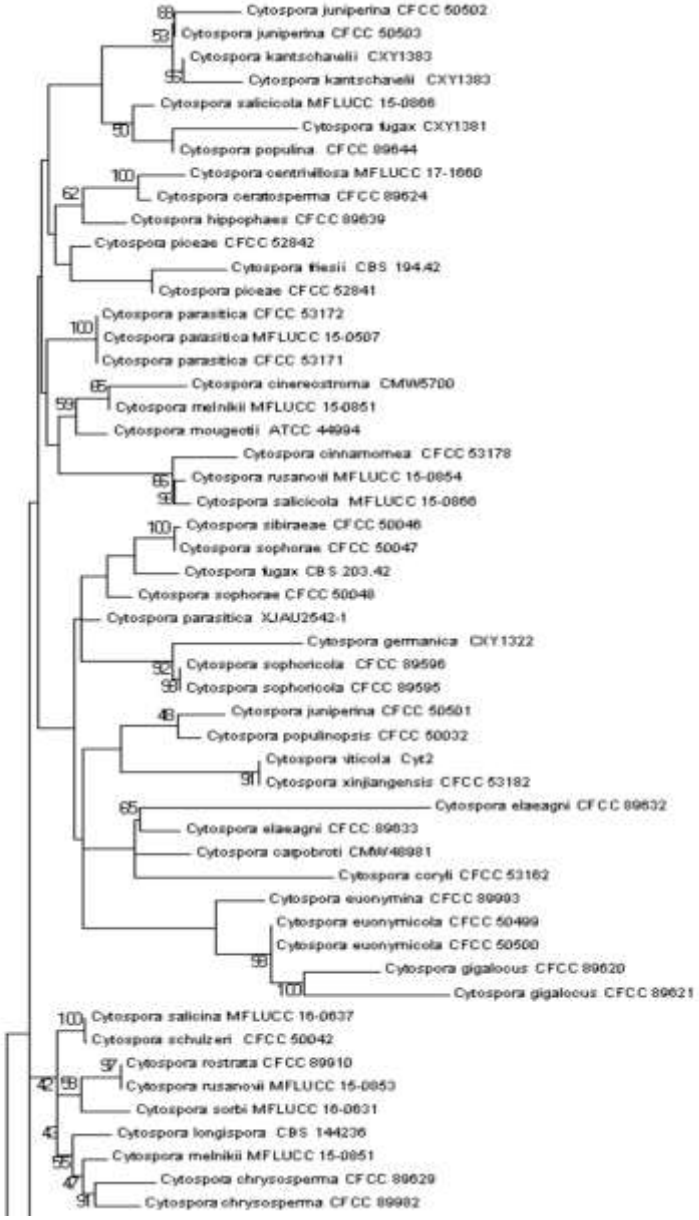
Figure 9: Phylogenetic tree based on ITS+ACT+TUB regions using the model TN93+G+I in maximum likelihood analysis

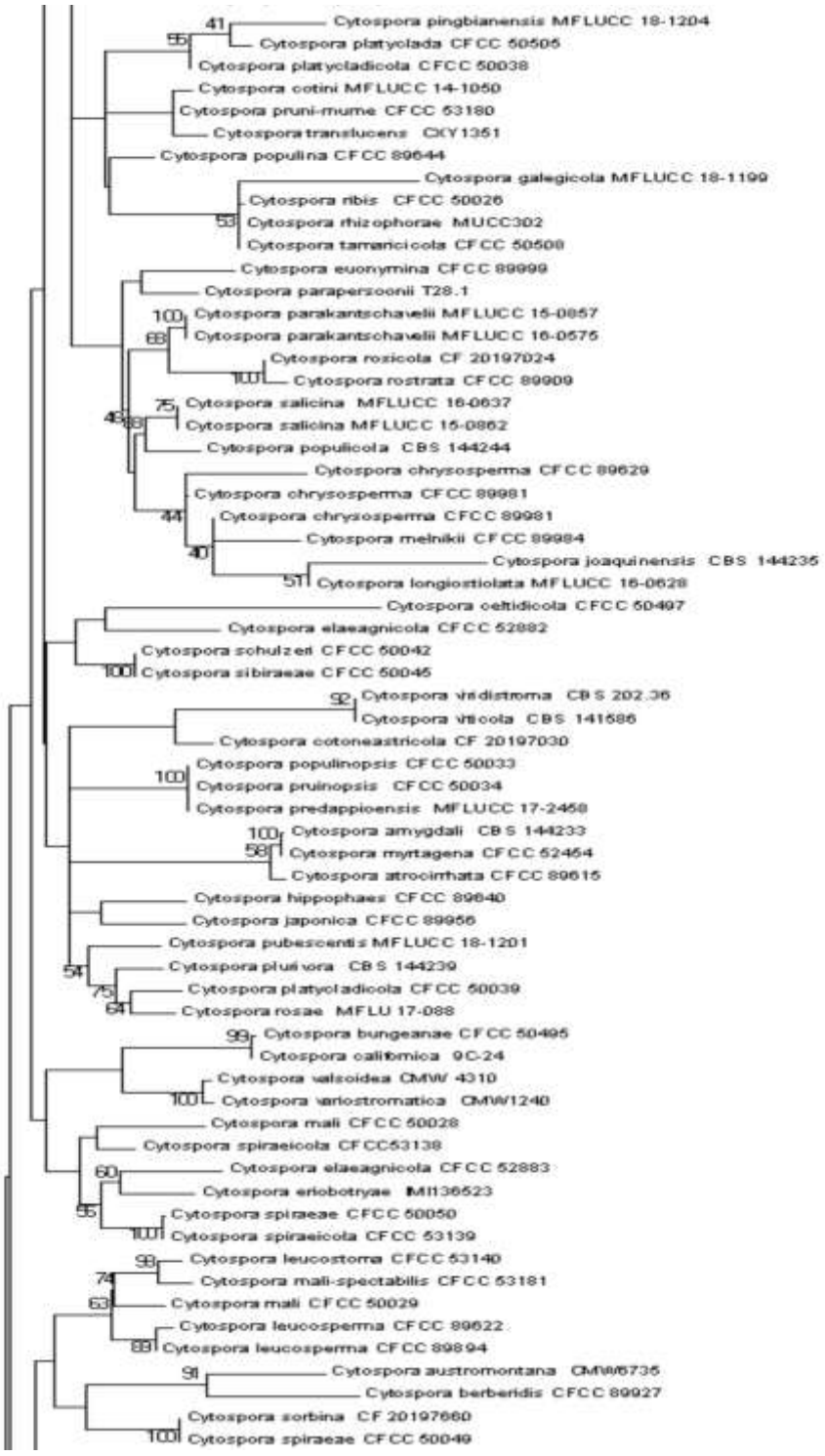
Table 10: Non separated species using ITS+ACT+TUB regions

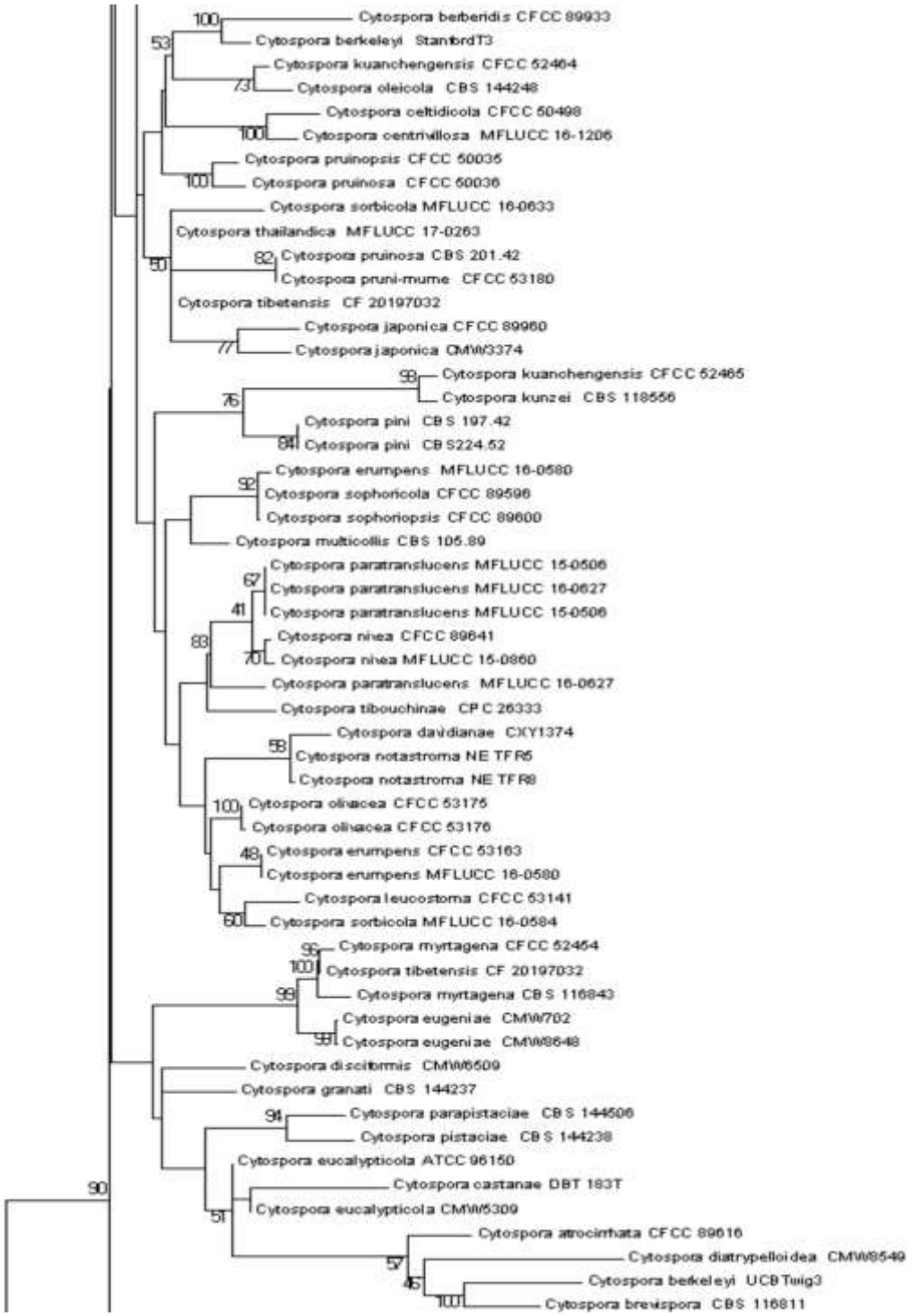
C. berkeleyi	*C. californica*
C. chrysosperma	*C. pingbianensis*
C. curvata	*C. centrivillosa*
C. fugax	*C. ulm*
C. kunzei	*C. ceratosperma*
C. populina	*C. platyclada*
C. rostrate	*C. viticola*
C. rusanovii	*C. bungeanae*
C. viridistroma	*C. californica*
C. xinjiangensis	*C. pingbianensis*
C. fraxinigena	

Combined ITS + ACT+RPB2 +TUB Regions

In the phylogenetic tree derived from the combination of ITS + ACT+ RPB2 + TUB regions (Figure 10), a total of 125 clades was identified. This combination succeeded in effectively separating 91% of the studied species from other taxa, a fact elucidated in Table 11. Species with multiple sequences used in the analysis showcased high bootstrap values within a unified clade.




Cytospora pingbianensis MFLUCC 18-1204
Cytospora platyclada CFCC 50505
Cytospora platycladicola CFCC 50038
Cytospora cotini MFLUCC 14-1050
Cytospora pruni-mume CFCC 53180
Cytospora translucens CXY1351
Cytospora populina CFCC 89644
Cytospora galegicola MFLUCC 18-1199
Cytospora ribis CFCC 50026
Cytospora rhizophorae MUCC302
Cytospora tamaricicola CFCC 50508
Cytospora euonymina CFCC 89999
Cytospora paraperzoonii T28.1
Cytospora parakantschavelii MFLUCC 15-0857
Cytospora parakantschavelii MFLUCC 16-0575
Cytospora rozicola CF 20197024
Cytospora rostrata CFCC 89909
Cytospora salicina MFLUCC 16-0637
Cytospora salicina MFLUCC 15-0862
Cytospora populicola CBS 144244
Cytospora chrysosperma CFCC 89629
Cytospora chrysosperma CFCC 89981
Cytospora chrysosperma CFCC 89981
Cytospora melnikii CFCC 89984
Cytospora joaquinensis CBS 144235
Cytospora longiostiolata MFLUCC 16-0628
Cytospora celtidicola CFCC 50497
Cytospora elaeagnicola CFCC 52882
Cytospora schulzeri CFCC 50042
Cytospora sibiraeae CFCC 50045
Cytospora viridistroma CBS 202.36
Cytospora vticola CBS 141586
Cytospora cotoneastricola CF 20197030
Cytospora populinopsis CFCC 50033
Cytospora pruinopsis CFCC 50034
Cytospora predappioensis MFLUCC 17-2459
Cytospora amygdali CBS 144233
Cytospora myrtagena CFCC 52454
Cytospora atrocirrhata CFCC 89615
Cytospora hippophaes CFCC 89640
Cytospora japonica CFCC 89956
Cytospora pubescentis MFLUCC 18-1201
Cytospora pluvivora CBS 144239
Cytospora platycladicola CFCC 50039
Cytospora rosae MFLU 17-088
Cytospora bungeanae CFCC 50495
Cytospora californica 9C-24
Cytospora valsoidea CMW 4310
Cytospora variostromatica CMW1240
Cytospora mali CFCC 50028
Cytospora spiraeicola CFCC53138
Cytospora elaeagnicola CFCC 52883
Cytospora eriobotryae IMI136523
Cytospora spiraeae CFCC 50050
Cytospora spiraeicola CFCC 53139
Cytospora leucostoma CFCC 53140
Cytospora mali-spectabilis CFCC 53181
Cytospora mali CFCC 50029
Cytospora leucosperma CFCC 89622
Cytospora leucosperma CFCC 89894
Cytospora austromontana CMW6735
Cytospora berberidis CFCC 89927
Cytospora sorbina CF 20197660
Cytospora spiraeae CFCC 50049


Cytospora berberidis CFCC 89933
Cytospora berkeleyi StanfordT3
Cytospora kuanchengensis CFCC 52464
Cytospora oleicola CBS 144248
Cytospora celtidicola CFCC 50498
Cytospora centrivillosa MFLUCC 16-1206
Cytospora pruinopsis CFCC 50035
Cytospora pruinosa CFCC 50036
Cytospora sorbicola MFLUCC 16-0633
Cytospora thailandica MFLUCC 17-0263
Cytospora pruinosa CBS 201.42
Cytospora pruni-mume CFCC 53180
Cytospora tibetensis CF 20197032
Cytospora japonica CFCC 89960
Cytospora japonica CMW3374
Cytospora kuanchengensis CFCC 52465
Cytospora kunzei CBS 118556
Cytospora pini CBS 197.42
Cytospora pini CBS224.52
Cytospora erumpens MFLUCC 16-0580
Cytospora sophoricola CFCC 89596
Cytospora sophoriopsis CFCC 89600
Cytospora multicollis CBS 105.89
Cytospora paratranslucens MFLUCC 15-0506
Cytospora paratranslucens MFLUCC 16-0627
Cytospora paratranslucens MFLUCC 15-0506
Cytospora nivea CFCC 89641
Cytospora nivea MFLUCC 15-0860
Cytospora paratranslucens MFLUCC 16-0627
Cytospora tibouchinae CPC 26333
Cytospora davidianae CXY1374
Cytospora notastroma NE TFR5
Cytospora notastroma NE TFR8
Cytospora olivacea CFCC 53175
Cytospora olivacea CFCC 53176
Cytospora erumpens CFCC 53163
Cytospora erumpens MFLUCC 16-0580
Cytospora leucostoma CFCC 53141
Cytospora sorbicola MFLUCC 16-0584
Cytospora myrtagena CFCC 52464
Cytospora tibetensis CF 20197032
Cytospora myrtagena CBS 116843
Cytospora eugeniae CMW702
Cytospora eugeniae CMW9648
Cytospora disciformis CMW6509
Cytospora granati CBS 144237
Cytospora parapistaciae CBS 144506
Cytospora pistaciae CBS 144238
Cytospora eucalypticola ATCC 96150
Cytospora castanae DBT 183T
Cytospora eucalypticola CMW5309
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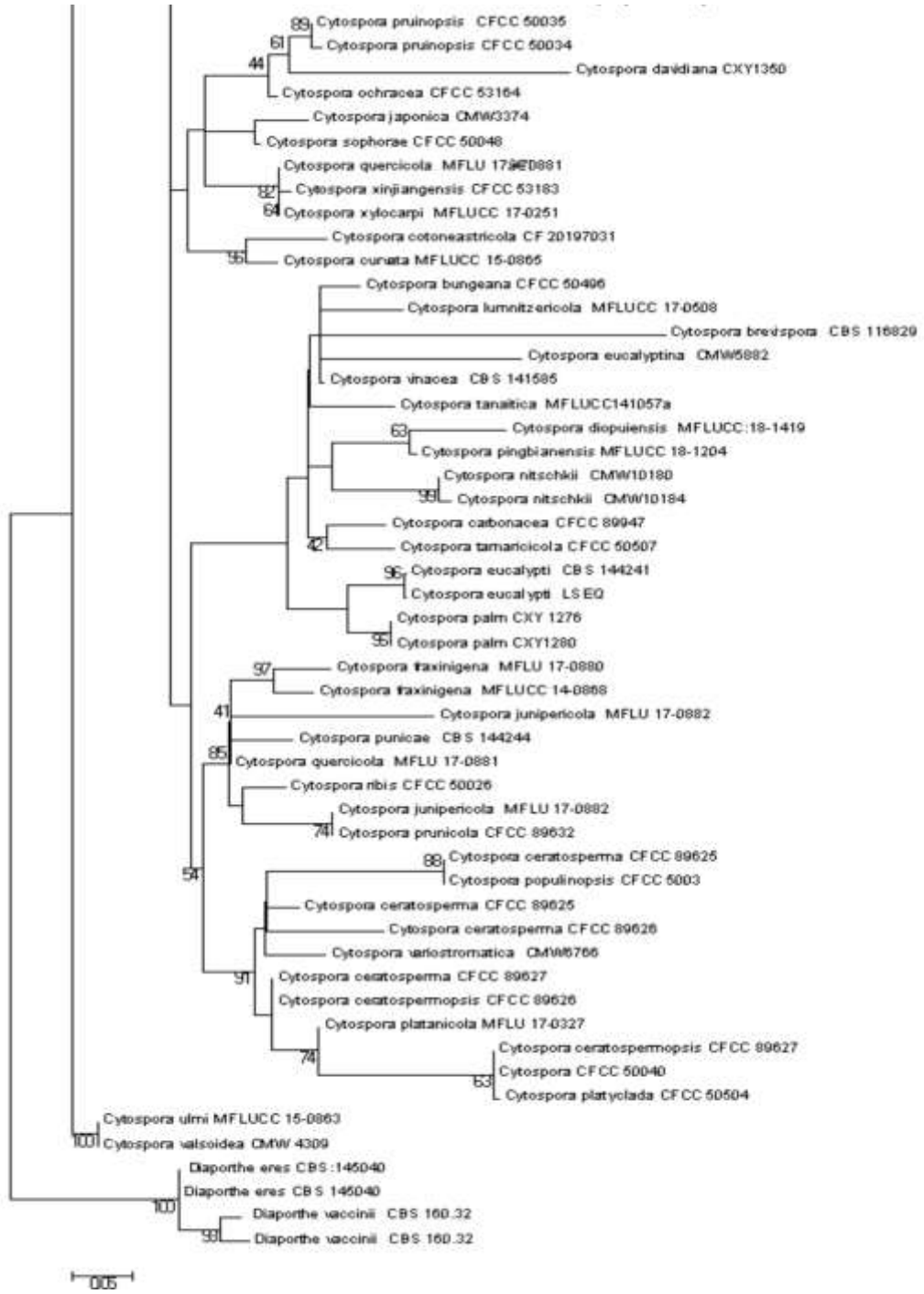

Figure 10: Phylogenetic tree based on ITS+ACT+RPB2+TUB regions using the model TN93+G+I in maximum likelihood analysis

Table 11: Non separated species using ITS+ACT+RPB2+TUB regions

C. rusanovii	*C. ceratosperma*
C. salicina	*C. populinopsis*
C. schulzer	*C. prunicola*
C. schulzeri	*C. rostrate*
C. sibiraeae	*C. ulmi*
C. viticola	*C. valsoidea*

Discussion

In this comprehensive study, a dataset comprising a total of 224 sequences from 138 distinct taxa, including outgroup taxa, was carefully collected. The subsequent phylogenetic analysis, performed using the maximum likelihood algorithm, aimed to reveal the evolutionary relationships among *Cytospora* species. As shown in Figures 1 to 10, both single-gene and multi-gene approaches were used for this purpose.The results from maximum likelihood analysis showcased consistent clustering patterns in both single-gene and multi-gene trees, affirming the compatibility of sequences within each region and the promise? of their synergistic application. These findings indicate that the genetic regions differ considerably in their phylogenetic resolution, which may be attributed to the evolutionary rates and selective constraints acting on each locus. The high variability of RPB2, in particular, provides strong phylogenetic signals for distinguishing closely related species, as previously reported for Sordariomycetes by Tang et al.(2007) (Tang et al., 2007).

The concatenated sequence, representing all four investigated regions, encompassed 2491 nucleotide sites after accounting for nucleotide insertions and deletions. This composite sequence integrated 554, 736, 697, and 296 nucleotide sites derived from the ITS, TUB2, ACT, and RPB2 regions, respectively. Notably, within this alignment, 1459 sites were found to be conserved after excluding outgroup taxa, with the ITS region contributing 262 sites, TUB2 contributing 85 sites, ACT contributing 93 sites, and RPB2 contributing 295 sites. Conversely, there were 198 variable sites that lacked informative data for species differentiation, encompassing 46, 115, 21, and 84 nucleotide sites from the ITS, TUB2, ACT, and RPB2 regions, respectively. In addition, 1256 variable sites provided informative data that helped in species separation, including 212, 551, 171, and 233 nucleotide sites from the ITS, TUB2, ACT, and RPB2 regions, respectively. In the context of single-gene phylogenetic analysis based on the individual gene regions, a distinct number of clades emerged, with 68, 34, 43, and 63 clades derived from the ITS, TUB2, ACT, and RPB2 regions, respectively. Notably, the concatenated dataset, integrates all four regions, yielded the highest number of clusters, containing125 high-confidence clusters. Conversely, the combined region analysis of ITS+ACT and ITS+RPB2 generated 86 clusters, representing the lowest clustering.

In the ITS region-based phylogenetic tree, only 43% of the strains successfully segregated from other species, forming low-confidence clusters. Although ITS remains the primary barcode for fungal identification, its limited discriminatory power within Cytospora agrees with findings by Lücking et al. (2020), who highlighted that ITS alone is often insufficient for species delimitation in closely related taxa (Lücking et al., 2020). This limitation stems from its high rate of intra-specific variability and alignment ambiguities in certain clades. Remarkably, the TUB region-based phylogenetic tree exhibited a separation success rate of 76% for the studied species, encompassing 23% of the total species. The relatively high resolution of TUB2 may be linked to its moderate evolutionary rate and balanced variation level. As reported by Conti et al. (2021), single-copy protein-coding genes with medium taxonomic resolution often provide optimal discrimination for species-level analyses, which aligns with the present findings for Cytospora. The RPB2 region-based phylogenetic tree showcased the most robust separation, with 90% of the studied species successfully distinguished, constituting 47% of the total. This strong resolving power of RPB2 may result from its slower saturation rate and balanced substitution pattern across codon positions, which preserve informative phylogenetic signals. Such performance is consistent with prior multilocus studies in Sordariomycetes and Cytospora (Tang et al., 2007), confirming RPB2 as a robust locus for delineating closely related specie. This high percentage of formed clusters underscored the reliability of this region for separation. In the case of the ACT region, the phylogenetic tree separated 53% of the studied species, representing 36% of the total. These findings align with previous studies (Pan et al., 2020; Wang et al., 2015), confirming the reliability of

RPB2 and TUB regions for species delimitation in Cytospora.

The phylogenetic trees constructed from combinations of regions, including ITS+ACT, ITS+RPB2, ITS+TUB, ITS+ACT+RPB2, ITS+ACT+TUB, and ITS+RPB2+ACT+TUB, displayed relatively similar clustering patterns. While the combined regions demonstrated effectiveness in separating most studied species, specific variations were observed in their ability to segregate certain species. It's noteworthy that species which were representatives of two or more strains consistently formed clusters with high values. However, several species, such *as C. ulmi, C. rostrate, C. populinopsis, C. viticola,* and *C. sibiraeae*, remained inseparable from other species across all phylogenetic trees based on the combined regions. Such unresolved clusters could be attributed to low interspecific divergence and limited sampling, as observed in other studies (Pan et al. 2021). It is also possible that incomplete lineage sorting or recent speciation events contribute to this lack of resolution within the genus. However, some species remained unresolved, likely due to low interspecific divergence or limited sampling, consistent with observations by (Reeb et al., 2004). Furthermore, species like *C. rusanovii, C. ceratosperma, C. valsoidea,* and *C. schulzeri* were successfully separated in single-region-based phylogenetic trees but not in the phylogenetic tree based on the combined regions.

Recent investigations in fungal population studies have emphasized the efficacy of phylogenetic analysis based on the combined sequences of multiple housekeeping genes, preferably encompassing non-coding gene regions (Dettman et al., 2003). In this study, four regions, namely ITS, TUB, RPB2, and ACT, were meticulously explored both individually and in combination to assess the phylogenetic separation of *Cytospora* species. Single-gene phylogenetic analyses indicated that the ITS, TUB, RPB2, and ACT regions independently enabled the separation of 58, 104, 109, and 72 species, respectively, from a total of 137 validated species within the *Cytospora* genus. Hence, these single regions can successfully distinguish and support the identification of up to three-quarters of the known Cytospora species. It's essential to highlight that this separation, as per a single-gene analysis, should align with the results of morphological investigations.

However, in situations where resource constraints necessitate amplification of only one single region, the RPB2 gene emerged as the most promising choice and provided optimal results. For studies with the capacity to amplify and analyze two regions, the combination of the TUB and RPB2 genes yielded the most reliable results. These results highlight that while multi-locus analyses enhance resolution, combining genetic data with morphological features remains essential for accurately distinguishing closely related species. Basically, to ensure the precise identification and differentiation of *Cytospora* species, sequence and phylogenetic analyses of the ITS, RPB2, and TUB regions, along with morphological investigations are essential. These methodologies collectively contribute to robust and accurate species identification. It's worth notingto acknowledge that while genetic analyses significantly aid in the species separation process, morphological features remain crucial in resolving sibling species and are particularly valuable for refining the final identification and differentiation of closely related *Cytospora* species.

This study enhances our understanding of *Cytospora* fungi by conducting extensive genetic analyses on four gene regions: ITS, TUB, RPB2, and ACT. The results reveal the effectiveness of the RPB2 and TUB regions for species differentiation. Morphological characteristics remain essential for resolving closely related species. The study distinguishes up to 90% of *Cytospora* species and provides valuable support for fungal taxonomy, benefiting researchers and mycologists. A combined genetic and morphological approach enables accurate species identification and enhances ecological and evolutionary research in this genus. Overall, these analyses reinforce the importance of integrating multiple gene regions and morphology in Cytospora taxonomy and provide guidance for future biodiversity studies. This research emphasizesthe

importance of integrating genetic and morphological data in fungal taxonomy for more accurate classifications.

Conflict of Interest

The authors declare no conflicts of interest.

Ethical Considerations

All ethical principles and standards were fully observed in the conduct of this research.

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بیماری‌های گیاهی، دوره ۶۰، شماره ۳، صفحات ۳۲۷-۲۸۲

تحلیل جامع ژنومی به منظور شناسایی دقیق گونه‌های *Cytospora* یافته‌های مبتنی بر نواحی ژنی *ITS*، *TUB*، *RPB2* و *ACT*

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چکیده

شناسایی و طبقه‌بندی گونه‌های *Cytospora* به‌دلیل محدودیت روش‌های مورفولوژیکی سنتی همواره با چالش همراه بوده است. بررسی ویژگی‌های ژنتیکی این قارچ‌ها می‌تواند راهکار مؤثری برای شناسایی دقیق‌تر و بازنگری در رده‌بندی آن‌ها فراهم کند. در این پژوهش، به‌منظور بررسی روابط فیلوژنتیکی میان گونه‌های مختلف *Cytospora*، چهار ناحیه ژنی ITS، *ACT*، *TUB* و *RPB۲* مورد مطالعه قرار گرفت. در ناحیه *ITS*، تعداد ۶۸ کلاد شناسایی شد و این بخش تنها قادر بود ۴۳ درصد از گونه‌های بررسی‌شده را از یکدیگر متمایز سازد. در این ناحیه، یک کلاد پایه‌ای شامل *C. valsoide* مشاهده شد. ناحیه *ACT* شامل ۴۵ کلاد بود و توانست ۵۳ درصد از گونه‌ها را تفکیک کند. این ناحیه برای گونه‌هایی مانند *C. populinopsis*، *C. japonica* و *C. leucostoma* مقادیر بوت‌استرپ بالاتری نشان داد. ناحیه *TUB* دقت بیشتری داشت و با ۳۴ کلاد، ۷۶ درصد از گونه‌ها را از هم جدا کرد. مقادیر بوت‌استرپ بالا در این بخش برای *C. chrysosperma*، *C. davidiana* و *C. brevispora* مشاهده شد. ناحیه *RPB۲* نیز با ۶۳ کلاد، ۸۰ درصد از گونه‌های بررسی‌شده را به‌خوبی از یکدیگر تمایز داد و گونه‌های دارای چندین توالی ثبت شده ، خوشه‌های منسجم با بوت‌استرپ بالا تشکیل دادند. ترکیب نواحی ژنی نتایج دقیق‌تری به‌همراه داشت. در ترکیب‌های ITS+*ACT*، ITS+*RPB۲*، ITS+*TUB*، *ITS+ACT+TUB* و ITS+*TUB+RPB۲+ACT* به‌ترتیب ۹۷، ۹۷، ۱۰۰، ۱۱۶ و ۱۲۵ گونه از مجموع ۱۳۷ گونه از هم متمایز شدند. نتایج این مطالعه نشان می‌دهد که استفاده از چند ناحیه ژنی برای شناسایی گونه‌های *Cytospora* دقت بالاتری ایجاد می‌کند. در میان نواحی مورد بررسی، ژن‌های *RPB۲* و *TUB* بیشترین کارایی را داشتند و میتوانند به عنوان گزینه‌های مناسب برای مطالعات فیلوژنتیکی به‌کار روند. به‌طور کلی، یافته‌های این پژوهش درک بهتری از تنوع ژنتیکی و روابط درون‌جنسی در *Cytospora* فراهم کرده و می‌تواند به بهبود دقت در طبقه‌بندی این جنس قارچی کمک کند.

کلیدواژگان: *بررسی تک ژنی، چندژنی، تجزیه و تحلیل تبارزایشی، شناسایی گونه*

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