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Dating divergence of *Polystigma* and other *Sordariomycetes*

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Abstract: Studies on the evolutionary history of ascomycetes in terms of time scale will help to understand historical patterns that shape their biodiversity. Until now most of dating studies of ascomycetes have focused on major events in fungal evolution but not on divergence events within smaller groups of fungi *e.g*. within *Sordariomycetes*. We used molecular dating to estimate the time of separation of *Polystigma* from other groups of *Sordariomycetes* with a Bayesian approach using a relaxed clock model and secondary calibration. Sequences from ITS region and SSU gene of rDNA were used for this purpose. We inferred evolutionary dates in *Sordariomycetes* particularly for *Xylariomycetidae*. Dating analyses showed that *Polystigma* diverged from *Xylariales* approximately 90 Million years ago in the late Cretaceous in which most other diversification events occurred. Our results also suggest that *Polystigma amygdalinum* and *P. rubrum* diverged in early Eocene concurrently with the divergence of their hosts, also providing a base for speculation on the location of evolution of these pathogens.

Key words: Evolution, molecular clock, rDNA sequences

INTRODUCTION

Based on morphological characteristics Canoon (1996) classified *Polystigma* DC. in the order *Phyllachorales*, in the *Sordariomycetidae*, while Habibi et al. (2015) by using ITS, LSU and SSU regions showed that they are close to the *Xylariales* in the *Xylariomycetidae*. *Polystigma* species are characterized principally by brightly colored stromata occurring on living leaves of *Prunus* spp. in the Euro-Asiatic regions. Leaves are infected in spring with ascospores, which are discharged from overwintering leaves just before flowering till fruit set. The first symptoms of infection are accompanied with the formation of stromata within the host tissue in which fruiting bodies and filiform spores develop. The disease symptoms continue to develop during the summer, followed by the development of perithecia during the autumn and winter in the fallen leaves on the ground. *Polystigma amygdalinum* P.F. Cannon and *Polystigma rubrum* (Pers.) DC. are the causal agents of almond red leaf blotch and plum red leaf spot diseases respectively, and often cause premature defoliation of their hosts (Banihashemi 1990; Saad and Masannat 1997).

The evolutionary history of *Polystigma* genus and the related lineages in *Sordariomycetes* is still unknown and no study has tried to infer a timing of their origin in the fungal tree of life. Until now, most dating studies and molecular clock analyses of dating evolutionary divergences in the fungal tree of life have focused on major events (Beimforde et al. 2014; Berbee and Taylor 1993, 2010; Lücking et al. 2009; Padovan et al. 2005; Prieto and Wedin 2013) but not on divergences at lower levels *e.g.* within the *Sordariomycetes*. The divergence of Basidiomycota and Ascomycota has been estimated between 390 Mya (million years ago) to 1.5 Bya (billion years ago) based on different calibration points used for analyses (Taylor and Berbee 2006). Prieto and Wedin (2013) estimated diversification dates for major clades in the *Pezizomycotina*. They showed that the *Pezizomycotina* started to segregate in the Cambrian, and radiations in the Jurassic and Cretaceous caused the diversity of the main modern groups. In addition, these authors provided estimates for diversification dates of major classes, orders and some families of lichenized and non-lichenized groups of *Pezizomycotina*. Beimforde et al. (2014) extended the initiation of diversification of *Pezizomycotina* to have started in the Ordovician and continued throughout the Phanerozoic. They discussed the evolutionary history of main lineages in ascomycetes but not within the smaller groups of ascomycetes, *i.e.* orders and families of *Sordariomycetes*.

Systematics seeks to construct an accurate "time tree" of life showing both the organismic relationships and their dates of origin (Benton & Ayala 2003; Pyron

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2011). Understanding the major processes that had shaped fungal biodiversity requires connecting biological evolution with climate changes, geological evolution and other historical patterns (Parham et al. 2011). In a recent study, we have determined the phylogenetic placement of *Polystigma* spp. in the fungal tree of life (Habibi et al. 2015).

However, the evolutionary history of *Polystigma* spp. in relation to the related lineages in the *Xylariomycetidae* of *Sordariomycetes* is unclear. This study was aimed at estimating the divergence time of *Polystigma* from other *Sordariomycetes*, discussing the evolutionary history of the genus and possible morphological and biogeographic factors underlying the diversification events in *Xylariomycetidae*. This study used a relaxed clock model and a secondary calibration to perform a molecular clock analysis to species level in *Polystigma* in relation to related lineages.

MATERIALS AND METHODS

Taxon sampling

The small ribosomal subunits (nuSSU) and ribosomal internal transcribed spacers (ITS) were used in this study. Sequences were obtained from various fresh and dried specimens representing *P. amygdalinum* and *P. rubrum* which were collected from infected almond and plum orchards in various parts of Iran (Habibi and Banihashemi 2015) and from Genbank (NCBI) which were extracted from reliable studies (Table 1).

DNA Amplification

Freeze-dried infected plant material, was homogenized using sea sand (Fluka, Darmstadt, Germany) and plastic disposable pestles. Cells were lysed using CTAB solution and DNA was extracted using DNG™-plus DNA extraction solution (Cinaclon, Iran) (Mostowfizadeh-Ghalamfarsa and Mirsolaeimani 2012). DNA concentrations were estimated by a NanoDrop spectrophotometer (NanoDrop Technologies, USA). DNA extractions were each diluted to 20 $ng.mL^{-1}$ in sterile distilled water for use as template DNA in PCR. Primers PyITS1 (Green et al. 2004) and ITS4 (White et al. 1990) were used to amplify ITS regions, and NS1 and NS4 primers (White et al. 1990) were used to amplify small subunit regions. Twenty-five µL PCR reactions contained 1 × reaction buffer, 0.4 mM of each primer, 200 mM dNTPs, 2.5 mM $MgCl_2$, 20 ng of DNA and 1 unit of Taq polymerase. PCR was carried out in a CG1-96 thermo cycler (Corbett Research) and cycling conditions consisted of 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min followed by 5 min at 72°C. Sequencing was performed by Elim Biopharmaceuticals, Inc. (USA). ITS and SSU sequences were deposited in GenBank and the accession numbers were obtained (Table 1).

Initial phylogenetic analysis

Datasets for each genomic region (SSU and ITS) were aligned separately using Geneious version 7 (Biomatters, USA). Models of sequence evolution were evaluated for each dataset and model parameter estimates obtained with JModeltest 2.1.1 (Darriba et al. 2012) and models were chosen according to the Bayesian information criterion (BIC, Schwarz 1978). The Bayesian information criterion supported the TIM+G model with equal base frequencies, six substitution rate parameters (11.0000, 2.1366, 1.0000, 1.0000, 4.4032, 1.000) and gamma distributed rates (shape parameter 0.7130) for SSU and the TIM2ef+I+G model with equal base frequencies, six substitution rate parameters (1.3892, 1.4482, 1.3892, 1.0000, 2.4398, 1.000) and gamma distributed rates (shape parameter 0.6960) for ITS. Topological congruence of the datasets was assessed by visual comparison of phylogenetic trees obtained from maximum likelihood-based analysis heuristic searches in PAUP v. 4.0a133 (Swofford 2002). Bayesian analyses were carried out using Markov Chain Monte Carlo (MCMC) approach in the software package MrBayes v3.2.2 (Ronquist et al. 2012) to generate a reasonable starting tree for subsequent analyses of divergence date estimates in BEAST. For analyses, a general time-reversible model of evolution was used. Rate heterogeneity across sites was modelled with a gamma distribution. Four chains starting with a random tree were run for 10,000,000 generations, retaining each 1000th tree and the first 25% of each analysis were discarded as burn-in.

Molecular clock analysis

For calibration we used the divergence time estimation of 247 Mya for the *Sordariomycetes* and *Leotiomycetes* split, inferred in recent molecular dating analysis by Prieto and Wedin (2013) who used six fossils reliable in age and identification including *Paleopyrenomycites devonicus* Taylor, Hass, Kerp, M. Krings & Hanlin as the oldest calibration point.

A Bayesian Markov Chain Monte Carlo algorithm was applied for estimating divergence times using data from the ribosomal DNA internal transcribed spacer region (ITS) and the 18S small-subunit of ribosomal DNA (SSU rDNA). The analyses were carried out using BEAST v1.8.0 software package (Drummond and Rambaut 2007). The tree topology and divergence time were estimated. Using BEAUTI (BEAST package) priors were set for the analyses and the necessary XML input for BEAST was produced. An HKY model of amino acid substitution and a gamma site heterogeneity model with four rate categories as priors were used. An uncorrelated relaxed clock model used to allow rates of molecular evolution to be uncorrelated across the tree Yull speciation process which specifies a constant rate of species divergence was applied. For the *Leotiomycetes* and *Sordariomycetes*

divergence, a prior normal distribution with a mean of 247 Mya and a standard diviation of 10 Mya was assigned to the node age. Beast analyses were run 10 milion generations, sampling parameters and trees every 1000 generations. The BEAST output was analyed with Tracer v1.6 (Rambaut and Drummond 2009). One hundred trees were removed from each run as burn-in and the rest of the trees were used to generate a maximum clade credibility tree which is the tree with the highest product of the posterior probability of all its nodes using TreeAnnotator v1.8.0 (BEAST package).

RESULTS AND DISCUSSION

The maximum clade credibility tree obtained from BEAST analysis (Fig. 1) was topologically identical to the best tree obtained with our maximum likelihood and bayesian analyses. By few exeptions the topologies resulted from BEAST analysis were congruent with the results of other phylogenies of *Sordariomycetes* reported by Zhang et al. (2006) and Eriksson (2006). The phylogenetic placement of *Polystigma* within the *Xylariomycetidae* was consistent with previous study (Habibi et al. 2015) and *Polystigma* built a sister group to the *Xylariales* clade in our analyses.

Divergence time estimates of *Sordariomycete* groups using 247 Mya calibration point for the *Sordariomycetes* and *Leotiomycetes* split, are shown in Fig. 1. Age estimates with means and 95% confidence intervals for some of the major *Sordariomycete* group splits are summarized in Table 2. According to our results, the first divergence within *Sordariomycetes* ([*Xylariomycetidae* + *Sordariomycetidae*] and *Hypocreomycetidae*) took placed in the early Jurassic (node 2; 201 Mya; 149-240 Mya credibility interval) (Fig. 1). The *Xylariomycetidae* and *Sordariomycetidae* split took placed in the Cretaceous (node 5; 172 Mya; 115-222 Mya credibility interval).

Table 1. Accession numbers of fungal species included in the study.

Species	Order	GenBank Accession No.	
		ITS rDNA	18S rDNA
Camarops ustulinoides	*Boliniales*	AY908991	DQ470989
Chaetosphaeriacurvispora	*Chaetosphaeriales*		AY502933
Diaporthe phaseolorum	*Diaporthales*	KC343180	AY779278
Valsella melostoma	*Diaporthales*	AF191184	
Valsella salicis	*Diaporthales*		DQ862057
Aspergillus niger	*Eurotiales*	FJ878652	KF225022
Erysiphefriesii	*Erysiphales*	AB000939	AB033478
Colletotrichum gloeosporioides	*Glomerellales*	DQ084498	JN940370
Glomerella miyabeana	*Glomerellales*		
Glomerella cingulata	*Glomerellales*	GQ373209	AY083798
Hypomyces chrysospermus	*Hypocreales*	HQ604858	AB027339
Kohlmeyeriella tubulata	*Lulworthiales*		AY878998
Lindra thalassiae	*Lulworthiales*		DQ470994
Lulworthia fucicola	*Lulworthiales*		AY879007
Lulworthia lignoarenaria	*Lulworthiales*		
Haloguignardia irritans	*Lulworthiales*	AY581943	AY566252
Magnaporthe salvinii	*Magnaporthales*	JF414838	DQ341477
Meliola centellae	*Meliolales*	KC252606	
Meliola niessleana	*Meliolales*		AF021794
Microascus cirrosus	*Microascales*	JQ906771	M89994
Coccodiella melastomatum	*Phyllachorales*		U78543
Coccodiella toledoi	*Phyllachorales*		CTU78544
Phyllachora graminis	*Phyllachorales*		AF064051
Sphaerodothis acrocomiae	*Phyllachorales*		SAU76340
Ophiodothella vaccinii	-		OVU78777
Polystigma amygdalinum	-	KC756360[a]	KM111539[a]
Polystigma rubrum	-	KC966927[a]	
Cercophora caudate	*Sordariales*	AY999135	DQ368659
Chaetomium elatum	*Sordariales*	HF548695	M83257
Chaetomium globosum	*Sordariales*	AY429056	JN939003
Farrowia longicollea	*Sordariales*		AF207685
Gelasinospora tetrasperma	*Sordariales*	AY681178	DQ471032
Lasiosphaeria ovina	*Sordariales*	GQ922528	AY083799
Neurospora crassa	*Sordariales*	AY681193	X04971
Sordaria fimicola	*Sordariales*	FN392318	AY545724
Nigrospora oryzae	*Trichosphaeriales*	JN198503	FJ176838
Xylaria acuta	*Xylariales*	JQ862676	JQ419764
Xylaria hypoxylon	*Xylariales*	DQ491487	NG_013136

[a]sequences generated in this study

Empty spaces mean that sequences were not available.

Table 2. Divergence time estimates of *Sordariomycetes* lineages obtained from Bayesian analysis. For each divergence, the median and range (95% credibility intervals) are provided. Divergence times are provided in millions of years (Mya). The node numbers correspond to numbers used in Fig. 1 to show their placement in the chronogram.

Nodes		Geological period	Time (Mya)	Confidence interval (mya)
1	*Hypocreomycetidae* crown group	Jurassic	172	116-222
2	*Hypocreomycetidae-Sordariomycetidae* and *Xylariomycetidae*	Jurassic	201	149-240
3	*Sordariomycetidae* crown group	Jurassic	148	94-199
4	*Leotiomycetes–Sordariomycetes*	Permian	247	225-265
5	*Sordariomycetidae -Xylariomycetidae*	Jurassic	172	115-222
6	*Sordariales* crown group	Cretaceous	74	36-121
7	Polystigma spp.- *Xylariales*	Cretaceous	92	34-169
7	*Xylariomycetidae* crown group	Cretaceous	92	34-169

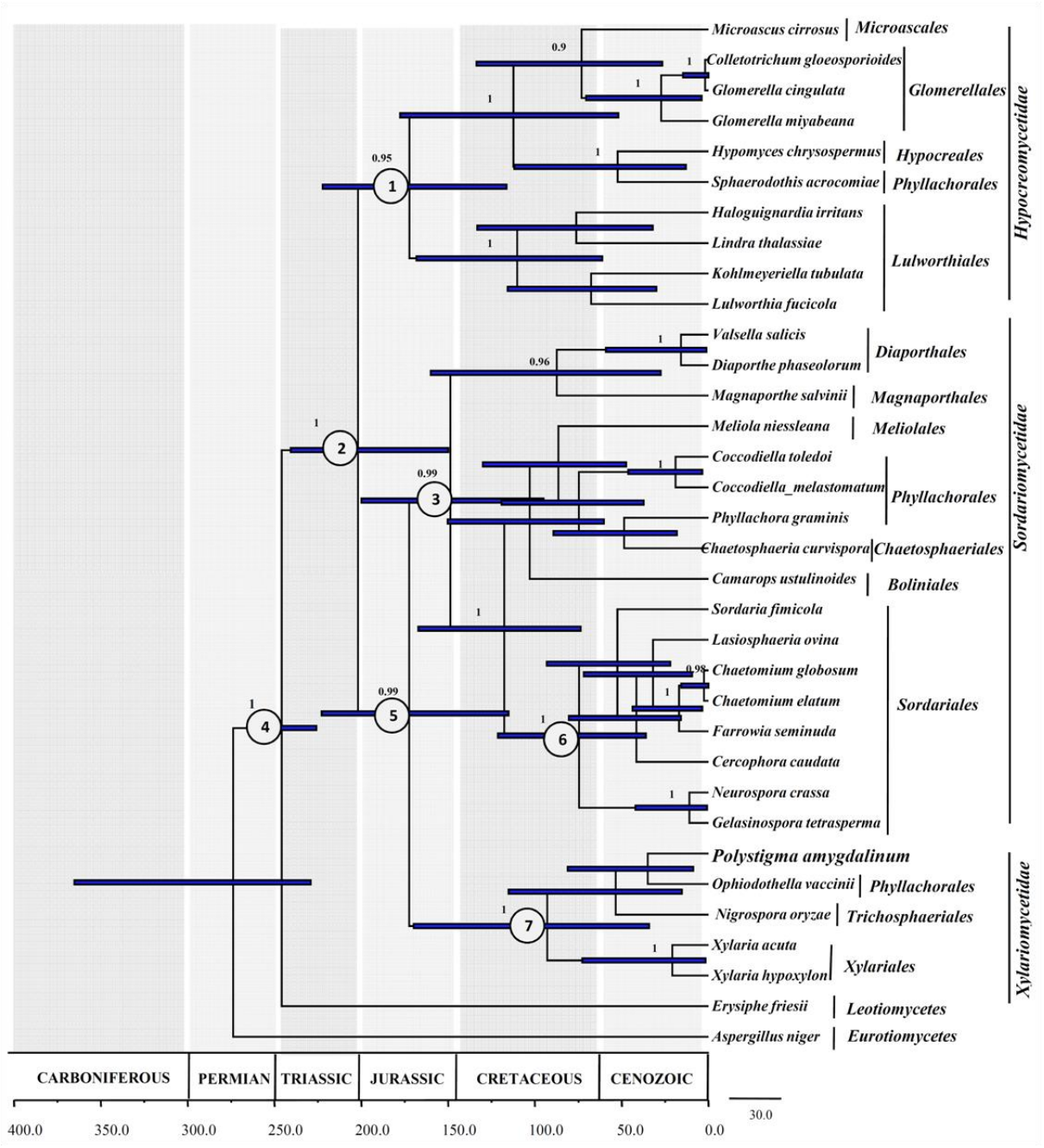

Fig. 1. Maximum clade credibility chronogram for the *Sordariomycetes* based on SSU rDNA. The chronogram is the result from BEAST analysis. Each node represents the mean divergence time estimate and bars show their associated 95% credibility interval. Bayesian posterior probabilities (probabilities %) are shown next to the branch points.The scale bar represents the number of changes per sites. Numbers corresponding to dated groups shown in table 2 are written at the nodes in circles.

Our analyses show that successive radiations in the Jurassic and mostly Cretaceous have generated the diversity in the main *Sordariomycetes* groups. Most *Sordariomycete* orders and families originated in the Cretaceous. The first diversification of *Sordariomycete* groups occurred after the Permian-Triassic (P-T) mass extinction, which had a profound effect on terrestrial and marine ecosystems.

Extreme abundances of fungal remains have been reported in sedimentary organic matter associations from the P–T boundary (Taylor 2004; Visscher et al. 1996). This diversification of fungi may be a response to a large amount of dead plant and organic material. On the other hand, Bell et al. (2005, 2010) estimated that the origin of angiosperms was in Lower Jurassic to Lower Cretaceous which could explain the main diversification events of *Sordariomycetes*. The high level of diversity and radiation in *Sordariomycete* groups in the Cretaceous could be influenced by several new environments dominated by the new diversity of angiosperms, which could host these fungi.

The results of this study were similar to those by Prieto and Wedin (2013) and Gueidan et al. (2011). Prieto and Wedin (2013) showed that most families of Ascomycetes have diverged in the Cretaceous–Paleocene. It is not surprising that our results would not significantly depart from the ones from Prieto and Wedin (2013), as we used one of their estimates as secondary calibration. However, Beimforde et al. (2014) suggested an earlier initiation for the diversification of *Pezizomycotina* starting in the Ordovician and continuing throughout the Phanerozoic. They suggested that the diversification was unaffected by mass extinctions due to ecological diversity within each lineage.

Our results suggest that *Polystigma* diverged from the *Xylariales* approximately 90 Mya in the late Cretaceous in which most diversification events occurred (Fig 1). It appears that *Polystigma* and *Xylariales* may have originated from endophytic ancestors as they both have endophytic behavior in common. There is a hypothesis for the role of endophytic *Xylaria* species which states that the fungi are simply waiting for their host to senesce (or perhaps to accelerate it), at which times they can begin to decompose cell walls (Davis et al. 2003; Petrini et al. 1995). This is similar to how *Polystigma* spp. treat their hosts. *Polystigma* species are known to initially have biotrophic nutrition. They develop on green plant tissue with little evidence of antagonistic relationship and the leaf tissue around the ascomata seems green and healthy (Cannon 1997). Cannon (1991) considered the relationship between this group of fungi and their hosts at least in some instances mutualistic, similar to what has been demonstrated for other endophytic fungi. Endophytes employing of this strategy, would have an advantage over competing saprophytes, having occupied the tissue before decomposition begins. Thus, we suggest that *Polystigma* species and endophytic *Xylariales* may have evolved from a common endophytic ancestor.

The analyses show in addition that *P. amygdalinum* and *P. rubrum* diverged in the early Eocene (49 Mya; Fig. 2). The fossil evidence for *Polystigma* spp. is very scanty. However, according to materials from fossil palms in western Canada, these fungi date back to Eocene (50Mya; Cannon, 1997). This divergence time is consistent with the divergence time estimates of the *Prunus* hosts, inferred by Chin et al. (2014). These authors showed that the genus *Prunus* appeared ~61 Mya in eastern Asia and diversification of all major lineages may have occurred in early Eocene, triggered by the global warming in early Eocene known as Paleocene–Eocene Thermal Maximum and Early Eocene Climate Optima events. The Cenozoic Era is the period of mammals and Angiosperms. The Era begins with the Paleogene Period containing three successive epochs, Paleocene, Eocene and Oligocene. The Paleocene started with a warm climate, which continued to the early Eocene [ca 50 Mya] (Crane et al. 2000). In addition, Chin et al. (2014) suggested that tectonic collision of the Indian plate with the Eurasian plate (~50 Mya) resulting in the orogenic uplifts of the Tibetan plateau likely contributed to this diversification by exerting vicariance forces. The forces are the processes by which the geographical range of an individual taxon, or a whole biota, is split into discontinuous parts by the formation of a physical or biotic barrier to gene flow.

Because *Polystigma* species are obligate biotrophs of *Prunus* spp., their niche is strictly confined to the leaves of the living hosts. Geographic distributions of the early hosts of *Polystigma* species in the Paleogene period, when the first radiation occurred, may provide a base to speculate on the location of the evolution of these pathogens. The center of diversity for the major *Prunus* crop species is Eurasia (Watkins 1976). The restriction of *P. amygdalinum* and *P. rubrum* distribution to the Euro–Asiatic region may correspond to a coevolution with the hosts in these regions.

Despite the increasing number of the studies on the origin, diversification and evolutionary history of different fungal groups, we still suffer from the lack of information on the evolutionary history of Ascomycetes, particularly within smaller groups such as orders, families and genera. In this study, using a Bayesian approach and a relaxed clock model, we provided the information which expanded the knowledge of the evolutionary dates in *Sordariomycetes* particularly within *Xylariomycetidae*. We also infered that most *Sordariomycete* orders and families originated in the Cretaceous. Our analyses suggest that the divergence between *P. amygdalinum* and *P. rubrum* occurred during the early Eocene concurrently with the divergence of their *Prunus* hosts. Inferring phylogenies and divergence times provided a base to speculate on the evolution of these pathogens.

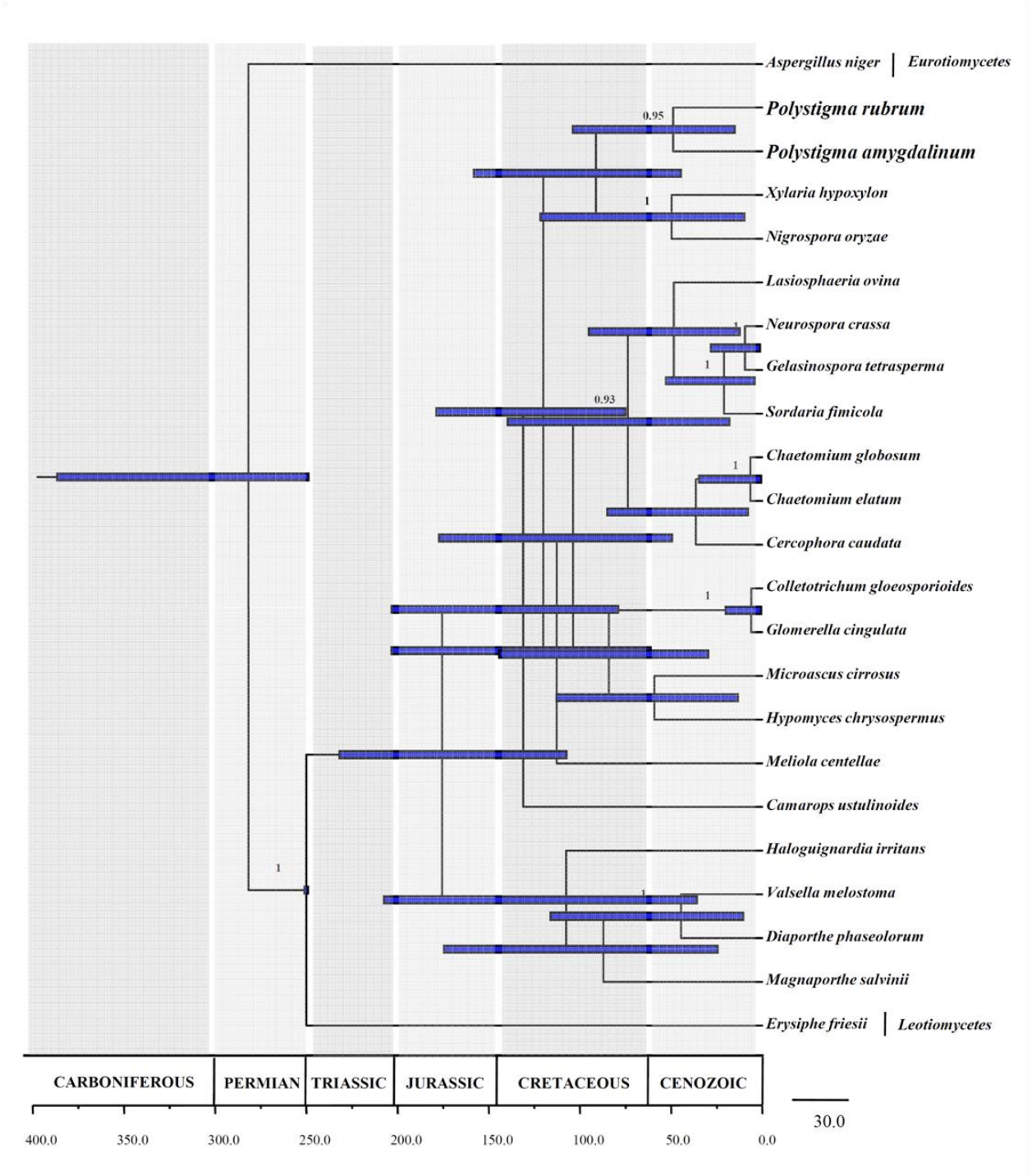

Fig. 2. Maximum clade credibility chronogram for the *Sordariomycetes* based on ITS rDNA region. The chronogram is the result from BEAST analysis. Each node represent the mean divergence time estimate and bars show their associated 95% credibility interval. Bayesian posterior probabilities (probabilities %) are shown next to the branch points. The scale bar represents the number of changes per sites.

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تعیین زمان واگرایی *Polystigma* از سایر آرایه های *Sordariomycetes*

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چکیده: مطالعه تاریخچه تکاملی آسکومیست‌ها از لحاظ مقیاس زمانی به درک الگوهای تاریخی شکل دهنده تنوع زیستی این گروه از قارچ‌ها کمک خواهد کرد. تا کنون بیشتر مطالعات تعیین زمان واگرایی در آسکومسیت‌ها روی وقایع اصلی تکامل قارچی متمرکز شده است و وقایع منجر به واگرایی بین گروه‌های کوچک‌تر قارچی مانند گروه‌های *Sordariomycetes* کمتر بررسی شده است. برای تخمین زمان واگرایی *Polystigma* از سایر گروه‌های *Sordariomycetes*، تعیین زمان مولکولی با کمک رهیافت بیسی و با استفاده از مدل ساعت مولکولی آسان (relaxed clock model) و کالیبراسیون ثانویه، انجام شد. توالی‌های ناحیه ITS و SSU از rDNA برای واکاوی‌ها مورد استفاده قرار گرفت. تاریخ‌های تکاملی در بین برخی گروه‌های *Sordariomycetes* خصوصاً در *Xylariomycetidae* مورد واکاوی قرار گرفت. واکاوی‌های تعیین تاریخ نشان داد که واگرایی *Polystigma* از *Xylariales* تقریباً ۹۰ میلیون سال پیش و در اواخر کرتاسه و همزمان با اکثر وقایع واگرایی بین گروه‌های قارچی رخ داده است. همچنین، نتایج نشان داد که واگرایی *P. amygdalinum* و *P. rubrum* دراوایل ائوسین رخ داده است و همزمان با واگرایی بین دودمان‌های اصلی میزبانشان، جنس *Prunus*، بوده است. با توجه به این مطلب می‌توان در مورد مکان تکامل این بیمارگرها نیز استنتاج کرد.

کلمات کلیدی: تکامل، ساعت مولکولی، توالی‌های rDNA

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