Universidade de Aveiro Departamento de Biologia
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MAT genes as phylogenetic markers in the genus *Lasiodiplodia*

Genes *MAT* como marcadores filogenéticos no género *Lasiodiplodia*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Anabela Rodrigues Lopes, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro e do Doutor Artur Jorge da Costa Peixoto Alves, Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro do FEDER através do programa COMPETE e do POR Lisboa e da FCT através de fundos nacionais no âmbito do projeto ALIEN.

PTDC/AGR-PRO/2183/2014
POCI-01-0145-FEDER-016788
LISBOA-01-0145-FEDER-016788

Para os meus pais,

Tudo o que alcancei até agora foi graças a vocês, não existem palavras que demonstrem nem metade da vossa grandeza.

Do fundo do coração, um obrigado.

o júri

presidente

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agradecimentos

Começo por agradecer ao Professor Artur Alves, por ter ajudado com a escolha do tema. Só tenho a agradecer por me ter aceite no seu grupo, e por toda a ajuda disponibilizada.
À Dr. Anabela Lopes. Por toda a disponibilidade e paciência que teve para comigo durante este longo ano, e por todos os conhecimentos preciosos que me transmitiu.
À minha Avó Teresa. Um dos meus maiores pilares. Obrigado por seres quem és e todo o apoio que me dás.
À Sofia Martins. O meu amor, sem ti não sei se seria capaz de terminar este trabalho. És a minha motivação, sem ti nada disto fazia sentido.
Por último gostava de agradecer à Filipa Lopes. Uma grande amiga que me ajudou incansavelmente durante este ano.

palavras-chave

Lasiodiplodia, espécies crípticas, genes *MAT*, estratégias sexuais, filogenia, sistemática.

resumo

Espécies de *Lasiodiplodia*, pertencentes à família *Botryosphaeriaceae,* são fungos patogénicos ou endofíticos, que podem infetar diferentes tipos de plantas globalmente. Este género contém mais de 50 espécies descritas até a data com base em caracteres morfológicos e análise filogenética. Em *Botryosphaeriaceae,* e em *Lasiodiplodia*, as espécies crípticas são comuns e em alguns casos pode ocorrer hibridação. Estes dois fenómenos levam a que diferenciação de espécies com base em caracteres morfológicos seja pouco confiável. Por esta razão, a delimitação de espécies é feita com base no critério GCPSR (General Concordance Phylogenetic Species Recognition), onde a delimitação é com base numa análise *multi-locus*. Os genes *mating type* (*MAT*) são os reguladores chave da reprodução sexual dos fungos, onde controlam várias funções como, diferenciação celular ou determinação da compatibilidade sexual. Neste estudo foram desenvolvidos conjuntos específicos de *primers* para a amplificação e sequenciação dos genes *MAT* nas espécies de *Lasiodiplodia* estudadas. Foi desenvolvido um ensaio de PCR padronizado específico para estes genes. O heterotalismo foi a estratégia sexual mais comum encontrada nas espécies estudadas. Foi também avaliado o potencial dos genes *MAT* como marcadores filogenéticos para delimitar espécies em *Lasiodiplodia*. Estes foram comparados com marcadores mais convencionais, nomeadamente a região *internal transcribed spacer* (ITS) do cluster ribossomal e o gene que codifica para o fator de alongamento da transcrição 1-alfa (*tef1-α*). Os resultados obtidos mostram que os genes *MAT* são capazes de discriminar entre espécies com um poder de resolução superior aos marcadores convencionais usados. Os genes *MAT* representam assim uma ferramenta útil para diferenciar entre espécies crípticas dentro do género *Lasiodiplodia*.

keywords

Lasiodiplodia, cryptic species, *MAT* genes, mating strategies, phylogeny, systematics.

abstract

Lasiodiplodia species, members of the family *Botryosphaeriaceae*, are pathogenic or endophytic fungi that can infect a broad range of plants, some with high economic value. This genus comprises more than 50 species described to date, based on morphological and phylogenetic analysis. In *Botryosphaeriaceae*, and in *Lasiodiplodia*, cryptic speciation is common and hybridization may occur, which makes species delimitation based on morphological characters unreliable. For this reason, species delimitation is based on the General Concordance Phylogenetic Species Recognition (GCPSR) criterium using a multi-locus phylogenetic analysis. Mating type (*MAT*) genes are the master regulators of sexual reproduction in fungi, they control several functions like mating combability or cellular differentiation. In this study specific sets of primers were designed for amplification and sequencing of *MAT* genes in all *Lasiodiplodia* species studied. A PCR-based mating type diagnostic assay was developed. Heterothallism was the most common state among all isolates. The potential of mating type gene sequences for species delimitation was also evaluated. These were compared with conventional molecular markers, namely, ribosomal internal transcribed spacer region (ITS) and partial sequence of the translation elongation factor 1-alpha gene (*tef1-α*). The results obtained show that *MAT* genes are able to delimit species in *Lasiodiplodia* with higher resolution power than the conventional molecular markers. Thereby, *MAT* genes represent a useful tool to differentiate cryptic species in the genus *Lasiodiplodia*.

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tef1-α – Translation elongation factor 1-alpha

ITS rDNA – Nuclear ribosomal internal transcribed spacer

tub – tubulin gene

MAT – mating-type

PCR – Polymerase chain reaction

ML – Maximum likelihood

NJ - Neighbour-Joining

ORF – Open reading frame

PDA – Potato-dextrose agar

TES – Tris-EDTA-SDS buffer

TE – Tris-EDTA buffer

TAE – Tris-acetate-EDTA buffer

EDTA – Ethylenediaminetetraacetic acid

BLAST - Basic Local Alignment Search Tool

Cox-VIa - Cytochrome c oxidase subunit VIa

GCPSR - Genealogical concordance phylogenetic species recognition

MEGA - Molecular evolutionary genetics analysis

PIM - Putative integral membrane protein

APN2 - DNA lyase

dNTP – Deoxynucleotide triphosphates

HMG - High mobility group

NCBI - National Center for Biotechnology Information

MSR – Morphological species recognition

PSR – Phylogenetic species recognitio

Introduction

The family *Botryosphaeriaceae*

Members of the family *Botryosphaeriaceae* have a cosmopolitan distribution. They infect a wide number of hosts across the world, affecting mostly tropical and subtropical regions with the exception of polar regions (due to environmental factors) (Crous et al. 2006; Crous et al. 2017; Dissanayake 2018). This family encompasses a wide amplitude of morphologically diverse fungi that can be pathogens, endophytes or saprobes, mostly on woody hosts, including gymnosperms and angiosperms. From dieback to canker, their frequent association with plant diseases has stimulated substantial interest in these fungi (Crous et al. 2017; Nagel et al. 2018).

These fungi interact with a very large number of hosts (Ismail et al. 2012; Yan et al. 2013). Usually, species that infect a wide range of hosts are more aggressive pathogens due to the fact that, wider interaction with different plants, allows a species to develop multi-resistance and permits the establishment in a new area much more easily (Galagan et al. 2005).

The taxonomy of this family was confused for a long time due to identification being based only on morphology, including size, shape, colour and wall thickness of the conidia (Crous et al. 2006; Groenewald et al. 2013; Yang et al. 2017). Often, species delimitation with basis on the mature conidia can cause uncertainty due to high similarity between them, which can lead to a wrong identification (Figure 1). Cryptic species are two or more species, undistinguishable by morphological characters, and therefore erroneously classified in the same species. Cryptic speciation is very common in *Botryosphaeriaceae* and a serious issue in terms of species identification (Alves et al. 2008; Liu et al. 2012; Lopes et al. 2018).

L. crassispora

L. parva

L. theobromae

Figure 1- Adapted images of *Lasiodiplodia crassispora*, *L. parva* and *L. theobromae*. All the images represent mature conidia of each species, to demonstrate how differentiation based on morphological characters can be unreliable.

Advances in DNA-based molecular techniques have allowed a better resolution and understanding of *Botryosphaeriaceae* family members. These DNA–based molecular tools started to be used and phylogenetic markers such as the internal transcribed spacer (ITS) region of the cluster coding for ribosomal RNA genes and translation elongation factor 1-alpha (*tef1-α*) had a huge impact in species delimitation (Alves et al. 2007; Philips et al. 2008; Groenewald et al. 2013).

The number of new species identification in *Botryosphaeriaceae* rapidly increase thankfully to these phylogenetic markers and a huge number of cryptic species has been identified. However, even these are not flawless which makes the search for alternative loci with higher resolving power a research area of extreme importance.

Different genera within the *Botryosphaeriaceae,* such as *Diplodia*, *Lasiodiplodia* and *Neofusicoccum* have been reported to contain a high number of cryptic species (Burgess et al. 2019; Lopes et al. 2017; Lopes et al. 2018). This study focused on the genus *Lasiodiplodia*, a well-known plant pathogen.

The genus *Lasiodiplodia*

The genus *Lasiodiplodia* belongs to the family *Botryosphaeriaceae* (Botryosphaeriales, Dothideomycetes, Ascomycota), and contains more than 30 species that have been described based on morphological and phylogenetic characters (Coutinho et al. 2017; Rodríguez-Gálvez et al. 2017; Burgess et al. 2019).

Lasiodiplodia species are cosmopolitan, very common in tropical and subtropical regions. Infection by *Lasiodiplodia* species occurs on broad range of hosts (monocotyledonous, dicotyledonous and gymnosperm) where these pathogens can survive as saprophytes or endophytes within seeds and other living tissues (Abdollahzadeh et al. 2010; Alves et al. 2007). They are associated with a diversity of symptoms, from shoot blights, stem cankers, fruit rots, and dieback to gum exudation and necrotic lesions, which could lead to the obstruction of vascular tissues and ultimately plant death (Abdollahzadeh et al. 2010; Crous et al. 2006; Coutinho et al. 2017).

As already mentioned above, cryptic speciation is very common in the family *Botryosphaeriaceae* and in the genus *Lasiodiplodia*, making species identification by morphological characters unreliable (Alves et al. 2008). It is clear, with the support from a reasonable amount of studies, that neither morphology (Fig. 1 – example of mature conidia in three distinct species, showing insignificant morphological differences between them) nor single locus sequence data are sufficient to define taxa (Taylor et al. 2000).

The differentiation of *Lasiodiplodia* species relies on the General Concordance Phylogenetic Species Recognition (GCPSR) by analyses of multigene phylogenies proposed by Taylor et al. 2000. For a long time *Lasiodiplodia* genus, was based only on *Lasiodiplodia theobromae* due to identification only by morphology (Alves et al. 2008). The application of GCPSR in *Lasiodiplodia* allowed the identification of high number of cryptic species that relies inside this genus (Alves et al. 2008; Marques et al. 2013). *Lasiodiplodia plurivora* was the first *Lasiodiplodia* species to be described containing very identical morphological characters with *L. theobromae* but different sequence data in a multi-locus analysis using the combination of internal transcribed spacer

(ITS) region and Translation Elongation Factor 1-alpha (*tef1-α*) (Damm et al., 2007). Shortly after, Alves et al. (2008) in a multi-locus analysis of a collection of isolates identified as *L. theobromae,* using the combination of the same loci, described *L. parva* and *L. pseudotheobromae* as the first cryptic species in *Lasiodiplodia*.

Apart from cryptic species, hybridization between species was also shown to occur in the genus *Lasiodiplodia* (Cruywagen et al. 2017). In general, fungal individuals of different species should have an isolated reproduction (Taylor et al. 2000), but there is an increasing of evidence that fungi are capable of out-cross and effectively reproduce to produce hybrids. Cruywagen et al. (2017) using a multi locus analysis describes *L. brasiliense*, *L. laeliocattleyae, L. missouriana* and *L. viticola* as hybrid species.

Thus far more than 50 species have been described in *Lasiodiplodia* on the basis of morphological characters combined with sequences from ITS region, *tef1-α* and tub2 (Rodríguez-Gálvez et al. 2017). Although the multi-locus analysis with the previously mentioned molecular markers (ITS and *tef1-α*) has been proved to delimit a large number of species it contains some shortcomings. The analysis of these molecular markers, many times, delimits two or more species that represent isolates of a single species, reminding that Intra-variability among individuals of the same species is common, and few nucleotide differences can occur within isolates of the same species. Doing so, it would be of extreme importance to study alternative loci with higher resolving power.

A good example of another type of phylogenetic markers that already has been used in the *Botryosphaeriaceae* (*Diplodia, Neofusicoccum*) with better results than these conventional markers are the mating type (*MAT*) genes (Lopes et al. 2017; Lopes et al. 2018).

Mating type loci and *MAT* genes

Mating type (*MAT*) genes play important roles in the biology and evolution of fungi (Bihon et al. 2014; Lopes et al. 2018) . From determining the mating compatibility to meiosis and cell differentiation, *MAT* genes are the master regulators of sex in fungi (Nagel et al. 2018; Thynne et al. 2017). These genes encode proteins that typically possess DNA binding domains, where the same proteins act as transcription factors that regulate the expression of genes related to sex (Bihon et al. 2014; Lopes et al. 2018).

These mating type genes, control the sexual reproduction in *Lasiodiplodia* and are located in the *MAT* locus (Bihon et al. 2014; Nagel et al. 2018). This *MAT* locus contains two alternate forms (or idiomorphs – different allelic variants of the same gene) *MAT1-1* and *MAT1-2.* The *MAT1-1* idiomorph is defined as containing the *MAT1-1-1* gene that possesses an alpha box protein domain. Otherwise, The *MAT1-2* idiomorph contains the *MAT1-2-1* gene that codes for a protein from the high mobility group (HMG) domain. In addition to these two main genes, other *MAT* genes have been located in *Lasiodiplodia MAT* locus such as *MAT1-1-8* in *MAT1-1* idiomorph, and *MAT1-2-5* in *MAT1-2* idiomorph (Bihon et al. 2014; Ni et al. 2010).

A high number of fungi are known to be sexual organisms, containing diverse patterns of sexual recombination, which occur throughout the phyla (Galagan et al. 2005; Ni et al. 2010). Sexual reproduction in fungi includes similar steps to sexual reproduction in multicellular eukaryotes. For instance, the first step requires two compatible mating partners that can recognise each other and undergo cell fusion (plasmogamy) (Ni et al. 2010). In Ascomycota, and subsequently in *Lasiodiplodia*, these compatible mating partners are controlled by the presence, of *MAT* genes in the genome. There are two main sexual strategies in fungi based on the presence of these genes, homothallism and heterothallism (Lopes et al. 2017; Thynne et al. 2017).

Both strategies have advantages, heterothallic species are characterized by individuals containing only one of the idiomorphs (either *MAT1-1* or *MAT1-2*) present in the *MAT* locus and requires an individual of the opposite idiomorph for sexual reproduction to be possible (Bihon et al. 2014; Ni et al. 2010). Heterothallism is better suited to situations where the probability of encounter a

viable mating partner is high and the fitness cost for selfing is unworthy (Lopes et al. 2017). Otherwise to heterothallism, homothallic species are self-compatible containing both *MAT1-1* and *MAT1-2* idiomorphs present in the *MAT* locus, by other means homothallism is characterized by indistinct mating (Nieuwenhuis et al. 2016). There are another two types of homothallic reproduction, pseudo-homothallism and secondary homothallism. Secondary homothallism is usually observed in yeasts (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) where the homothallic behaviour results from mating type switching and the presence of three *MAT* loci in the same genome, one is active and the other two are silent (Haber 2012). Pseudo-homothallic species are in fact true heterothallic individuals, they equally need 2 genetically-compatible partners so sexual reproduction can occur, the main difference is that pseudo-homothallic species can produce self-fertile mycelium in which the 2 different nuclei carrying the compatible mating types are present (Grognet et al. 2015).

MAT gene sequences have been used as phylogenetic marker inside *Botryosphariaceae*. These phylogenetic markers are a high valuable population genetic tools for studying the presence or absence of sexual reproduction in plant pathogenic fungi (Lopes et al. 2017; Lopes et al. 2018; Nagel et al. 2018). Two studies performed on the genera *Neofusicoccum* and *Diplodia* showed that phylogenetic analysis based on *MAT* genes sequence had superior resolution in species delimitation, then the combination of internal transcribed spacer (ITS) region of the rDNA cluster with translation Elongation Factor 1-alpha (*tef1-α)* and beta-tubulin (β-tubulin) (Lopes et al. 2017; Lopes et al. 2018). Due to their fast evolution rates, high interspecific variation and low dissimilarity within species, *MAT* genes could be a great successor for phylogenetic analysis of closely related species, giving better resolution than conventional markers as previously mentioned (Lopes et al. 2018). The breakthrough on technology and DNA-based techniques, like next generation genome sequencing, allowed the identification of different *MAT* loci in the available genomes of *Neofusicoccum, Diplodia, Lasiodiplodia, Botryosphaeria* (Nagel et al. 2018) (Fig. 2 – *MAT* locus of *Lasiodiplodia theobromae).*

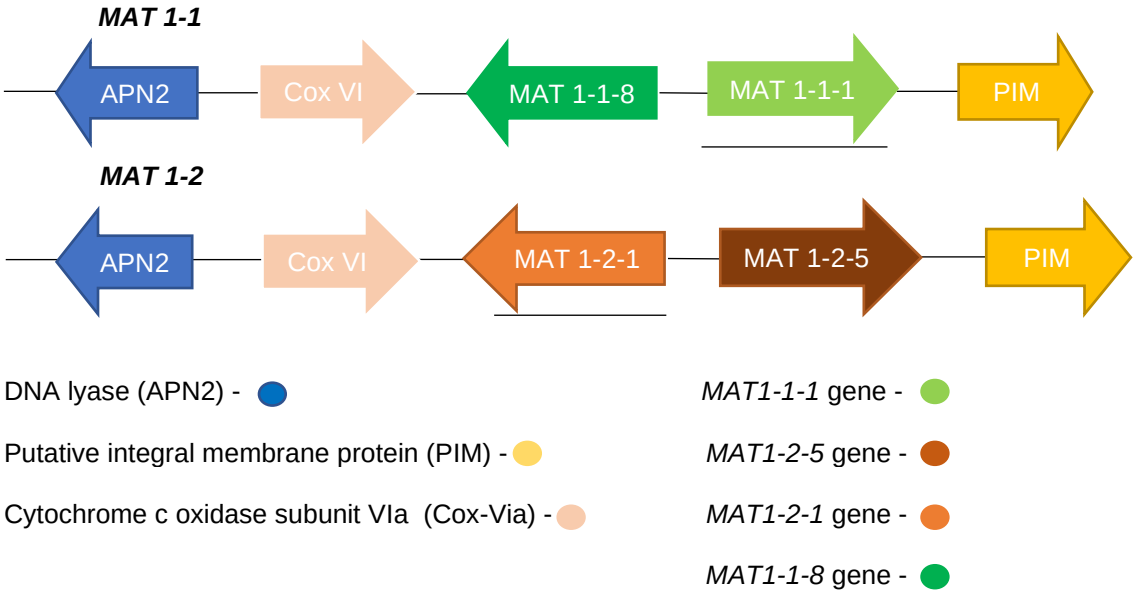

Figure 2- Structure of the mating type locus of the heterothallic species *Lasiodiplodia theobromae* CMW40942 for *MAT1-1* and *Lasiodiplodia theobromae* CSS001 for *MAT1-2*. Arrows indicate gene orientation (5'-3'). Image adapted from Nagel et al .2018). MAT 1-2-1 and MAT 1-1-1 genes are highlighted with a black line beneath them.

MAT genes have been sequenced, not only to investigate their reproduction mode, but also to resolve phylogenetic history and species boundaries (Nagel et al. 2018). The analysis of these genes has lead to a whole new idea on how asexual fungi reproduce, due to, apparently asexual fungi were found containing these genes (Ni et al. 2010). Studies on thes genes also provide knowledge on how plant pathogens reproduce and what is their frequency, which is relevant to epidemiology and control of plant diseases (responsible for significant losses to the agricultural, horticultural and forestry industries globally) (Coutinho et al. 2017). Knowledge obtained from genetic analysis of plant pathogen populations may be relevant for breeding and fungicide resistance management programs (Marques et al. 2013).

Doing so, the main objective of this study was to determine if *MAT* genes can be used as phylogenetic markers in species delimitation in the genus *Lasiodiplodia*. Additionally, it was aimed to develop a "universal" set of primers capable of amplifying the *MAT* region in all *Lasiodiplodia* species and to infer about their mating strategy.

Methods and Materials

Fungal strains used

A total of 41 fungal isolates representing 20 species were used in this study.

Table 1 - Fungal species and isolates used in this study.

Species	Isolate nr	GenBank accession nr	
		ITS	*tef1- α*
L. gonubiensis	CBS 115812	DQ458892.1	DQ458877.1
L. gonubiensis	CBS 116355	AY639594.1	DQ103567.1
L. venezuelensis	LASID3	KU507480	KU507447
L. crassispora	CBS 110492	EF622066.1	EF622066.1
L. crassispora	CBS 118741	DQ103550	EU673303
L. rubropurpurea	CBS 118740	DQ103553	DQ103571
L. parva	CBS 494.78	EF622084.1	EF622064.1
L. parva	CBS 495.78	EF622085.1	EF622065.1
L. parva	CBS 456.78	EF622083.1	EF622063.1
L. parva	CBS 356.78	EF622082.1	EF622062.1
L. mediterranea	B6	EF622082.1	EF622062.1
L. mediterranea	B4	KP178596.1	KP178599.1
L. mediterranea	B5	KP178594.1	KP178597.1
L. mediterranea	CBS 124060	KP178595.1	KP178598.1
L. mediterranea	CAA012	HQ607897.1	KU695584.1
L. plurivora	STE-U 4583	KU578250.1	KU695584.1
L. brasiliensis	LAYAP1	EF445362.1	EF445396.1
L. iraniensis	RB31	KU507473	KU507440
L. hormozganesis	CBS 339.90	KY052971.1	KY024641.1
L. laeliocattleayae	LACICI	EF622072.1	EF622052.2
L. laeliocattleayae	LAREPI	KU507462	KU507428
L. laeliocattleayae	CBS167.28	KU507484	KU507451
L. euphorbicola	Bot 32	KU507487	KU507454
L. euphorbicola	Bot 33	*	
L. pontae	Lasio A	*	
L. pontae	Lasio B	*	
L. gravistriata	Bot 55	*	
L. jatrophicola	Bot 27	*	
L. jatrophicola	Bot 157	*	
L. caatinguensis	Bot 22	*	
Lasiodiplodia sp.	LACAM1	*	
L. theobromae	CSS001	KU507469	KU507436
L. theobromae	CBS 164.96	MDYX00000000	

* - sequences that have not been deposit in GenBank database

Table 1 – Fungal species and isolates used in this study **(continuation)**

Species	Isolate nr	GenBank accession nr	
		ITS	*tef1- α*
L. pseudotheobromae	CBS 304.79	NR_111174.1	
L. pseudotheobromae	CBS 374.54	EU101311.1	EU101355.1
L. pseudotheobromae	CBS 447.62	MH858207.1	EU673255.1
L. pseudotheobromae	CBS 116459	EF622077.1	EF622057.1
L. exigua	BL 104	KJ638317.1	KJ638336.1
L. exigua	BL 184	KJ638318.1	KJ638337.1
L. exigua	BL 185	KJ638319.1	KJ638338.1
L. exigua	BL 186	KJ638320.1	KJ638339.1

* - sequences that have not been deposit in GenBank database

Analyses of *Lasiodiplodia gonubiensis, L. theobromae and L. pseudotheobromae MAT* sequences

Currently there are available in GenBank public data base, three *MAT* loci from *Lasiodiplodia gonubiensis* CBS 115812 (KX787887), *L. theobromae* CBS 164.96 (type strain - KX787889) and *L. pseudotheobromae* CBS116459 (KX787888) (fig 3 – a), c), d)) and the first set of primers to amplify *MAT1-1-1* and *MAT1-2-1* genes were designed based on them. Another *MAT* sequence from *L. theobromae* CSS001 was used (fig 3 – b), but only the genome was available (MDYX00000000). The genome was checked for the presence of both *MAT* genes (*MAT1-1-1* and *MAT1-2-1*), flanking regions were predicted with the FGENESH tool (Solovyev et al. 2006) in the BLAST software package (www.softberry.com) using *MAT* sequences from *L. gonubiensis,* the only homothallic species available. The analysis of *L. gonubiensis, L. theobromae and L. pseudotheobromae MAT* loci has been done previously (Fig. 3) (Nagel et al. 2018). *MAT1-1-1* and *MAT1-2-1* were the only genes amplify with the specific set of primers, in spite of *Lasiodiplodia MAT* loci contains other *MAT* genes like *MAT1-1-8* for *MAT1-1* idiomorph and *MAT1-2-5* for *MAT1-2* idiomorph.

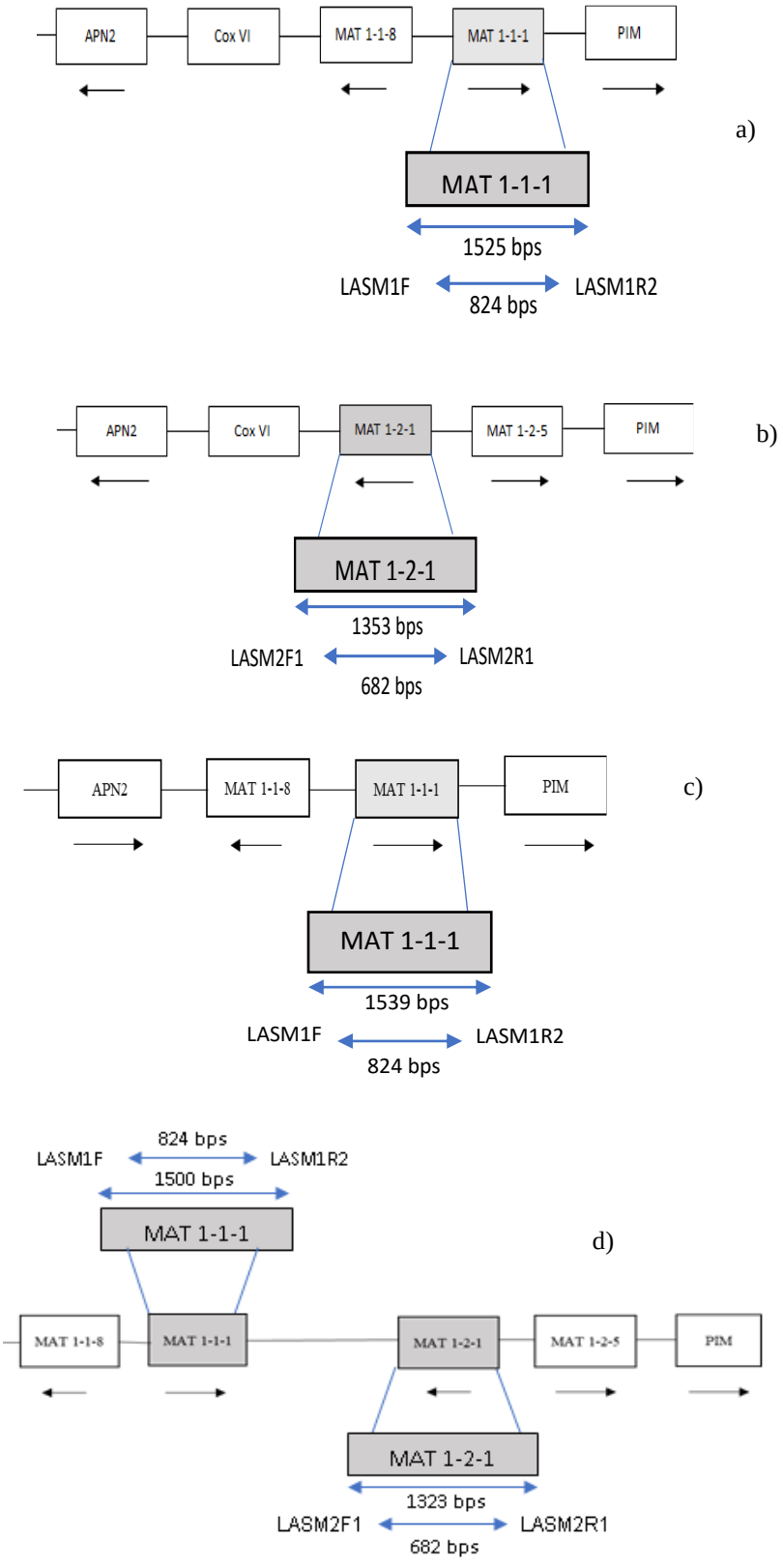

Figure 3 - Structure of the mating type locus of a) *Lasiodiplodia theobromae* CBS 164.96, b) *L. theobromae* CSS01, c) *L. pseudotheobromae* CBS 116459, d) *L. gonubiensis* CBS 115812. Arrows below the genes indicate genes orientation (5'-3'). Primers used to amplify partial *MAT* genes are noted with respective fragment lengths. Distances and sizes are not drawn to a scale. Adapted from Nagel et al., 2018.

Primer design, DNA extraction and PCR amplification

The first set of primers used for amplification of the *MAT1-1-1* and *MAT1-2-1* genes (Table 2 - a) *MAT1-1-1* primers, LASM1F and LASM1R2; b) MAT 1-2-1 primers LASM2F1 and LASM2R1) have been previously designed (Nagel et al. 2018) based on the *MAT* sequences mentioned above. Since the first set was not able to amplify *MAT genes* for all isolates, a second set of primers was designed based on the new *MAT* sequences obtained with the amplification using the first set.

Both sets were designed with the help of OligoCalc: Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html) and Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/pcr_primer_stats.html). where characteristics such as length, GC content, melting temperature, potential haairpin formation, complementarity and potential self-annealing sites were automatically checked with the softwares. These were synthesized by STAB Vida Lda (Lisbon, Portugal), reconstituted and diluted (10 pmol) in Tris-EDTA buffer, and stored at -20°C.

Genomic DNA was extracted from fresh mycelium grown on half-strength PDA plates for 5 days at approximately 23°C, according to Alves et al. 2004. All PCR reactions were carried out in 25 µL (1 µL DNA, 1 µL of each primer, forward and reverse) reaction mixtures with NZYTaq 2 × Green Master Mix (2.5 mM MgCl2; 200 mM dNTPs; 0.2 U/µL DNA polymerase) (Lisbon, Portugal), in a Bio-Rad C-1000 Touch™ Thermal Cycler (Hercules, CA, USA). Negative controls with sterile water instead of template DNA were used in every PCR reaction.

Thermal conditions for primers of *MAT1-1-1* gene were denaturation at 95 ºC for 3 min; 35 cycles at 94 ºC for 30 s, 52 ºC for 30 s, and 72 ºC for 1 min 10 s; final extension at 72 ºC for 10 min. For *MAT1-2-1* amplification the set of primers (table 2 – b)) were used with the following thermal conditions: denaturation at 95 ºC for 3 min; 35 cycles at 94 ºC for 30 s, 52 ºC for 30 s, and 72 ºC for 1 min 10 s; final extension at 72 ºC for 10 min. Both thermal conditions were used in all set of primers.

After amplification, 2 µL of each PCR product were separated by electrophoresis in 1.5 % agarose gels at 90 V for 1 h in 1 × TAE buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0). A GeneRuler DNA Ladder Mix (Thermo Scientific, USA) was also included. Gels were stained with ethidium bromide and visualized on a BioRad Molecular Imager Gel Doc™ XR+ to assess PCR amplification. The amplified PCR fragments were purified with the DNA Clean & Concentrator™-5 kit (Zymo Research, CA, USA) before sequencing.

Both strands of the PCR products were sequenced at GATC Biotech (Cologne, Germany). The nucleotide sequences were read with FinchTV v.1.4.0 (Geospiza Inc. http://www.geospiza.com/finchtv). All sequences were checked manually, and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. The primers used for amplification of *MAT* genes are listed in Table 2.

Table 2 - Primers used in PCR amplification, a) *MAT1-1-1* primers, LASM1F and LASM1R2 are the first set of primers based on *L. gonubiensis*, *L. theobromae* and *L. pseudotheobromae MAT* sequences; Lasio_MAT1_356F and Lasio_MAT1_1023R are the set of primers based on *MAT* sequences that were amplified with the first set. b) *MAT1-2-1* primers, LASM2F1 and LASM2R1 are the first set of primers based on *L. gonubiensis*, *L. theobromae* and *L. pseudotheobromae MAT* sequences; Lasio_MAT2_734F and Lasio_MAT2_1246R are the set of primers based on *MAT* sequences that were amplified with the first set.

Locus	***Primer name***	***Primer sequence 3' 5'***	**Reference**
MAT 1-1-1 a)	LASM1F	AACTGCTTCGTTGCCTTCC	Nagel et al. 2018
	LASM1R2	TTGACCCTGACCTGTCTAC	Nagel et al. 2018
	Lasio_MAT1_356F	TCGACAAGTTCCAGCAGAAG	This study
	Lasio_MAT1_1023R	GGTGTCKTGAGTCAGCTTGT	This study
MAT 1-2-1 b)	LASM2F1	ACCGCAGGGACAACCAC	Nagel et al. 2018
	LASM2R1	CTGCTTCCCAGCGCAAC	Nagel et al. 2018
	Lasio_MAT2_734F	CAGATCATTGGCGCGATGTGG	This study
	Lasio_MAT2_1246R	GTGAGAGCTGTTGAAGAGC	This study

Phylogenetic analysis

Sequences were aligned with ClustalX v. 2.1 (Thompson et al. 1997), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25 %). Alignments were checked and edited with BioEdit Alignment Editor v. 7.2.5 (series and 1999 n.d.). Phylogenetic analyses of sequence data were performed with MEGA7 v. 7.026 (Tamura et al. 2012), all gaps were included. The model of DNA sequence evolution to be applied in each analysis was determined by the software. Maximum Likelihood (ML) analysis was performed on a Neighbour-Joining (NJ) starting tree automatically generated by the software. The bootstrap values (1000 replicates) were analyzed to estimate the consistency of each node of the trees.

Phylogenetic informativeness of the loci used in phylogenetic analyses

Profiles of phylogenetic informativeness for each locus were obtained using PhyDesign (Townsend 2007). This allows a comparison of the different loci through calculation of the informativeness per base pair as well as net phylogenetic informativeness, which takes into account sequence length. Since the species are in majority putative heterothallic, comparisons between each *MAT* gene and the other loci used in phylogenetic analyses were done separately and comparisons between *MAT1-1-1* and *MAT1-2*-1 were therefore not possible.

Results:

***Mating type genes* primers test**

Nagel et al. (2018) had already studied the *MAT* locus of *Lasiodiplodia gonubiensis* CBS 115812, *L. theobromae* CBS 164.96, *L. theobromae* CSS001 and *L. pseudotheobromae* CBS 116459, which facilitated the first part of this work, where specific primers were design to amplify *MAT1-1-1* and *MAT1-2-1* genes and these were the first set of primers tested in this work. (Table 2 - a) *MAT1-1-1* primers, LASM1F and LASM1R2; b) *MAT 1-2-1* primers LASM2F1 and LASM2R1)

This initial set of primers was then tested in all 41 isolates of *Lasiodiplodia* (Table 3), for both *MAT1-1-1* and *MAT1-2-1* genes. Every time a PCR product generates an amplicon, the products were stored for later sequencing. Fifteen isolates amplified for *MAT1-2-1* gene and eleven for *MAT 1-1-1*, for the isolates *L. venezuelensis, L. parva* (all isolates)*, L. mediterranea* B5, *L. laeliocattleayae* (LACIC1 and LAREP1), *L. brasiliensis* LAYAP1*, L. pseudotheobromae CBS447.62, L. exígua* (all isolates), *L. rubropurpurea* CBS 118740*, L. pontae* LASIO B and *L. gravistriata* Bot 55 the primers were not able to generate an amplicon.

Since these primers did not raise an amplicon for the isolates previously mentioned, a second set of primers was designed with basis on the *MAT* sequences obtained by amplification and sequencing using the first set (Table 2 – a) *MAT1-1-1* Lasio_MAT1_356F and Lasio_MAT1_1023R; b) *MAT1-2-1* LASIO_MAT2_734F and LASIO_MAT2_1246R). The new primers were submitted to same procedure as the first set, where they were tested in all *Lasiodiplodia* isolates (Table 4) and later sequenced. *L. venezuelensis L. parva, L. mediterranea* B5*, L. brasiliensis* LAYAP1*, L. laeliocattleayae* (LACIC1 and LAREP1)*, L. pseudotheobromae* CBS 447.62*, L. pontae Lasio B, L. rubropurpurea* CBS 118740 and *L. gravistriata* Bot 55 did not raise an amplicon with the second set, so combinations between the two sets was performed in order to achieve a “Universal” set capable of amplify *MAT* gene sequences for all *Lasiodiplodia* species.

The first combination tested in this study was with forward primer from the first set and reverse from the second set, for both *MAT1-1-1* and *MAT1-2-1*

genes (Table 5). This combination was tested on the isolates that did not generate an amplicon using the first and second primers set, *L. venezuelensis, L. parva, L. mediterranea* B5*, L. brasiliensis* LAYAP1*, L. laeliocattleayae* (LACIC1 and LAREP1)*, L. pseudotheobromae* CBS 447.62*, L. pontae, Lasio B, L. rubropurpurea* CBS 118740 and *L. gravistriata* Bot 55 plus other isolates that already amplified with the two first sets as a positive control to make sure that the combination of primers were working. This procedure was made in order to achieve an "universal" set of primers capable of amplify both *MAT* genes in all *Lasiodiplodia* species.

Lastly, a second combination with forward primer from the second set and reverse from the first, was used (Table 6) with the same objective as the first combination. This combination was the most successful among our isolates, nine isolates amplify for *MAT1-1-1* and eighteen for *MAT1-2-1* (Table 6). Even though this combination amplifies for most of the isolates including *L. brazieliensis* LAYAP1, *L. pseudotheobromae and L. mediterranea* B5, there were still six isolates that did not generate an amplicon namely *L. venezuelensis, L. gravistriata* Bot 55, *L. rubropurpurea* CBS 118740, *L. pontae Lasio B, L. parva* CBS 494.78 and *L. jatrophicola* Bot 157

A "universal" set of primers capable of amplifying both *MAT* genes in all *Lasiodiplodia* species was not possible to reach, yet the combination with forward primer from the first set and reverse from the second, works for most of the isolates.

Table 3 – First set of primers represented as well all results from PCR amplification from these primers. These set was designed based on three *MAT* gene sequences namely, *L. gonubiensis*, *L. theobromae* and *L. pseudotheobromae.*

		1st Set of primers	
		MAT 1-1-1	*MAT 1-2-1*
Species	**Isolate nr**	LASM1F x LASM1R2	LASM2F1 x LASM2R1
L. gonubiensis	CBS 115812	✓	✓
L. gonubiensis	CBS 116355	✓	✓
L. venezuelensis	LASID3	×	×
L. crassispora	CBS 110492	×	✓
L. crassispora	CBS 118741	✓	×
L. rubropurpurea	CBS 118740	×	×
L. parva	CBS 494.78	×	×
L. parva	CBS 495.78	×	×
L. parva	CBS 456.78	×	×
L. parva	CBS356.78	×	×
L. mediterranea	B6	×	✓
L. mediterranea	B4	×	✓
L. mediterranea	B5	×	×
L. mediterranea	CBS 124060	✓	×
L. mediterranea	CAA012	✓	×
L. plurivora	STE-U 4583	×	✓
L. brasiliensis	LAYAP1	×	×
L. iraniensis	RB31	×	✓
L. hormozganesis	CBS 339.90	×	✓
L. laeliocattleayae	LACICI	×	×
L. laeliocattleayae	LAREPI	×	×
L. laeliocattleayae	CBS 167.28	✓	×
L. euphorbicola	Bot 32	×	✓
L. euphorbicola	Bot 33	×	✓
L. pontae	Lasio A	×	✓
L. pontae	Lasio B	×	×
L. jatrophicola	Bot 27	×	✓
L. jatrophicola	Bot 157	✓	×
L. gravistriata	Bot 55	×	×
L. caatinguensis	Bot 22	✓	×
Lasiodiplodia sp.	LACAM1	×	✓

✓- Primer generate an amplicon

X – Primer did not generate amplicon

Table 3 – First set of primers represented as well all results from PCR amplification from these primers. These set was designed based on three *MAT* gene sequences namely, *L. gonubiensis*, *L. theobromae* and *L. pseudotheobromae* (continuation).

		1st Set of primers	
		MAT 1-1-1	*MAT 1-2-1*
Species	**Isolate nr**	LASM1F x LASM1R2	LASM2F1 x LASM2R1
L. theobromae	CSS001	×	✓
L. theobromae	CBS 164.96	✓	×
L. pseudotheobromae	CBS 304.79	✓	×
L. pseudotheobromae	CBS 374.54	×	✓
L. pseudotheobromae	CBS 447.62	×	×
L. pseudotheobromae	CBS 116459	✓	×
L. exigua	BL 104	×	×
L. exigua	BL 184	×	×
L. exigua	BL 185	×	×
L. exigua	BL 186	×	×

✓- Primer generate an amplicon

X – Primer did not generate amplicon

Table 4 - Second set of primers represented as well all results from PCR amplification from these primers. These primers were designed based on the *MAT* sequences amplified with the first set.

		2nd Set of primers	
		MAT 1-1-1	*MAT 1-2-1*
Species	**Isolate nr**	Lasio_MAT1_356F x Lasio_MAT1_1023R	Lasio_MAT2_734F x Lasio_MAT2_1246R
L. gonubiensis	CBS 115812	✓	✓
L. gonubiensis	CBS 116355	✓	✓
L. venezuelensis	LASID3	×	×
L. crassispora	CBS 110492	×	✓
L. crassispora	CBS 118741	✓	×
L. rubropurpurea	CBS 118740	×	×
L. parva	CBS 494.78	×	×
L. parva	CBS 495.78	×	×
L. parva	CBS 456.78	×	×
L. parva	CBS 356.78	×	×

✓- Primer generate an amplicon

X – Primer did not generate amplicon

Table 4 - Second set of primers represented as well all results from PCR amplification from these primers. These primers were designed based on the *MAT* sequences amplified with the first set. (Continuation)

		2nd Set of primers	
		MAT 1-1-1	*MAT 1-2-1*
Species	**Isolate nr**	Lasio_MAT1_356F x Lasio_MAT1_1023R	Lasio_MAT2_734F x Lasio_MAT2_1246R
L. mediterranea	B6	×	✓
L. mediterranea	B4	×	✓
L. mediterranea	B5	×	×
L. mediterranea	CBS 124060	✓	×
L. mediterranea	CAA012	✓	×
L. plurivora	STE-U 4583	×	✓
L. brasiliensis	LAYAP1	×	×
L. iraniensis	RB31	×	✓
L. hormozganesis	CBS 339.90	×	✓
L. laeliocattleayae	LACICI	×	×
L. laeliocattleayae	LAREPI	×	×
L. laeliocattleayae	CBS 167.28	✓	×
L. euphorbicola	Bot 32	×	✓
L. euphorbicola	Bot 33	×	✓
L. pontae	Lasio A	×	✓
L. pontae	Lasio B	×	×
L. gravistriata	Bot 55	×	×
L. jatrophicola	Bot 27	×	✓
L. jatrophicola	Bot 157	✓	×
L. caatinguensis	Bot 22	✓	×
Lasiodiplodia sp.	LACAM1	×	✓
L. theobromae	CSS001	×	✓
L. theobromae	CBS 164.96	✓	×
L. pseudotheobromae	CBS 304.79	✓	×
L. pseudotheobromae	CBS 374.54	×	✓
L. pseudotheobromae	CBS 447.62	×	×
L. pseudotheobromae	CBS 116459	✓	×
L. exigua	BL 104	×	✓
L. exigua	BL 184	×	✓
L. exigua	BL 185	×	✓
L. exigua	BL 186	×	✓

✓- Primer generate an amplicon

X – Primer did not generate an amplicon

Table 5 – Combinations between the two set of primers, for *MAT1-1-1* forward from the first set and reverse from the second, for *MAT1-2-1* the same. The remaining species were not tested with these combinations of primers.

		1st Combination of primers	
		MAT 1-1-1	*MAT 1-2-1*
Species	**Isolate nr**	LASM1F X Lasio_MAT1_1023R	LASM2F1 X Lasio_MAT2_1246R
L. venezuelensis	LASID3	×	×
L. crassispora	CBS 118741	×	×
L. parva	CBS 356.59	×	✓
L. rubropurpurea	CBS 118740	×	×
L. gravistriata	Bot 55	×	×
L. mediterranea	B6	×	✓
L. mediterranea	B4	×	✓
L. mediterranea	B5	×	✓
L. brasiliensis	LAYAP1	×	×
L. iraniensis	RB31	×	×
L. hormozganesis	CBS 339.90	×	✓
L. laeliocattleayae	LACICI	×	×
L. laeliocattleayae	LAREPI	×	×
L. pontae	Lasio B	×	×
L. jatrophicola	Bot 157	×	×
L. caatinguensis	Bot 22	✓	×
L. theobromae	CBS 164.96	×	×
L. pseudotheobromae	CBS 304.79	✓	×
L. pseudotheobromae	CBS 447.62	×	×
L. exigua	BL 185	×	✓

✓- Primer generate an amplicon

X – Primer did not generate an amplicon

Table 6 – Combinations between the two set of primers, for *MAT1-1-1* forward from the first set and reverse from the second, for *MAT1-2-1* the same. The remaining species were not tested with these combinations of primers.

		2nd Combination of primers	
		MAT 1-1-1	*MAT 1-2-1*
Species	**Isolate nr**	Lasio_MAT1_356F x LASM1R2	Lasio_MAT2_734F X LASM2R1
L. gonubiensis	CBS 115812	✓	✓
L. venezuelensis	LASID3	×	×
L. crassispora	CBS 110492	×	✓
L. crassispora	CBS 118741	✓	×
L. rubropurpurea	CBS 118740	×	×
L. parva	CBS 494.78	×	×
L. parva	CBS 495.78	×	✓
L. parva	CBS 456.78	×	✓
L. mediterranea	B6	×	✓
L. mediterranea	B4	×	✓
L. mediterranea	B5	×	✓
L. mediterranea	CBS 124060	✓	×
L. mediterranea	CAA012	✓	×
L. plurivora	STE-U 4583	×	✓
L. brasiliensis	LAYAP1	✓	×
L. iraniensis	RB31	×	✓
L. hormozganesis	CBS 339.90	×	✓
L. laeliocattleayae	LACICI	×	✓
L. laeliocattleayae	LAREPI	×	✓
L. laeliocattleayae	CBS 167.28	✓	×
L. euphorbicola	Bot 32	×	✓
L. pontae	Lasio A	×	✓
L. pontae	Lasio B	×	×
L. gravistriata	Bot 55	×	×
L. jatrophicola	Bot 27	×	✓
L. jatrophicola	Bot 157	×	×
L. caatinguensis	Bot 22	✓	×
Lasiodiplodia sp.	LACAM1	×	✓
L. theobromae	CBS 164.96	✓	×
L. pseudotheobromae	CBS 304.79	✓	×
L. pseudotheobromae	CBS 447.62	×	✓
L. exigua	BL 185	×	✓

✓- Primer generate an amplicon

X – Primer did not generate an amplicon

Mating strategies in *Lasiodiplodia*

After testing all primers and obtaining the *MAT* gene sequences from the isolates that generate an amplicon, it was possible to infer about *Lasiodiplodia* mating strategy. The presence of the different idiomorph in each genome is going to determine if the species are either homothallic or heterothallic (Nagel et al. 2018).

From the initial 41 isolates, it was only possible to obtain 37 *MAT* gene sequences*. L. pontae* Lasio B, *L. gravistriata* Bot 55, *L. rubropurpurea* CBS 118739*, L. venezuelensis* LASID3 did not generate an amplicon with any primers and combinations tested and by consequent it was not possible to obtain the *MAT* gene sequences for these isolates, meaning that it was not possible to infer about their mating strategy.

Heterothallism was the predominant mating strategy. In the 36 isolates, representing 18 species of *Lasiodiplodia*, only *Lasiodiplodia gonubiensis* was confirmed to be homothallic (table 7) (Nagel et al. 2018) due to the presence of both idiomorphs, *MAT1-1* and *MAT1-2* in the genome.

L. crassispora, *L. mediterranea*, *L. laeliocattleyae*, *L. jatrophicola*, *L. theobromae* and *L. pseudotheobromae,* species were considered to be heterothallic, due to the fact that in all isolates, there was at least two isolates, one containing the *MAT1-1-1* gene and another containing *MAT1-2-1 gene.*

For *L. parva*, *L. plurivora*, *L. brasiliensis*, *L. iraniensis*, *L. hormozganensis*, *L. euphorbicola*, *L. caatinguensis*, *Lasiodiplodia* sp., *L. exigua* and *L. pontae,* only one of both idiomorphs was found in the genome (either *MAT1-1-1 or MAT1-2-1*), and due to this, these species were considered to be putative heterothallic.

Table 7 – Mating strategy of all *Lasiodiplodia* species studied, as well as the correspondent gene that amplified either set of primers.

Species	**Isolate nr**	**Mating strategy**	***MAT* Gene**	
			MAT1-1	*MAT1-2*
L. gonubiensis	CBS 115812	Homothallic	*MAT1-1-1 ; MAT1-2-1*	
L. gonubiensis	CBS 116355	Homothallic	*MAT1-1-1 ; MAT1-2-1*	
L. crassispora	CBS 110492	Heterothallic		*MAT1-2-1*
L. crassispora	CBS 118741	Heterothallic	*MAT1-1-1*	
L. parva	CBS 494.78	Heterothallic*		*MAT1-2-1*
L. parva	CBS 495.78	Heterothallic*		*MAT1-2-1*
L. parva	CBS 456.78	Heterothallic*		*MAT1-2-1*
L. mediterranea	B6	Heterothallic		*MAT1-2-1*
L. mediterranea	B4	Heterothallic		*MAT1-2-1*
L. mediterranea	B5	Heterothallic		*MAT1-2-1*
L. mediterranea	CBS 124060	Heterothallic	*MAT1-1-1*	
L. mediterranea	CAA012	Heterothallic	*MAT 1-1-1*	
L. plurivora	STE-U 4583	Heterothallic*		*MAT 1-2-1*
L. brasiliensis	LAYAP1	Heterothallic*	*MAT 1-1-1*	
L. iraniensis	RB31	Heterothallic*		*MAT 1-2-1*
L. hormozganesis	CBS 339.90	Heterothallic*		*MAT 1-2-1*
L. laeliocattleayae	LACICI	Heterothallic		*MAT 1-2-1*
L. laeliocattleayae	LAREPI	Heterothallic		*MAT 1-2-1*
L. laeliocattleayae	CBS167.28	Heterothallic	*MAT 1-1-1*	
L. euphorbicola	Bot 32	Heterothallic*		*MAT 1-2-1*
L. euphorbicola	Bot 33	Heterothallic*		*MAT 1-2-1*
L. pontae	Lasio A	Heterothallic*		*MAT 1-2-1*
L. jatrophicola	Bot 27	Heterothallic		*MAT 1-2-1*
L. jatrophicola	Bot 157	Heterothallic	*MAT 1-1-1*	
L. caatinguensis	Bot 22	Heterothallic*	*MAT 1-1-1*	
Lasiodiplodia sp.	LACAM1	Heterothallic*		*MAT 1-2-1*
L. theobromae	CSS001	Heterothallic		*MAT 1-2-1*
L. theobromae	CBS 164.96	Heterothallic	*MAT 1-1-1*	
L. pseudotheobromae	CBS 304.79	Heterothallic	*MAT 1-1-1*	
L. pseudotheobromae	CBS 374.54	Heterothallic		*MAT 1-2-1*
L. pseudotheobromae	CBS 447.62	Heterothallic		*MAT 1-2-1*
L. pseudotheobromae	CBS 116459	Heterothallic	*MAT 1-1-1*	
L. exigua	BL 104	Heterothallic*		*MAT 1-2-1*
L. exigua	BL 184	Heterothallic*		*MAT 1-2-1*
L. exigua	BL 185	Heterothallic*		*MAT 1-2-1*
L. exigua	BL 186	Heterothallic*		*MAT 1-2-1*

*- Putative Heterothallic species (species only amplified one of the two idiomorphs)

Phylogenetic Analysis

Whit the obtained *MA*T sequences, from the 37 isolates that did generate an amplicon with the previous sets of primers, it was possible perform a phylogenetic analysis using MEGA7 v. 7.026. According to Taylor et al. (2000), phylogenetic species recognition can avoid the incongruence of determining the limits of a species by relying on the concordance of more than one gene genealogy, so a phylogenetic analysis of the combined internal transcribed spacer (ITS) region of the rDNA cluster with translation Elongation Factor 1-alpha (*tef1-α)* was also performed for comparison.

Phylogenetic relationships based on the combined sequence data from ITS-*tef1-α* was inferred, using the Maximum Likelihood method based on the ***Kimura-2 parameter***. The analysis of ITS-*tef1-α* phylogenetic tree differentiate fifteen clades plus another one cointaing *Lasiodiplodia sp.* LACAM1, a possible hybrid described by Rodrígues-Gálvez et al. (2016) (Figure 4). The clade containing *L. theobromae* CSS 01s, *L. theobromae* CBS 164.96 and *L. pontae* Lasio A can bring doubt about the delimitaion due to the proximity on the tree and the low bootstrap values.

Phylogenetic relationships of *Lasiodiplodia* species based on the sequence data from *MAT1-1-1* gene sequences was inferred using the method Maximum Likelihood based on the ***Tamura-3 parameter***. Only ten isolates amplified for *MAT1-1-1* gene, and by the analysis of *MAT1-1-1* phylogenetic tree, these form six consistent clades with strong bootstrap values, that represents nine *Lasiodiplodia* species. Most species were clearly distinguished by *MAT1-1-1* phylogenetic tree analysis*. L. theobromae* CBS 164.96, *L. brasiliensis* LAYAP1 and *L. laeliocattleyae* formed a clade suggesting to be a single species*. L. jatrophicola* Bot 157 and *L. pseudotheobromae* CBS 116459 also formed a clade that suggest that could be to a single species.

Phylogenetic relationships of *Lasiodiplodia* species based on the sequence data from *MAT1-2-1* gene sequences was inferred using the method Maximum Likelihood based on the ***Tamura-3 parameter***. *MAT1-2-1* phylogeny reveals eight well supported clades, *L. parva* (all isolates) *L. euphorbicola* (all isolates), *Lasiodiplodia sp.* LACAM1 formed a single clade with high bootstrap values. *MAT1-2-1* phylogeny also groups *L. jatrophicola* Bot 27 with *L.*

pseudotheobromae CBS 44762 showing congruence with *MAT1-1-1* phylogeny. The same thing occurs in the clade containing *L. laeliocattleyae* LAREP1, *L. pontae* Lasio A, *L. theobromae* CSS01s and *L. laeliocattleyae* LACIC1, where *MAT1-1-1* phylogeny also groups *L. laeliocattleyae* and *L. theobromae* together.

In both *MAT* phylogenies there was clearly congruence between the trees and the bootstrap values are clearly higher than the combined dataset of the regions ITS rDNA and *tef1-α*,

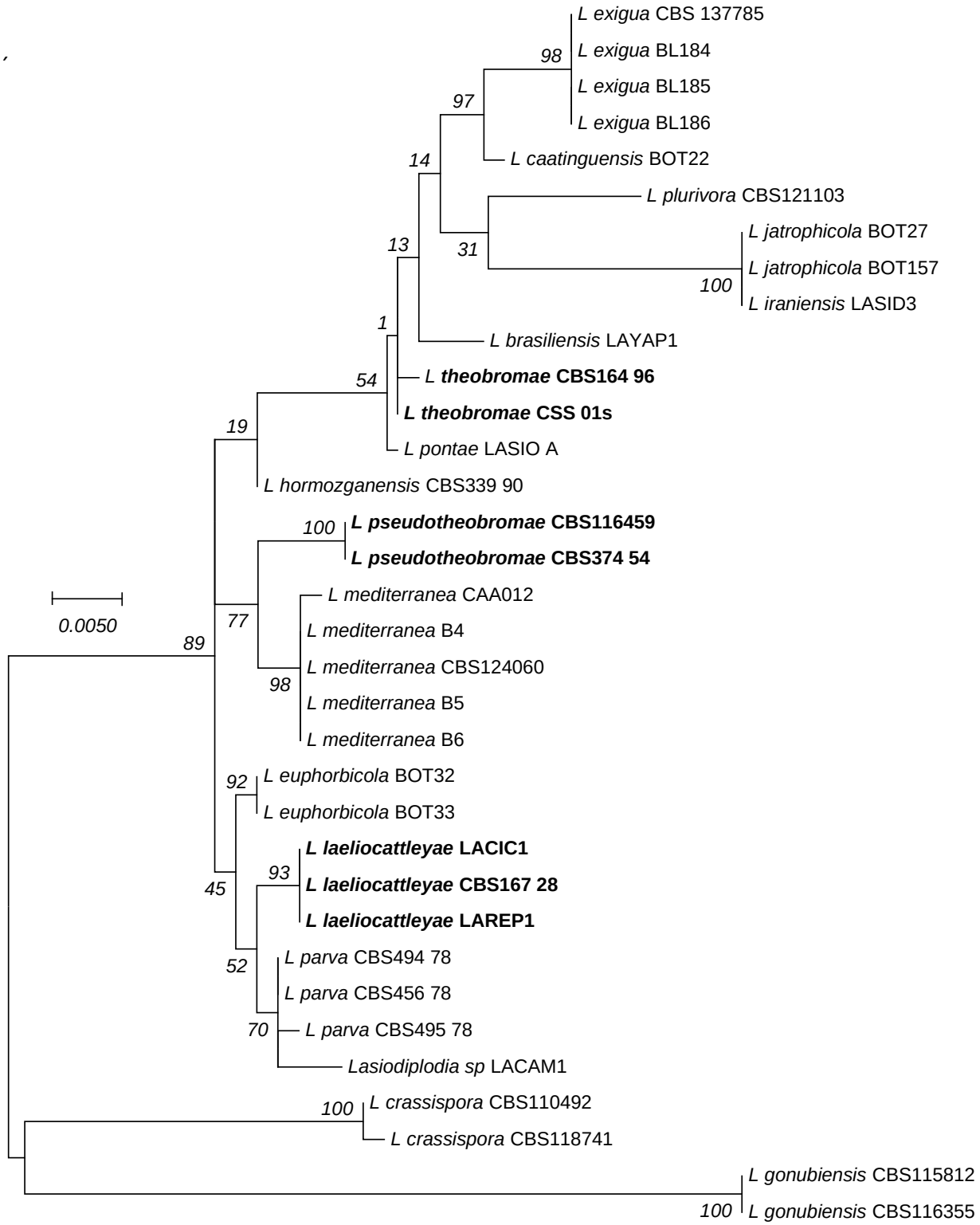

Figure 4 – Phylogenetic relationships of *Lasiodiplodia* isolates based on the combined sequence data from ITS and *tef1-α.* The method used was Maximum Likelihood based on the Kimura 2-parameter model.

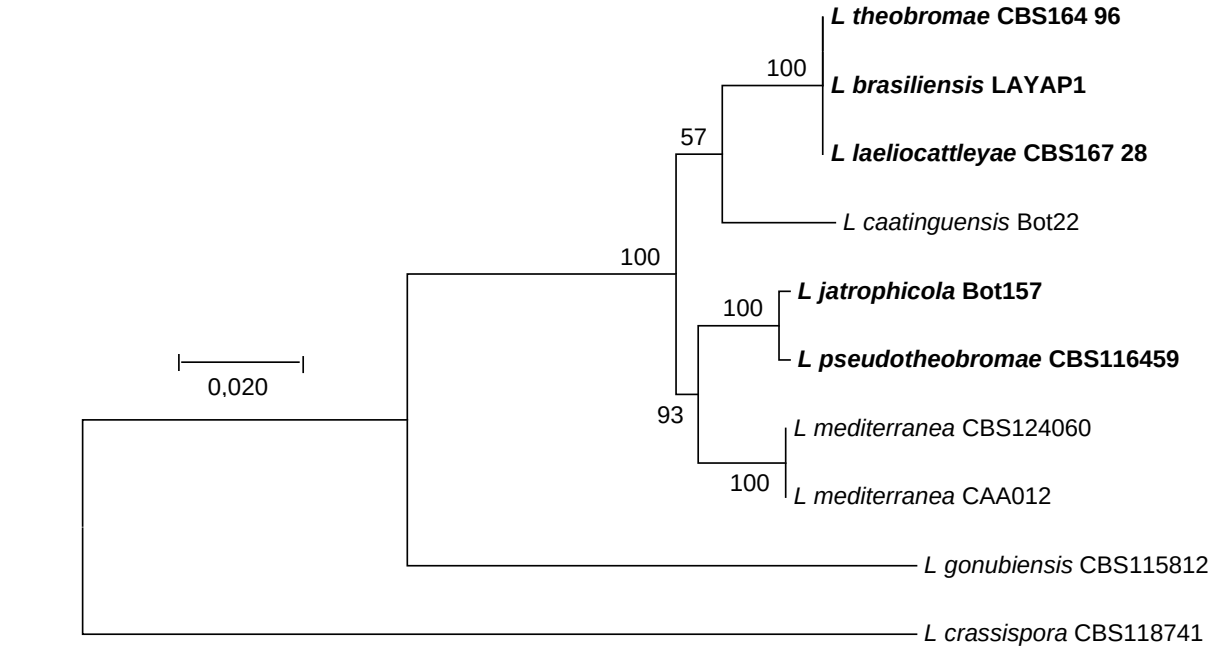

Figure 5 – Phylogenetic relationship of *Lasiodiplodia* species based on the sequence data from *MAT1-1*-1 gene sequences. The method used was Maximum Likelihood based on the *Tamura-3 parameter*.

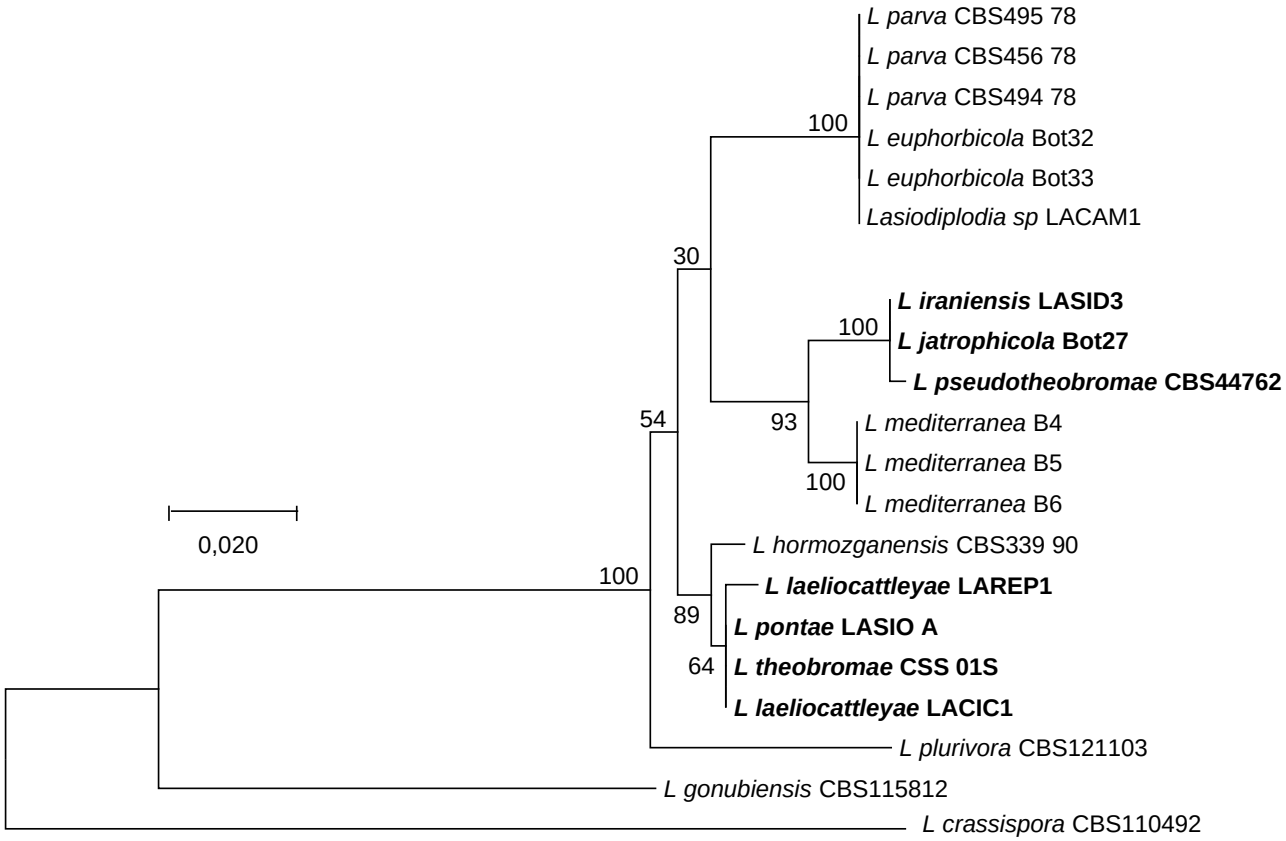

Figure 6 - Phylogenetic relationships of *Lasiodiplodia* species based on the sequence data from *MAT1-2-1* gene sequence. The method used was Maximum Likelihood based on the *Tamura-3 parameter*.

Phylogenetic informativeness of the loci used in the phylogenetic analyses

With the analysis of figures 7 and 8, it is clear that *MAT* genes are the highest contributors to both net and per-site phylogenetic informativeness from all loci studied. On the other hand, ITS sequences are the least informative to resolve *Lasiodiplodia* species (Fig. 7 – a); Fig. 8 – a)).

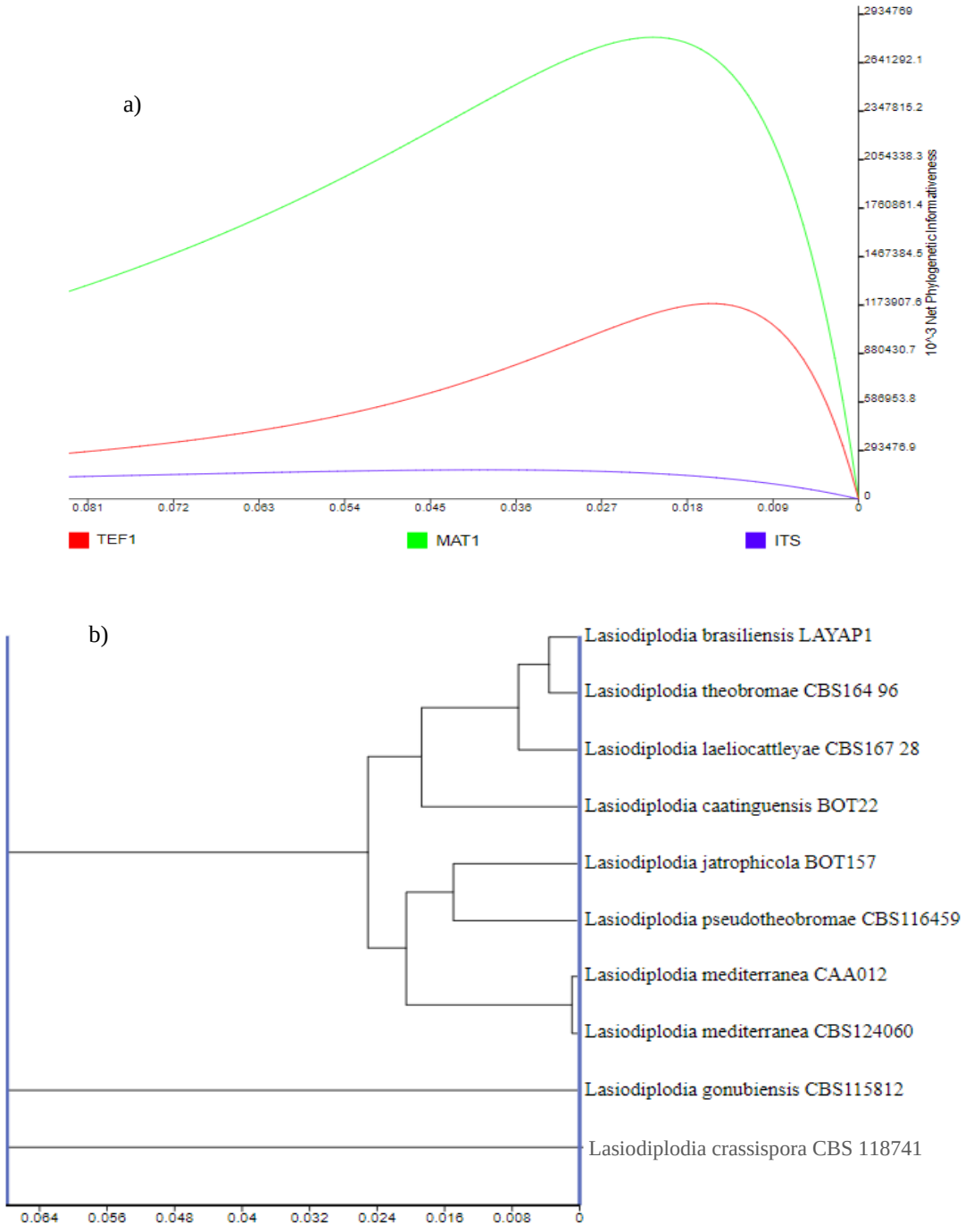

Figure 7 – Profiles of phylogenetic informativeness for the loci ITS, *tef1-α*, *MAT1-1-1.* a) graphic representing time vs phylogenetic informativeness of the tree loci; b) phylogenetic tree representing the evolutive scale across time.

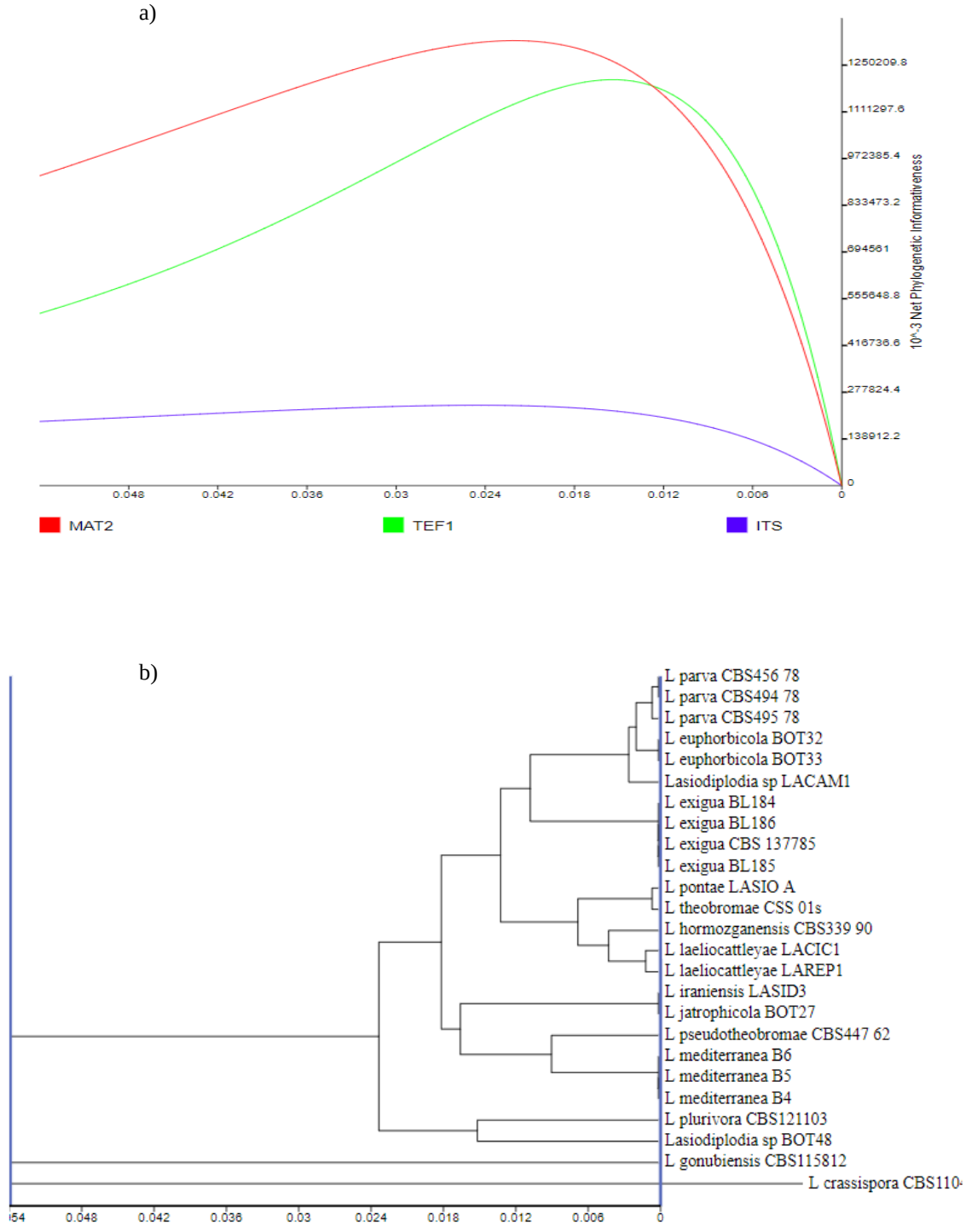

Figure 8 - Profiles of phylogenetic informativeness for the loci ITS, *tef1-α*, *MAT1-2-1.* a) graphic representing time vs phylogenetic informativeness of the tree loci; b) phylogenetic tree representing the evolutive scale across time.

Discussion

It was fundamental for this study the availability of specific primers for *MAT1-1-1* and *MAT1-2-1 genes*, previously design by Nagel et al. (2018) with basis on sequences from *Lasiodiplodia gonubiensis* CBS 115812 (KX787887), *L. theobromae* CBS 164.96 (type strain - KX787889) *L. theobromae* CSS001 and *L. pseudotheobromae* CBS 116459 (KX787888). The study of *Lasiodiplodia MAT* locus was also performed by Nagel et al. (2018) showing the existence of other *MAT* genes in this locus, *MAT1-1-8* for the *MAT1-1* idiomorph and *MAT1-2-5* for *MAT1-2* idiomorph. These genes have been reported in other genus in the same family (Lopes et. al 2017; Lopes et al. 2018; Nagel et al. 2018), but the main targets of this study were *MAT1-1-1* and *MAT1-2-1*, since these have been proved to be good phylogenetic markers inside *Botryosphaeriaceae (*Bihon et al. 2014; Lopes et al. 2017; Lopes et al. 2018; Nagel et al. 2018;).

The primers designed by Nagel et al. (2018) were the first set test in our study. From all forty-one isolates only twenty-six generated an amplicon, sixteen for *MAT1-2-1* and eleven for *MAT1-1-1*. The obtained results allowed us to sequencing the *MAT* genes of the twenty-seven isolates, and redesign the primers in order to obtain a “universal” set capable of amplify all isolates. The second set of primers was able to amplify 30 isolates but unfortunately, neither set of primers were capable of amplify all *Lasiodiplodia* isolates, and even with different combinations between them this achievement was not possible. The fact that neither set of primers or combinations could amplify all *MAT* genes can be seen as a consequence of the enormous variability that relies among different species (Taylor et al. 2000; Nagel et al. 2018). *MAT* genes are semi-conserved which means that there is a high variability among different species, but quite conserved among isolates of the same species (Lopes et al. 2018). From all the primers used, including combinations, the better results were with combination Lasio_MAT1_356F x LASM1R2 for *MAT1-1-1* and Lasio_MAT2_734F x LASM2R1 for *MAT1-2-1,* but still *for L. rubropurpurea* CBS 118740, *L. pontae* Lasio B, *L. gravistriata* Bot 55, *L. parva* CBS 356.59, *L. venezuelensis* LASID3, *L. crassispora* CBS 118741 an amplicon was not generated.

Similar to what has been reported for the genus *Neofusicoccum* and *Diplodia* (Lopes et al. 2017; Lopes et al. 2018), *MAT* gene sequence alignments from *Lasiodiplodia* are more similar in the internal regions of the genes than the

regions closer to the end. Sequences of both genes, among isolates of the same species are almost identical.

When it comes to mating strategies, apart from *L. gonubiensis* (the only homothallic species found), all the species studied were found to be heterothallic. The same has been described by Lopes et al. (2018) in *Diplodia,* a close related genus member of *Botryosphaeriaceae*. These majority of heterothallism as mating strategy is an important detail with implications on the genetic variability and evolution of species (Lopes et al. 2018). According to Nagel et al. (2018), the evolution of thallism inside *Botryosphaeriaceae* can be explained by two hypotheses, the first requires a homothallic ancestor and two independent deletion events, where the *MAT* genes are a leftover of a deletion event involved in shift from homothallism to heterothallism. The second hypothesis requires and heterothallic ancestor and at least two unequal recombination events, where the mating type genes appear as a result of recombination between idiomorphs in heterothallic species. The most reported in *Botryospheriaceae* is the shift from heterothallism to homothallism, making the heterothallism the ancestral state. In this study we cannot draw any precise conclusions regarding *Lasiodiplodia* ancestral sate, we can only conclude that heterothallism is the most common state among our isolates.

Working on heterothallic species using *MAT* genes for phylogeny can be harsh, due to putative heterothallic species. When all isolates from a species, contains only the same idiomorph (either *MAT1-1-1 or MAT1-2-1),* that species is defined as putative heterothallic. This means that, a species that is considered to be heterothallic can actually be homothallic and the specific primers were not able to amplify the other idiomorph. This is why is so important to have several isolates from the same species with both idiomorphs, the big limitation is that most of the time only one isolate is available, making uncertainties about the mating strategies. On this study, 9 species were considered to be putative heterothallic species, namely, *L. venezuelenzis, L. plurivora, L. brasiliensis, L. iraniensis, L. hormozganensis, L. euphorbicola, L. caatinguensis, L. exigua* and *L. pontae* as previously said, we cannot draw any conclusions about their mating strategies and a phylogenetic comparison between the two idiomorphs on these species is also not possible since only one idiomorph is available.

The sequences of the *MAT* genes obtained with the specific primers allowed us to perform a phylogenetic analysis. The Genealogical Concordance Phylogenetic Species Recognition (GCPSR) concept proposed by Taylor et al. (2000) was applied, where comparison of the phylogeny of *MAT* genes with ITS and *tef1-α* regions was accomplished. The GCPSR relies on determining the concordance between multiple gene genealogies and delimiting species by the branches of multiple trees displaying congruence (Taylor et. al. 2000; hybridization). The obtained phylogenies, driven from the *MAT* sequences revealed congruence between them, this was consistent with the support from other studies suggesting that phylogenies of mating type genes are generally consistent.

On the *ITS-tef1-α, L. iraniensis* and *L. jatrophicola* appear in the same clade with high bootstrap value. According to Rodrígues-Gálvez et al. (2016), *L. jatrophicola* is considered a synonym of *L. iraniensis*, and with basis on the present results we agree that they are the same species.

A possible hybrid was evaluated too. *Lasiodiplodia sp*. LACAM1 was described by Rodrígues-Gálvez et al. (2016) where morphologically, it closely resembled with *L. parva* and in the combined *ITS-tef1-α* phylogenetic analysis it was placed between *L. citricola* and *L. parva.* In this study, due to the limitation of only one isolate of *Lasiodiplodia sp*. LACAM1 and only one parental species are available (*L. parva*), few can be concluded about the hybrid, we can only point out that *Lasiodiplodia sp*. LACAM1 appears as a close related species to *L. parva* in the analysis of the combined *ITS-tef1-α* region and in the *MAT 1-2-1* phylogeny.

When comparing the *MAT* phylogenies with the *ITS-tef1-α* few incongruences shows up, analysing the clade containing *L. laeliocattleyae* on both *MAT* gene trees, its notable that, in both trees this species is aggregated with *L. theobromae* suggesting that can be the same species, in the phylogenetic tree used with *ITS* and *tef1-α* the resolution was different where *L. laeliocattleyae* appears to be a single species apart from *L. theobromae.* By nucleotide analysis of the *ITS* region of those two specific species isolates, it is clear that the differences are few (table 7) , reminding that in phylogenetic analyses, minor genetic variations between isolates of the same species are common due to intraspecific variability it is possible that these two species are actually the same (Cruywagen et al. 2017). The same happens with *L. jatrophicola and L.*

pseudotheobromae where they appear aggregate in both MAT phylogenies but separated in the combined *ITS-tef1-α* and by analysis of the nucleotide sequences of these species in the *ITS* region the differences are also few (table 8). Recombination among individuals of the same species is common which can lead to a conflict between multi-gene genealogies. According to Taylor et al. 2000 the transition from concordance to conflict determines the species boundaries, which suggest that actually *L. theobromae*, *L. laeliocattleyae* and *L.jatrophicola*, *L. pseudotheobromae* could be two single species.

Table 8 – Nucleotide differences in ITS region alignment between *L. laeliocattleayae* LACIC1, *L. laeliocattleayae* LAREP1, *L. laeliocattleayae* CBS516728, *L. theobromae* CBS 16496, *L. theobromae* CSS01s

	Location (bp)												
Species	**550**	**551**	**552**	**553**	**554**	**555**	**571**	**572**	**577**	**578**	**579**	**581**	**582**
L. laelliocattleae LACIC1	T	G	A	A	G	A	T	T	A	A	C	C	C
L. laelliocattleae LAREP1	T	G	A	A	G	A	T	T	A	A	C	C	C
L. laelliocattleae CBS 167.28	T	G	A	A	G	A	T	T	A	A	C	C	C
L. theobromae CSS001	G	T	G	C	A	C	A	A	C	C	T	G	A
L. theobromae CBS 164.96	G	T	G	C	A	C	A	A	T	C	T	G	A
L. brasiliensis LAYAP1	G	T	G	C	A	C	A	A	T	C	T	G	A
L. pontae LASIO A	G	T	G	C	A	C	A	A	T	C	T	G	A

Table 9 – Nucleotide differences in ITS region alignment between *L. pseudotheobromae* CBS116459, *L pseudotheobromae* CBS44762, *L jatrophicola* Bot 157, *L jatrophicola* Bot 27.

	Location (bp)												
Species	**550**	**551**	**552**	**553**	**554**	**555**	**571**	**572**	**577**	**578**	**579**	**581**	**582**
L. pseudotheobromae CBS116459	T	G	A	A	G	A	T	T	A	A	C	C	C
L pseudotheobromae CBS44762	T	G	A	A	G	A	T	T	A	A	C	C	C
L jatrophicola Bot157	G	T	G	C	A	C	A	A	C	C	-	G	A
L jatrophicola Bot27	G	T	G	C	A	C	A	A	C	C	-	G	A

Also, the test for phylogenetic informativeness showed that both *MAT* genes are highly effective in opposition to *ITS* which is the least effective. Thus, in the resolution of *Lasiodiplodia s*pecies we strongly recommend the use of *MAT*

genes due to their high resolving power as well their high net and per-site phylogenetic informativeness.

Some studies have point out that *MAT1-2-1* could be a better phylogenetic marker (Amorim et al. 2017; Lopes et al. 2018) but, in this study, that conclusion could not be afford it, as mentioned above, the existence of putative heterothallic species does not allow a full comparison between *MAT1-1-1* and *MAT1-2-1* genes. Nonetheless, based on the individual results of each *MAT* phylogeny, both idiomorphs had equal efficacy in resolving species. In spite of both *MAT* genes had good results in PCR amplification, *MAT1-2-1* contains a larger number of species that had been evaluated than *MAT1-1-1,* which suggest that this gene is a more reliable phylogenetic marker when it comes to species differentiation.

Conclusions

All the results accomplished here are a mark in the knowledge about *MAT* genes in the genus *Lasiodiplodia* as well as their true power as phylogenetic markers. The PCR-based assay developed here will be an important tool to assess and score the mating types of this genus in a fast and more reliable way.

The lack of availability of *MAT gene* sequences on any database from other isolates of *Lasiodiplodia* is a big limitation that compromises several results like mating strategy or phylogenetic analyses. Due to this issue, a full comparison between *MAT* phylogenies from all isolates was not possible, however this study shows that the individual phylogenies of *MAT genes* presented a higher resolution power when compared to the *ITS* - *tef1-α* which makes *MAT* gene analysis a good approach for accurate and reliable species differentiation within the genus *Lasiodiplodia*, either alone or in combination with other loci such as the *tef1-α.*

To our knowledge this is the second study on *Lasiodiplodia* using *MAT* genes as phylogenetic markers. The data provided here will contribute to the increase knowledge of *MAT* genes, as well as, the mating strategy inside *Lasiodiplodia* which can facilitate future studies

Future Perspectives

It would be of extreme importance that in the future more information about *MAT* gene flanking regions would be gather and increase the analyses of *MAT loci* in other species of *Lasiodiplodia.* This future knowledge will provide information to better understand the species delimitation based on these genes and also to better understand putative heterothallics and confirm their true mating strategy.

Future studies should focus on spreading these knowledges to more genus inside *Botryosphaeraceae* in order to get the more information possible across this family to see if the same genes present the same congruence in all *Botryosphaeraceae* genus. All the information about *MAT* genes and thallism should be then deposited on a viable database.

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