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Endophytic *Phomopsis* Strains from Leaves of *Guarea guidonia* (Meliaceae).

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ABSTRACT.—The genus *Phomopsis* has been long recognized as harboring high levels of character variability. Species delimitation is confusing since nomenclature is mainly based on host affiliation. Our purpose was to compare grouping of fungal endophytes according to morphological and molecular data. We studied endophytic populations of *Phomopsis* isolated from leaves of a tropical timber tree (*Guarea guidonia*: Meliaceae), from two sites within the Luquillo region (Puerto Rico). Individuals were grouped into morphotypes according to traditional morphological and culture criteria (color, colony morphology, and growth rate). Five morphotypes emerged according to this grouping, a high number of *Phomopsis* isolates per host. Morphological and culture data were compared with molecular data obtained from restriction digests of the Internal Transcribed Spacer (ITS) region of the nuclear ribosomal DNA. Partial agreement between morphological and molecular data was found: terminal clades were the same with both methods. However, trees based on restriction patterns grouped together individuals phenotypically different (assigned to different morphotypes), and resulting clades were composed of a mixture of different morphotypes from both study sites. The ITS of one isolate was sequenced and by a BLAST search shown to be related to *P. oryzae* and *Diaporthe phaseolorum*. Both morphological and ITS restriction site data were highly variable, and further studies are needed to identifying a taxonomic character, either molecular or morphological, useful for the entire group.

Keywords.—Endophytes, endophytic fungi, *Guarea guidonia*, ITS, morphotypes, *Phomopsis*, rDNA.

INTRODUCTION

Species of *Phomopsis* are widespread plant pathogens and endophytes (Boddy and Griffith 1989). *Phomopsis* has been reported in both cultivated plants such as mango (*Mangifera indica*), papaya (*Carica papaya*), avocado (*Persea americana*) (Ploetz et al. 1994), coconut (*Cocos nucifera*) (Mariano et al. 1997), blueberry and cranberry (Farr et al. 2002), and *Stylosanthes* sp. (Pereira et al.1993), and in wild plants in the families *Sapotaceae* (Lodge et al. 1996, Bayman et al. 1998), *Ericaceae* (Bills et al. 1992, Okane et al. 1998), *Arecaceae* (Rodrigues 1994), and *Meliaceae* (Gamboa 1998; Gamboa and Bayman 2001). *Phomopsis* has traditionally been considered highly pathogenic (Rehner and Uecker 1994), but some *Phomopsis* species are thought to be mutualistic endophytes (Webber and Gibbs 1984; Carroll 1986).

Species of *Phomopsis* produce interesting secondary metabolites, which include mycotoxins that affect the nervous system of vertebrates (Bills et al. 1992), and some toxic alkaloids with pharmacological potential, such as phomopsins (Cockrum et al. 1994).

The coelomycete genus *Phomopsis* corresponds to the imperfect state of *Diaporthe* (*Pyrenomycetes*: *Ascomycotina*). Although the generic circumscription is good, species delimitation is difficult due to character plasticity. Problems include lack of production of β-conidia and teleomorphs by many species (Rehner and Uecker 1994), and morphological and growth rate variation within and among isolates (Parmeter 1958; Rehner and Uecker 1994). Species delimitation in *Phomopsis* based on morphological and cultural characters has been shown to be inadequate (van der Aa et al. 1990).

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In the past, species names in *Phomopsis* were based on the host plant name. Given that some species and isolates can infect more than one host (Brayford 1990; Farr et al. 2002), this strategy is currently questioned (Rehner and Uecker 1994; Says-Lesage 2002). In addition to morphological and cultural characters used to identify species and isolates, some authors have used alternative characters which include virulence (Vidic 1991), proteins and serology (Lee and Snow 1992; Velicheti et al. 1991), ITS sequences (Zhang et al. 1998) and enzymes and secondary metabolites (Shivas et al. 1991). Unfortunately, no character applicable to the entire group has emerged (Rehner and Uecker 1994). Some 800 valid specific epithets of *Phomopsis* have been published (Uecker 1988).

The ubiquity and importance of *Phomopsis* both as an endophyte and pathogen, its ability to produce physiologically active secondary metabolites, and its confusing taxonomy constitute strong stimuli to study this group. Here we compare the morphology and genetic variation of the ITS region of endophytic *Phomopsis* from leaves of a tropical timber tree from two sites within the Luquillo region in Puerto Rico. We try to determine if there is agreement between cultured and morphological versus molecular characters for grouping individuals.

Materials and Methods

Isolation of fungi

Colonies of *Phomopsis* were isolated from trees of *Guarea guidonia* (*Meliaceae*), from opposite sides of the Luquillo mountains, Puerto Rico, at El Verde (EV) and Las Piedras (LP). Leaves were collected during the morning and processed the same day. They were washed in 0.01% Tween20 and surface-sterilized in ethanol 75% (1 min), commercial bleach 10% (3 min; 0.5% sodium hypoclorite), and ethanol 75% (30 s), then cut into 2x5 cm pieces and placed in petri dishes containing potato dextrose agar (PDA, Difco® potato dextrose broth, 12 g/L and agar 20 g/L with 50 ppm each penicillin, streptomycin and tetracycline added after autoclaving) (Gamboa and Bayman 2001). *Phomopsis* isolates were transferred individually to petri dishes and after clean, pure cultures were obtained they were transferred to PDA, and grown in a chamber at a 12 h light/dark cycle at 20-22 °C.

Isolates of *Phomopsis* were assigned to morphotypes according to three morphological traits: color, colony morphology and velocity of growth (Table 1, columns 2-4). Other measurements were diameter of the glomeruli at their widest point, hyphal width, setae length, and length and width of alpha and beta conidia (Table 1, columns 5-9) (Rosskopf et al. 2000). At least five measurements were taken for each character. These data were used in a principal component analysis (Table 3).

DNA extraction, amplification and sequencing

Total DNA was extracted from mycelia grown in PD broth and lyophilized, using phenol:chloroform extraction and ethanol precipitation (Lee and Taylor 1990). The ITS region was amplified using primers ITS1 and ITS4 (White et al. 1990). The ITS of isolate *P*1 was sequenced in both directions on a LICOR automatic sequencer. The most similar sequences in GenBank were identified by BLAST searches.

Restriction analysis

PCR products were cut with the restriction enzymes *Hae*III, *Msp*I, *Rsa*I, *Taq*I, and restriction bands were visualized with ethidium bromide in 2% agarose gels. A presence/absence matrix was constructed for each band (available upon request), and putative relationships among individuals were estimated by using PAUP (Swofford 1985). A distance matrix for the two populations of *Phomopsis* morphotypes 1 (EV and LP) was based on Euclidean Distance (Krebs 1989) from the presence/absence matrix, and this distance matrix was used for constructing a Euclidean distance tree.

Results

Morphology in culture

Phomopsis was characterized by dark pycnidia at the periphery of the culture, of-

ten with long, black setae up to 5 mm, and abundant comma-shaped β-conidia, and ovoid α-conidia, immersed in a white-creamy liquid (see Sutton 1980). Mycelium was sparse, often white-yellowish, sometimes brown, wrinkled in appearance, and septate. Antagonism between colonies (as evidenced by pigmented zones of interaction) was common. Abundant dark pigments were deposited on the bottom of the plate. Twenty-three individuals were isolated.

Isolates were assigned to morphotypes based on culture characters as used in current literature (Sutton 1980; Rosskopf et al. 2000), including color, mycelium colony morphology, and growth rate. Color and colony morphology were recorded from mature colonies (1-2 months old). Growth rate was estimated as time to reach the periphery of the petri dish (r=45 mm). The fastest isolates took less than two weeks and the slowest ones more than four weeks (Table 1). T1

Five morphotypes were recognized based on colony color, colony morphology and growth rate (Table 2). Nevertheless, the other morphometric characters were very variable and were not useful for grouping isolates into morphotypes, either singly or combined (Table 1, columns 5-9). From the principal components analysis, it is clear that 80% of variance is found within the first four axes (vectors) (Table 3). T2 T3

DNA amplification and sequencing

The ITS of most isolates was about 590, 600 or 620 bp. One individual, *Phomopsis* 18 (*P*. 18), had a 720 bp band. BLAST searches showed the ITS sequence of isolate *P*. 1 was most similar to *Diaporthe phaseolorum* strain AK25A from soybean (AF 001025), and *Phomopsis oryzae* (Rosskopf *et al*. 2000, AF 079777). ITS sequence of isolate *P*. 1 is at GenBank (accession number AY940425).

Restriction analysi

Very few individuals were identical in restriction fragment profiles (e.g. *P*. 1 and *P*. 3). Although the relative position of some clades was constant among trees, including trees made after character sorting and change in order of data entry (data not shown), the bootstrap consensus tree based on restriction fragment profiles (Fig. 1), did not support the morphotypes based on culture characteristics. F1

Agreement between molecular and morphological data in placing individuals under the same morphotypes was constant for some isolates, i.e. *P*. 1, *P*. 3, and *P*. 9; *P*. 28 and *P*. 29; *P*. 23, *P*. 24 and *P*. 25. However morphotype 1 had individuals in different groupings (Fig. 1).

Analysis between populations of Phomopsis morphotype 1

This morphotype was the only one with enough individuals in both study sites (EV and LP), to allow a comparison between them. Phylogenetic analysis showed that there are two main clades (Fig. 2) in which a mixed set of individuals from both sites (EV and LP) occurred together, and these molecular data do not support the two populations of morphotype one (*P*. 1 to *P*. 7 and *P*. 8 to *P*. 14) as discrete entities. F2

DISCUSSION

Diversity of Phomopsis

Phomopsis is a very common endophytic fungal genus in tropical trees, and given the high character plasticity it harbors, systematics should be based on molecular characters as well as morphology (Rehner and Uecker 1994). About 70% of valid specific epithets of *Phomopsis* reflect the name of the host plant, and if we were to use this naming strategy at least four undescribed species would emerge from this study, since only one *Phomopsis* has been described as an endophyte of *G. guidonia* (Lodge et al. 1996). Restriction data support the existence of at least two sets of morphotype grouping that reasonably could be expected to correspond to biological entities with important genetic differences (Fig. 1). It is not possible to differentiate biological species with presented data, but it seems worth to study if these groups correspond to biological species.

TABLE 1. Morphological data for endophytic *Phomopsis* from *Guarea guidonia* leaves. Color key: 1 = yellow, 2 = dark yellow, 3 = white bone, 4 = bone, 5 = white, Colony morphology key: 1 = poppy, 2 = mucous, 3 = crustose, 4 = flat wrinkled, 5 = wrinkled.

Isolate	Morphotye	Color	Colony morpholog	Growth rate	Glomeruli diameter (μm)	Hyphase width (μm)	Filament length (mm)	α-Conidia (μm)	β-Conidia (μm)
P1	Sp1	1	1	Slow to medium	4.9 × 5.5-8.0 × 10.5	0.6-2.0	—	1 × 2-2 × 6	—
P2	Sp1	1	1	Slow to medium	4.3 × 4.9-11.7 × 14.2	0.6-2.0	5.0	—	0.4 × 18
P3	Sp1	1	1	Slow to medium	4.9 × 4.9-14.8 × 16.1	0.6-2.0	7.0	0.6 × 2-4 × 10	—
P6	Sp1	1	1	Slow to medium	6.8 × 6.8-7.4 × 8.0	2.0	4.0	0.6 × 2	1.2 × 16.2
P7	Sp1	1	1	Slow to medium	7.4 × 8.0-11.1 × 13.0	2.0	—	2 × 2-2 × 6	1.2 × 17.5
P8	Sp1	1	1	Slow to medium	24 × 26-100 × 108	2-4	—	2 × 5	0.6 × 14
P9	Sp1	1	1	Slow to medium	—	2.0	—	1 × 2-3.6 × 6	1 × 22
P13	Sp1	1	1	Slow to medium	4.9 × 4.9-11.7 × 12.4	0.18-0.31	4.0	1.2 × 1.8	0.6 × 18.7
P14	Sp1	1	1	Slow to medium	—	1.0-2.0	5.0	1 × 2-2 × 3	0.6 × 18
P15	Sp5	5	4	Slow	—	1.6-3.0	4.0	2 × 3	0.6 × 18
P17	Sp4	4	5	Medium	—	1.6-3	2.0	1 × 2-2 × 5	0.6 × 18
P18	Sp4	4	5	Medium	—	1.0-2.0	2.0	2 × 6	—
P19	Sp4	4	5	Medium	30 × 34-64 × 80	2.0	2.0	1 × 2-2 × 6	0.6 × 20
P20	Sp4	4	5	Medium	—	2.0	2.0	1 × 2-2 × 6	0.6 × 18
P21	Sp3	3	3	Medium to fast	50 × 62-84 × 100	1.6-2.4	4.0	2 × 6	0.4 × 20
P22	Sp3	3	3	Medium to fast	—	1.6-20	2.0	1 × 2	0.6 × 18
P23	Sp3	3	3	Medium to fast	26 × 38	1.8-3.8	2.0	2 × 4	0.6 × 20
P24	Sp3	3	3	Medium to fast	10 ×12-40 × 40	1.6-30	2.0-5.0	1 × 2	0.6 × 20
P25	Sp3	3	3	Medium to fast	—	1.8-3.8	2.0	1.6 × 4-2 × 6	0.6 × 20
P27	Sp2	2	2	Fast	—	1.6-2.0	1.0	2 × 6	0.6 × 14
P28	Sp2	2	2	Fast	24 × 26-52 × 66	1.6-20	—	1 × 1	—
P29	Sp2	2	2	Fast	—	1.6-3.0	—	1 × 1	—

TABLE 2. Number of individuals and location for each morphotypes of *Phomopsis*. sp1, . . . , 5 = morphotypes one throught five, EV = El Verde, LP = Las Piedras.

Phomopsis morphotypes	Number of individuals		
	EV	LP	Total
sp1	5	5	10
Sp2	2	1	3
sp3	4	1	5
sp4	4		4
sp5	1		1
Total	16	7	23

Since 80% of morphological variation is found within the first four eigenvalues, discussion about principal components analysis should be directed to their respective axes (vectors). Eigenvalues represent the variance along the principal axes (Sokal and Rohlf 1995), and factors with the highest absolute values within such axes are responsible for most of the observed variance. When a ponderate value is calculated for each factor (data not shown), color, colony morphology, growth rate, and hyphal width are the factors harboring most of the variance.

Given that values assigned to some factors were not quantitative (especially color and colony morphology), a principal components analysis was performed with morphometric characters only (columns 5 to 9 in Table 1). In this case characters such as area of α-conidia, filament length, and area β-conidia were the ones harboring most of the variance. We found no morphological characters that appear reliable for identifying species of the genus *Phomopsis*, and postulating/discarding these factors needs further works with well-established *Phomopsis* species. Unfortunately, there are few experts willing to identify *Phomopsis* isolates to species. Even a recent study that used DNA sequencing to group *Phomopsis* isolates did not identify any isolate to species (Rehner and Uecker 1994).

Length variation and sequence of the ITS region

The Internal Transcribed Spacer sequences of nuclear ribosomal DNA have been useful in reconstructing species relationships, both in plants (Hamby and Zimmer 1992; Sanderson and Doyle 1992), and fungi (Kohn 1992; Zambino and Szabo 1993; Hughes and Petersen 1998) especially at the interspecific and intergeneric levels. ITS can be used to identify species, as postulated for chantarelles which have particularly long ITSs (Feibelman et al. 1994). In this study the ITS region size was shown to be variable at the intrapopulation level and could be used as an additional character when studying confusing taxa.

The ITS sequence of *P*.1 was similar to two recognized species, both named according to the host they were found in (*Diaporthe phaseolorum* and *P. oryzae*). *Diaporthe phaseolorum* from soybean contains three subspecific taxa var. *caulivora*, var. *meridionalis* and var. *sojae* that constitute, along with *Phomopsis longicolla*, a plant-pathogenic species complex difficult to characterize (Fernández and Hanlin 1996; Zhang et al. 1998). Sequence similarity with our isolate is not an argument strong enough for assigning it to this complex.

Restriction analysis

The nucleotide sequence (Carbone and Kohn 1993; Rehner and Uecker 1994; Zhang et al. 1997; Jacobs and Rehner 1998), length (Feibelman et al. 1994), and restriction patterns (Hughes and Petersen 1998; Farmer and Sylvia 1998), of fungal ITS region were used to clarify relationships among species. Recently, an ITS sequence was used as a key character for naming a new species of *Phomopsis*, although it was not (phenotypically) very different from a named species (Rosskopf et al. 2000).

In our study, all clades with over 70% bootstrap support agree with morphological characters. However, morphotype 1 was divided amongst three separate groups, and in morphotypes 2 and 3, at least one isolate did not group with the others. Trees were very similar when changes were done in taxon order, character deletion and selection of outgroup. For both morphological and restriction data, the most unique isolate found was *P*. 18,

TABLE 3. Principal component analysis showing eigenvalues, vectors, and distribution of variance among vector/factor.

	Eigenvalues	Percent of variance	Cumulative percent of variance
1	2.41552	30.2	30.2
2	1.46236	18.3	48.5
3	1.31212	16.4	64.9
4	1.10582	13.8	78.7
5	0.81081	10.1	88.8
6	0.50423	6.3	95.1
7	0.35415	4.4	99.6

	Vectors						
Factor	1	2	3	4	5	6	7
α-Conidia area	0.0686	−0.0538	−0.4105	0.6268	0.6209	−0.0602	−0.2013
β-Conidia area	−0.0801	0.1693	0.7611	0.1922	0.1397	−0.2526	−0.5145
Glomeruli area	0.1830	0.3780	−0.1990	0.4742	−0.6749	0.1566	−0.2799
Hyphae wide	0.4212	0.3859	0.2399	0.2518	0.0868	−0.2901	0.6705
Filament length	−0.2284	−0.5647	−0.0244	0.3027	−0.3594	−0.6214	0.1306
Color	0.5456	−0.3926	0.1510	0.0170	−0.0465	0.1069	−0.0103
Growth	0.3644	0.2270	−0.3445	−0.4359	0.0191	−0.6239	−0.3420
Colony morphology	0.5435	−0.3900	0.1143	−0.0033	−0.0150	0.1924	−0.1812

and when used as the outgroup no change was observed in terminal grouping of clades. The only relevant change observed was the movement of the *P*. 19, *P*. 21, and *P*. 22 subgroup between clades A and B.

Some of the groups based on restriction data included individuals from different morphotypes (Fig. 1). Examples are *P*. 19 (morphotype 4) with *P*. 21 (morphotype 3), and *P*. 8, *P*. 10 (morphotype 1) with *P*. 28, *P*. 29 (morphotype 2). This agrees with findings in study with *Botryosphaeria* (Jacobs and Rehner 1998), and clearly shows that within *Phomopsis* there are high levels of both genetic variability and morphological plasticity. Restriction fragments of the same size are not necessarily homologous. Different gains or losses of restriction sites may result in restriction fragments of similar sizes, and very small restriction fragments may be hard to detect on gels.

Analysis between sites

No locality-dependent grouping of isolates was detected either using morphological or restriction data, which suggests that populations of *Phomopsis* from EV and LP are not isolated, discrete entities. The *P*. 28, *P*. 29 sub-clade (morphotype 2) was well supported in all trees, although its individuals are from LP and EV, respectively. In the strongly supported clade composed of *P*. 2, *P*. 6, *P*. 7, *P*. 13, there are individuals from both sites (Fig. 1). Although geographically apart, EV and LP seem within the range of distribution of this *Phomopsis* morphotype, which appears a highly variable, taxonomic complex.

Conclusions

From this study is clear that the genus *Phomopsis* is highly variable at various levels. First, the presence of five morphotypes in *G. guidonia* indicates that endophytic *Phomopsis* strains are diverse, and that the host-based naming strategy may underestimate fungal diversity. In fact, some species may remain cryptic due to the tendency to identify any isolate of some taxa as the most known, generally host-base named, described species (see Hawksworth and Rossman 1997). Second, there were few isolates highly similar in restriction profiles, suggesting high levels of genetic

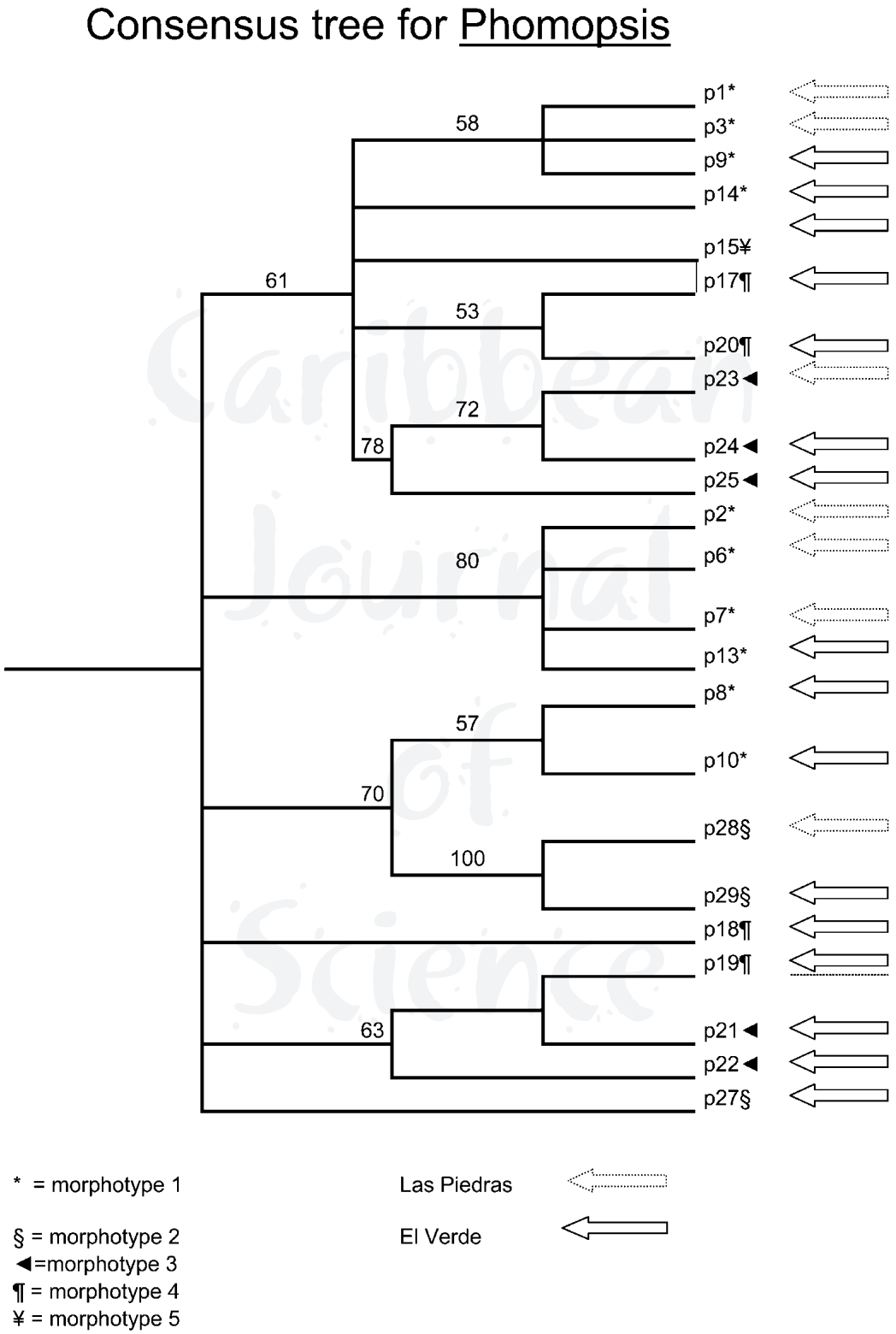

FIG. 1. Bootstrap consensus tree for morphotypes of *Phomopsis*. Numbers above the branches indicate the bootstrap (100 replicates). **MSI . . . MS5** = morphotypes one . . . morphotypes five. Populations from El Verde and Las Piedras are denoted by green and yellow arrows, respectively.

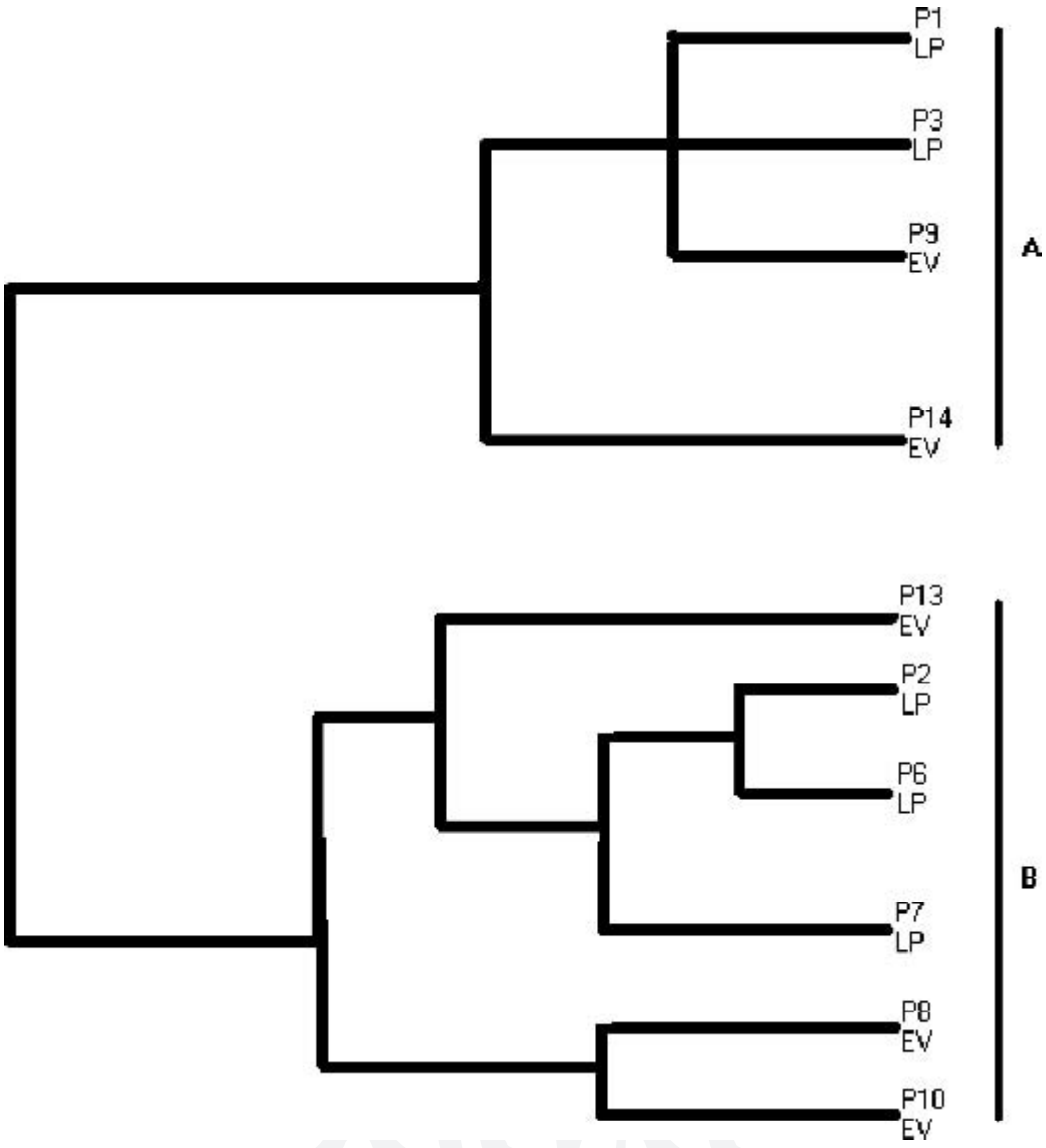

FIG. 2. Tree neighbor-joining of putative relationships among individuals of morphotypes one of *Phomopsis.* Length = 32.0, CI = 0.75 (length of branches not at scale, characters unordered, rooting at the base). EV = El Verde, LP = Las Piedras..

variation. Third, since EV and LP could not be separated as discrete populations by molecular or morphological data, *Phomopsis* isolates may belong to geographically extended populations.

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