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A preliminary overview of the Diaporthales based on large subunit nuclear ribosomal DNA sequences

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Abstract: The ascomycete order Diaporthales includes a number of plant pathogenic fungi such as *Cryphonectria parasitica*, the chestnut blight fungus, as well as many asexually reproducing fungi without known sexual states. Relationships among genera in the Diaporthales were evaluated as a basis for the recognition of families and to provide a taxonomic framework for the asexually reproducing diaporthalean fungi. Phylogenetic relationships were determined based on analyses of large subunit (LSU) nuclear ribosomal DNA (nrDNA) sequences. Within the Diaporthales 82 sequences representing 69 taxa were analyzed. Results suggest the presence of at least six major lineages within the Diaporthales recognized as the Gnomoniaceae *sensu stricto*, Melanconidaceae *sensu stricto*, *Schizoparme* complex including the anamorph genera *Coniella* and *Pilidiella*, *Cryphonectria-Endothia* complex, Valsaceae *sensu stricto*, and Diaporthaceae *sensu stricto*. In addition, six teleomorphic and anamorphic taxa fell within the Diaporthales but were not allied with any of the six lineages.

Key Words: canker fungi, *Cryphonectria*, *Diaporthe*, *Discula*, phylogeny, *Valsa*

INTRODUCTION

The ascomycete order Diaporthales includes a number of plant pathogenic fungi, the most notorious of which is *Cryphonectria parasitica* (Murrill) Barr, the chestnut blight fungus that altered the landscape of eastern North America (Anagnostakis 1987). Other diseases caused by members of this order include stem canker of soybeans (*Diaporthe phaseolorum* (Cooke & Ellis) Sacc. and its varieties), stem-end rot of citrus fruits (*Diaporthe citri* F.A. Wolf), and peach canker disease (*Phomopsis amygdali* (Del.) J.J. Tuset & T. Portilla) (Farr et al 1999). Some species produce secondary metabolites that result in toxicoses of animals such as lupinosis of sheep (*Diaporthe toxica* P.M. Will. et al) (Williamson et al 1994). A number of asexually reproducing plant pathogenic fungi also belong in the Diaporthales, such *Greeneria uvicola* (Berk. & Curt.) Punith., cause of bitter rot of grape, and *Discula destructiva* Redlin, cause of dogwood anthracnose, both of which are mitotic diaporthalean species with no known sexual state (Farr et al 2001, Zhang and Blackwell 2001).

As an order the Diaporthales is well-defined morphologically based on brown to black perithecial ascomata immersed in stromata or substrata, lack of true paraphyses at maturity, and unitunicate asci that often floar free within the centrum at maturity and have a refractive ring in the apex (Barr 1978, Samuels and Blackwell 2001). The known asexual states of members of the Diaporthales are generally coelomycetous bearing their phialidic, rarely annellidic, conidiogenous cells and conidia in acervuli or pycnidia with or without well-developed stromata. Molecular data have supported the Diaporthales as a distinct order within the Sordariomycetes, the class of ascomycetous fungi that generally produce their asci in perithecial ascomata (Farr et al 2001, Zhang and Blackwell 2001).

Within the Diaporthales eight families have been recognized by various authors over the past 25 yr. However, no single author has ever recognized all eight and the configuration of each family has varied considerably. Most recently, Eriksson et al (2001), presenting a synthesis of data from the literature with input from the mycological community, recognize three families in the Diaporthales, namely the Melanconidaceae G. Winter, Valsaceae Tul. & C. Tul., and Vialaeaceae P.F. Cannon. The familial classifications of the Diaporthales as recognized by Barr (1978, 1990), Hawksworth et al (1995), and Wehmeyer (1975) were summarized by Zhang and Blackwell (2001). They included the Gnomoniaceae G. Winter,

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Diaporthaceae Höhn. ex Wehm. and Pseudovalsaceae M.E. Barr as well as the Melanconidaceae and Valsaceae. Two additional families have been included in the Diaporthales. The Magnaporthaceae P.F. Cannon was shown to be extralimital to the Diaporthales (Berbee 2001, Farr et al 2001, Zhang and Blackwell 2001) as had been suggested by Vasilyeva (1993). The Sydowiellaceae Lar.N. Vassiljeva was established for the genus *Sydowiella* Petr. (Vasilyeva 1987). Of the 98 genera of plant-associated fungi in the Diaporthales 13 to 15 genera have not been referred to families (Eriksson et al 2001, Kirk et al 2001).

Generic concepts within the Diaporthales are based primarily on characteristics of the stromata, perithecia and ascospores. Stromatal characteristics used in defining genera in the Diaporthales are the extent and type of development, tissue type forming the stromata, and the relationship of the stromata to the host. The position of the perithecia relative to the host surface has been used to distinguish genera, as has the arrangement of the perithecia in the stromata and convergence or not of perithecial necks within the stromata. In addition to stromatal and perithecial characteristics, variations in the ascospore shape and septation have been used to define genera (Petrak 1966, Kobayashi 1970, Barr 1978, 1990, Monod 1983, Vasilyeva 1993). In some genera these distinctions are difficult to determine and the generic concepts have been unstable with many species transferred from one genus to another depending on the author.

Approximately 60% of the described species of plant-associated fungi reproduce asexually and lack any known sexual state (Rossman 1993). Although most are mitotic ascomycetes, their relationships to teleomorph taxa are generally unknown. Because these fungi include many serious plant pathogens, knowledge of their taxonomic affinities is crucial for developing measures to control the diseases they cause. With increased use of molecular sequence data for reconstructing fungal evolutionary relationships at all levels (Kohn 1992), the affinities of mitotic fungi with their sexually reproducing relatives can be determined. Recently *Greeneria uvicola*, a mitotic species with no known sexual affinities, was found to belong in the Diaporthales (Farr et al 2001). Since its emergence in the late 1970s, a sexual state for *Discula destructiva*, the cause of dogwood anthracnose, has been sought. Redlin (1991) suggested that it might have a teleomorph belonging to *Apiognomonia* Höhn. or *Gnomonia* Ces. & De Not. Zhang and Blackwell (2001) used molecular sequence data to infer the sexual state of *Discula destructiva* but were unable to come to any definitive conclusion except that it belonged in the Gnomoniaceae.

Knowledge of the relationships among genera within the Diaporthales is needed to serve as a basis for the recognition of families. In addition such knowledge will provide a taxonomic framework for determining relationships of teleomorph genera to asexually reproducing diaporthalean fungi. Thus, a study was undertaken to determine the major lineages within the Diaporthales based on a sequence analysis of the LSU nrDNA.

MATERIALS AND METHODS

Isolation, maintenance, and deposition of cultures and voucher specimens.—Newly sequenced isolates used in this study are listed in TABLE I. GenBank accession numbers for previously sequenced isolates are included with the species name in FIGS. 1–3. Fresh specimens were sent as air-dried collections primarily by the third and fourth authors to the second author. Isolates obtained from these specimens were grown from single ascospores or conidia that had been plated on 1.7% Difco Corn Meal Agar (CM) supplemented with 0.2% dextrose and antibiotics. Germinated spores were transferred to both 3.9% Difco Potato Dextrose Agar (PDA) and CM plates for observation. All isolates were maintained on CM agar slants and as plugs in 20% glycerol-water at 4 C (Burdsall and Dorworth 1994). Living cultures were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, and the original specimens from which isolates were obtained were deposited in the U.S. National Fungus Collections (BPI) as listed in TABLE I. For living cultures obtained from repositories such as ATCC (American Type Culture Collection), CBS and IMI (International Mycological Institute, now CABI), dried culture specimens were deposited in BPI if the culture sporulated.

Nucleic acid extraction and PCR amplification.—Mycelium for DNA extraction was grown in shaker flasks at 125 rpm for 5–10 d in 100 mL liquid CYM (Raper and Raper 1972) at room temperature under ambient light conditions. Mycelium was harvested by vacuum filtration on Whatman No. 1 filter paper and freeze-dried prior to DNA extraction. Alternatively, DNA was extracted directly from actively growing surface mycelium scraped from PDA plates. DNA was extracted with the Plant DNeasy Mini kit (Qiagen Inc., Chatsworth, California, USA) according to the manufacturer's instructions using approximately 15 mg dried tissue or 50 mg fresh mycelium.

The LSU nrDNA was amplified in 50 μL reactions on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, California, USA) under the following reaction conditions: 10–15 ng of genomic DNA, 200 mM each dNTP, 2.5 units Amplitaq Gold (Applied Biosystems, Foster City, California, USA), 25 pmoles each of primers LR0R and LR7 (Vilgalys and Hester 1990, Rehner and Samuels 1994) and the supplied 10× PCR buffer with 15 mM $MgCl_2$. The thermal cycler program was as follows: 10 min at 95 C followed

TABLE I. Newly sequenced taxa included in phylogenetic analyses

Species[a]	Locality	Host	Collector	Specimen No.[b]	Source[c]	GenBank No.
Apiognomonia errabunda (Roberge) Höhn. (anam. *Discula umbrinella* (Berk. & Broome) M. Morelet)	Switzerland	*Fagus sylvatica* L.	M. Monod	—	AR 2813 (= CBS 109747)	AF408334
Chromendothia citrina Lar. N. Vasiljeva	Russia	*Quercus mongolica* Fisch. ex Ledeb.	L. Vasilyeva	BPI 747935	AR 3446 (= CBS 109758)	AF408335
Coniella australiensis Petr.	South Africa	Leaf litter	K.T. van Warmelo	BPI 748425	IMI 261318	AF408336
***Coniella fragariae* (Oudem.) B. Sutton**	India	Soil	V.V. Bhatt	BPI 841767	IMI 081599	AF408391
Coniella musaiensis B. Sutton var. *hibisci* B. Sutton	?Africa	*Hibiscus* sp.	R.R. Cervantes	BPI 748426	AR 3534 (= CBS 109757)	AF408337
Cryphonectria cubensis (Bruner) Hodges	Cameroon	*Eucalyptus urophylla* S.T. Blake	I.A.S. Gibson	BPI 841768	CBS 101281	AF408338
Cryphonectria havanensis (Bruner) M.E. Barr	Zaire	*Eucalyptus saligna* Sm.	Unknown	BPI 748427	CBS 505.63	AF408339
Cryphonectria macrospora (Tak. Kobay. & Kaz. Itô) M.E. Barr	Russia	*Quercus mongolica*	L. Vasilyeva	BPI 748428	AR 3444 (= CBS 109764)	AF408340
Cryphonectria nitschkei (G.H. Otth) M.E. Barr	Russia	*Quercus mongolica*	L. Vasilyeva	BPI 748429	AR 3433 (= CBS 109776)	AF408341
***Cryptodiaporthe aesculi* (Fuckel) Petr.**	Austria	*Aesculus hippocastanum* L.	W. Jaklitsch	BPI 748430	AR 3580 ex WJ 1695 (= CBS 109765)	AF408342
Cryptodiaporthe corni (Wehm.) Petr.	USA: Maine	*Cornus alternifolia* L.f.	S. Redlin	BPI 747916	AR 2814 (= CBS 245.90)	AF408343
Cryptodiaporthe hystrix (Tode) Petr.	Austria	*Acer pseudoplatanus* L.	W. Jaklitsch	BPI 748431	AR 3565 ex WJ 1491 (= CBS 109759)	AF408344
Cryptodiaporthe salicella (Fr.) Petr.	Austria	*Salix* sp.	W. Jaklitsch	BPI 747938	AR 3455 ex WJ 1463 (= CBS 109775)	AF408345
***Cryptosporella hypodermia* (Fr.) Sacc.**	Austria	*Ulmus minor* Mill.	W. Jaklitsch	BPI 748432	AR 3552 ex WJ 1691	AF408346
Cryptosporella hypodermia	Austria	*Ulmus minor/laevis* Pall.	W. Jaklitsch	BPI 748433	AR 3566 ex WJ 1497 (= CBS 109753)	AF408347
Diaporthe decedens (Fr.) Fuckel	Austria	*Corylus avellana* L.	W. Jaklitsch	BPI 747942	AR 3459 ex WJ 1473 (= CBS 109772)	AF408348
Diaporthe detrusa (Fr.) Fuckel	Austria	*Berberis vulgaris* L.	W. Jaklitsch	BPI 748434	AR 3424 ex WJ 1445 (= CBS 109770)	AF408349
***Diaporthe eres* Nitschke**	Austria	*Acer campestre* L.	W. Jaklitsch	BPI 748435	AR 3538 ex WJ 1643 (= CBS 109767)	AF408350

TABLE I. Continued

Species[a]	Locality	Host	Collector	Specimen No.[b]	Source[c]	GenBank Number
Diaporthe fibrosa (Pers. : Fr.) Nitschke	Austria	*Rhamnus cathartica* L.	W. Jaklitsch	BPI 747929	AR 3425 ex WJ 1417 (= CBS 109751)	AF408351
Diaporthe oncostoma (Duby) Fuckel	Russia	*Robinia pseudoacacia* L.	L. Vasilyeva	BPI 747934	AR 3445 (= CBS 109741)	AF408353
Diaporthe padi G.H. Otth	Austria	*Prunus padus* L.	W. Jaklitsch	BPI 748436	AR 3419 ex WJ 1458 (= CBS 109784)	AF408354
Diaporthe pardalota (Mont.) Fuckel	Canada: British Columbia	*Epilobium angustifolium* L.	M. Barr	BPI 747946	AR 3478 ex MBB 10220 (= CBS 109768)	AF408355
Diaporthe perjuncta Niessl	Austria	*Ulmus glabra* Huds.	W. Jaklitsch	BPI 748437	AR 3461 ex WJ 1480 (= CBS 109745)	AF408356
Diaporthe pustulata (Desm.) Sacc.	Austria	*Acer pseudoplatanus*	W. Jaklitsch	BPI 747928	AR 3430 ex WJ 1428 (= CBS 109742)	AF408357
Diaporthe pustulata	Austria	*Acer pseudoplatanus*	W. Jaklitsch	BPI 748438	AR 3535 ex WJ 1628 (= CBS 109760)	AF408358
Discula destructiva Redlin	USA: Washington	*Cornus nuttallii* Audubon	M. Daughtrey	BPI 1107757	AR 2596 (= CBS 109771)	AF408359
***Ditopella ditopa* (Fr. : Fr.) J. Schröt.**	Austria	*Alnus glutinosa* (L.) Gaertn.	W. Jaklitsch	BPI 748439	AR 3423 ex WJ 1443 (= CBS 109748)	AF408360
***Gnomonia gnomon* (Tode : Fr.) J. Schröt.**	Italy	*Corylus avellana*	M. Ribaldi	—	CBS 199.53	AF408361
Gnomonia leptostyla (Fr. : Fr.) Ces. & De Not. (anam. *Marssonina juglandis* (Lib.) Magnus)	USA: Illinois	*Juglans nigra* L.	D. Neely	BPI 747976	FAU 543	AF408362
***Harknessia eucalypti* Cooke**	Australia	*Eucalyptus regnans* F. Muell.	Z-q. Yuan	—	CBS 342.97	AF408363
Harknessia lythri D.F. Farr & Rossman	USA: Minnesota	*Lythrum salicaria* L.	E. Katovich	BPI 747560	AR 3383 (= ATCC PTA-2756)	AF408364
***Hercospora tiliae* (Pers. : Fr.) Fr.**	Austria	*Tilia tomentosa* Moench	W. Jaklitsch	BPI 748440	AR 3526 ex WJ 1600 (= CBS 109746)	AF408365
Leucostoma auerswaldii Nitschke	Austria	*Frangula alnus* Mill.	W. Jaklitsch	BPI 748456	AR 3428 ex WJ 1424 (= CBS 109774)	AF408384
Leucostoma cincta (Fr. : Fr.) Höhn.	Russia	*Padus maackii* Rupr.	L. Vasilyeva	BPI 748441	AR 3415 (= CBS 109766)	AF408366
Leucostoma nivea (Hoffm. : Fr.) Höhn.	Austria	*Salix purpurea* L.	W. Jaklitsch	BPI 748442	AR 3512 ex WJ 1555 (= CBS 109743)	AF408367
Mazzantia napelli (Ces.) Sacc.	Austria	*Aconitum vulparia* Rchb.	W. Jaklitsch	BPI 748443	AR 3498 ex WJ 1531 (= CBS 109769)	AF408368
Melanconis alni Tul.	Austria	*Alnus viridis* (Vill.) Lam. & DC.	W. Jaklitsch	BPI 748444	AR 3500 ex WJ 1542 (= CBS 109773)	AF408371
Melanconis desmazierii Petr.	Austria	*Tilia* sp.	W. Jaklitsch	BPI 748445	AR 3525 ex WJ 1588 (= CBS 109780)	AF408372

TABLE I. Continued

Species[a]	Locality	Host	Collector	Specimen No.[b]	Source[c]	GenBank No.
Melanconis marginalis (Peck) Wehm.	Canada: British Columbia	*Alnus rubra* Bong.	M. Barr	BPI 748446	AR 3442 ex MBB 1021A (= CBS 109744)	AF408373
***Melanconis stilbostoma* (Fr.) Tul.**	Austria	*Betula pendula* Roth	W. Jaklitsch	BPI 748447	AR 3501 ex WJ 1543 (= CBS 109778)	AF408374
Ophiovalsa betulae (Tul. & C. Tul.) Petr. (anam. *Disculina betulina* (Sacc.) Hohn.)	Austria	*Betula pendula*	W. Jaklitsch	BPI 748448	AR 3524 ex WJ 1610 (= CBS 109763)	AF408375
***Ophiovalsa suffusa* (Fr.) Petr.** (anam. *Discula vulgaris* (Fr.) B. Sutton)	Austria	*Alnus incana* (L.) Moench	W. Jaklitsch	BPI 748449	AR 3496 ex WJ 1556 (= CBS 109750)	AF408376
***Phragmoporthe conformis* (Berk. & Broome) Petr.**	Canada: British Columbia	*Alnus rubra*	M. Barr	BPI 748450	AR 3632 ex MBB 10338 (= CBS 109783)	AF408377
***Pilidiella castaneicola* (Ellis & Everh.) Arx**	Korea	Unknown	K. S. Bae	BPI 748451	CBS 143.97	AF408378
Pilidiella granati (Sacc.) Aa	Cyprus	*Punica granatum* L.	R.M. Naurass	BPI 748452	CBS 152.33	AF408379
Pilidiella granati	Turkey	*Punica granatum*	N. Kaskalôglu	BPI 748453	CBS 814.71	AF408380
Plagiostoma conradii (Ellis) M.E. Barr	USA: New Jersey	*Hudsonia tomentosa* Nutt.	G. Bills	BPI 746482	AR 3488 (= CBS 109761)	AF408381
***Plagiostoma euphorbiae* (Fuckel) Fuckel**	Netherlands	*Euphorbia palustris* L.	Unknown	—	CBS 340.78	AF408382
Schizoparme botrytidis Samuels	Puerto Rico	Dead wood	S. Huhndorf	BPI 748454	SMH 1351 (= AR 3504)	AF408383
Valsa cenisia De Not.	Austria	*Juniperus communis* L.	W. Jaklitsch	BPI 748457	AR 3522 ex WJ 1583 (= CBS 109752)	AF408385
Valsa ceratosperma (Tode : Fr.) Maire	Russia	*Quercus mongolica*	L. Vasilyeva	BPI 748458	AR 3416 (= CBS 109756)	AF408386
Valsa ceratosperma	Austria	*Quercus robur* L.	W. Jaklitsch	BPI 748459	AR 3426 ex WJ 1425 (= CBS 109777)	AF408387
Valsella adhaerens Fuckel	Russia	*Betula* sp.	L. Vasilyeva	BPI 748460	AR 3549 (= CBS 109782)	AF408388
***Valsella salicis* Fuckel**	Italy	*Salix fragilis* L.	W. Jaklitsch	BPI 748461	AR 3514 ex WJ 1580 (= CBS 109754)	AF408389
Wuestneia molokaiensis Crous & J.D. Rogers	USA: Hawaii	*Eucalyptus robusta* Sm.	J. Rogers	BPI 748462	AR 3578 (= CBS 109779)	AF408390

[a] Type species of the genus in bold.

[b] BPI = U.S. National Fungus Collections.

[c] AR = Amy Rossman, second author; ATCC = American Type Culture Collection; CBS = Centraalbureau voor Schimmelcultures; FAU = maintained by second author; IMI = International Mycological Institute, now CABI, Inc.; MBB = Margaret Barr Bigelow, Sidney, British Columbia; SMH = Sabine M. Huhndorf, Field Museum, Chicago, IL; WJ = Walter Jaklitsch, third author.

by 35 cycles of 30 s at 94 C, 30 s at 55 C, 1 min at 72 C, with a final extension period of 10 min at 72 C. Following amplification, the PCR products were purified with QIAQuick columns (Qiagen Inc., Chatsworth, California, USA) according to the manufacturer's instructions. Amplified products were sequenced with the BigDye dye terminator kit (Applied Biosystems, Foster City, California, USA) on an ABI 310 or ABI 377 automated DNA sequencer using the following primers: LR0R, LR3R, LR5R, LR7, LR5, LR3 (Vilgalys and Hester 1990, Rehner and Samuels 1994).

Sequence analysis. Raw sequences were edited using Sequencher version 4.05 for Windows (Gene Codes Corporation, Ann Arbor, Michigan, USA). Alignments were manually adjusted using GeneDoc 2.6.001 (http://www.psc.edu/biomed/genedoc/). Two alignments were generated. Alignment 1 included sequences from 55 newly sequenced diaporthalean taxa, 27 diaporthalean sequences from GenBank for which only approximately 600 bp are available for some taxa with *Magnaporthe grisea* (T.T. Hebert) Yaegashi & Udagawa and *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier from the Magnaporthaceae as outgroup taxa. Alignment 1 was truncated to 650 aligned positions to minimize the effects of large amounts of missing data for some of the taxa in the analyses. Alignment 2 included only the 55 taxa newly sequenced for this study as well as 16 sequences recently reported in Farr et al (2001) for which approximately 1350 bp of the 5′ end of the LSU nrDNA were sequenced. The sequence alignments were deposited in TreeBASE as S815.

For both alignments, trees were inferred by the neighbor-joining (NJ) method (Kimura 2-parameter distance calculation) and by maximum parsimony (MP) using the heuristic search option with the random addition sequence (1000 replications) and the branch swapping (tree bisection-reconnection, TBR) option of PAUP* 4.0b8 (Swofford 1998). For both types of analyses, ambiguously aligned positions were excluded. All characters were unordered and given equal weight during the analysis. Gaps were treated as missing data in the parsimony analysis and the neighbor joining analysis; missing or ambiguous sites were ignored for affected pairwise comparisons. Heuristic searches for most parsimonious trees (MPT) with the MULTREES option in effect resulted in large numbers of trees and did not search to completion. Maximum likelihood analyses were not attempted due to the length of time required for a data set of this size.

All resulting MPT were compared using the Shimodaira-Hasegawa (S-H) test (Shimodaira and Hasegawa 1999) as implemented in PAUP* 4.0b8. The likelihood model was determined by Modeltest version 3.06 (Posada and Crandall 1998). Relative support for branches was estimated with 1000 bootstrap replications (Felsenstein 1985) with MULTREES and TBR off and 10 random sequence additions for the MP bootstraps.

Phylogenetic trees were also inferred for alignment 2 using Bayesian inference as implemented in MrBayes (http://morphbank.ebc.uu.se/mrbayes/) with the following commands: (i) exclude positions 75, 76, 116, 117, 475–488, 504, 505, 862–1227; (ii) likelihood settings (lset) number of substitution types (nst) = 6, a proportion of sites invariable and the rest drawn from the gamma distribution (rate = invgamma), base frequencies = estimate, rate matrix = (1.7034, 6.7182, 3.8375, 0.9578, 15.0004, 1.0000); (iii) number of generations = 500 000, sample frequency = 100, number of chains = 4, temperature = 0.5, save branch lengths = yes, starting tree = random. The first 100 000 generations were discarded as the chains were converging (burnin). Likelihood model assumptions were as determined with Modeltest version 3.06 (Posada and Crandall 1998): base frequencies A = 0.2708, C = 0.2196, G = 0.2847, T = 0.2249; number substitution types = 6; proportion of invariable sites = 0.719; gamma shape parameter = 0.6023, number rate categories = 4, mean average rate; rate matrix = 1.7034, 6.7182, 3.8375, 0.9578, 15.0004, 1.0000. Four independent analyses, each starting from a random tree, were run under the same conditions.

Phylogenetic trees corresponding to the most recent classification schemes of Barr (1990) and Eriksson et al (2001) were constructed by using taxa contained within each accepted family as a separate monophyletic constraint in MP analyses of alignment 2 using the heuristic search option (1000 random sequence additions, TBR and MULTREES off). The tree with the best −ln likelihood score resulting from each constrained analysis and all trees resulting from the unconstrained analysis were compared by the S-H test as described above (TABLE II), including all characters in the analysis except for ambiguously aligned positions and intron sequences. The following topologies were tested with the 372 equally parsimonious trees resulting from the unconstrained analysis: (i) Barr (1990) Gnomoniaceae, (ii) Barr (1990) Melanconidaceae, (iii) Barr (1990) Valsaceae, (iv) Eriksson et al (2001) Melanconidaceae, (v) Eriksson et al (2001) Valsaceae, and (vi) Bayesian topology. The range of −ln likelihood scores of trees from each constraint topology is shown. However, only the topology with the best −ln likelihood score from each constraint was tested against the unconstrained trees.

RESULTS

Sequence alignments.—Alignment 1 consisted of 650 total characters of which 20 ambiguously aligned positions were excluded. Of the remaining 630 characters, 132 were parsimony informative. Alignment 2 consisted of 1650 bases of which 366 positions were excluded due to the presence of introns in two of the sequences (*Cryptodiaporthe corni* AR 2814, *Ditopella ditopa* AR 3423) and 20 positions were excluded because of potentially ambiguous alignments, leaving 1264 positions of which 189 were parsimony informative.

Sequence analyses.—For MP analyses, heuristic searches resulted in excess of 5000 trees. When the MULTREES option was turned off, 210 and 372 equally parsimonious trees were generated for alignment 1 and alignment 2, respectively. A strict consensus of

trees generated with MULTREES on (MAXTREES = 5000) was identical to the strict consensus of trees generated from analyses with MULTREES off for both analyses (trees not shown).

Parsimony tree scores for alignment 1 were CI = 0.428, RI = 0.879, RC = 0.376, and length = 428. For alignment 2, tree scores were CI = 0.487, RI = 0.893, RC = 0.435 and length = 503. The MPT with the best −ln likelihood score for alignment 1 is shown in FIG. 1 and one of two MPT with the best −ln likelihood score is shown for the alignment 2 (FIG. 2), although neither tree was significantly better than others generated in each analysis ($P = 0.05$). Bootstrap values greater than 70 percent are indicated on FIGS. 1 and 2 above (MP) and below (NJ) the respective branches.

To determine if trees resulting from MP analyses with the MULTREES option = off, in general, reflected a good approximation of relationships within this group, Bayesian phylogenetic inference was used to construct a tree and determine the probabilities of a particular group existing in that tree (given the observed data). Bayesian analysis using Markov chain Monte Carlo algorithms is computationally more practical than bootstrapping and maximum likelihood. In addition, heuristic searches are not guaranteed to converge to the optimal tree (Larget and Simon 1999), whereas the Markov chain explores possible tree topologies and dimensions of other parameters of trees in proportion to their posterior probabilities (Lewis 2001). [For detailed explanations of this method, see Larget and Simon 1999, Huelsenbeck et al 2000, and Lewis 2001.]

FIGURE 3 shows the tree resulting from the Bayesian analysis with the highest −ln likelihood score with the numbers above the branches reflecting the probability that each group exists expressed as a percentage. Four independent analyses were run with each starting from a random tree and probabilities and topologies were similar in all analyses. Topologies from the four analyses differed only in the placement of the Valsaceae and Diaporthaceae as sister taxa in two of the four analyses. However, posterior probabilities of that placement were not particularly high (0.58 and 0.69).

Likelihood ratio tests.—Phylogenetic analysis of the diaporthalean taxa available for this study indicates the presence of at least six lineages within the Diaporthales. Although elements of many of the previously described families are present, these lineages do not entirely conform to taxonomic schemes that have been proposed. The S-H test results for constrained tree topologies corresponding to recently recognized families are presented in TABLE II.

Shimodaira-Hasegawa test results show that when analyses are constrained to conform to the placement of genera within the three families as recognized by Barr (1990) and Eriksson et al (2001), resulting trees from all of the constraints except for the Valsaceae as recognized by Barr (1990) are significantly worse than FIG. 2. Trees resulting from analyses constraining *Valsa* Fr., *Leucostoma* (Nitschke) Höhn., *Valsella* Fuckel, *Endothia* Fr. and *Chromendothia* Lar.N. Vassiljeva to be a monophyletic group could not be rejected as significantly worse explanations of the data than FIG. 2. These results suggest that two of the three major families in the Diaporthales as currently circumscribed (Eriksson et al 2001) are not monophyletic and that a greater number of families should be recognized.

DISCUSSION

Phylogenetic analyses.—Phylogenetic analysis of LSU nrDNA sequences for available taxa within the Diaporthales shows the presence of at least six lineages within this order. Likelihood ratio tests (Shimodaira and Hasegawa 1999) comparing the topologies obtained by constraining monophyletic groups that correspond to recent classifications schemes do not recognize these families as equally good explanations of the data when compared with FIG. 2, the result of an unconstrained analysis, except in the case of Barr's (1990) Valsaceae. Recent papers have pointed out that the Kishino-Hasegawa (K-H) test (Kishino and Hasegawa 1989) is not appropriate for making multiple comparisons or for trees resulting from the analysis of the data set and designated *a posteriori* (Shimodaira and Hasegawa 1999, Goldman et al 2000, Whelan et al 2001). However, there is some doubt about whether or not it is appropriate to use the S-H test for questions of monophyly (Goldman et al 2000, Whelan et al 2001). In light of these concerns, although the S-H test was performed, it may be more appropriate to compare only the −ln likelihood scores of each topology and not to evaluate their statistical significance.

The results of maximum parsimony, neighbor joining, and Bayesian inference analyses were all similar in topology as well as levels of support. Bootstrap support for groups was generally higher in NJ analyses than for MP analyses and support was higher in alignment 2 (1264 bp) than alignment 1 (630 bp). In order to determine if this observation might be a result of either the more limited taxon sampling or the larger number of analyzed characters in alignment 2, the first 650 bp, excluding 20 ambiguously aligned positions, of this alignment was analyzed by both MP and NJ bootstrap analyses as described in the mate-

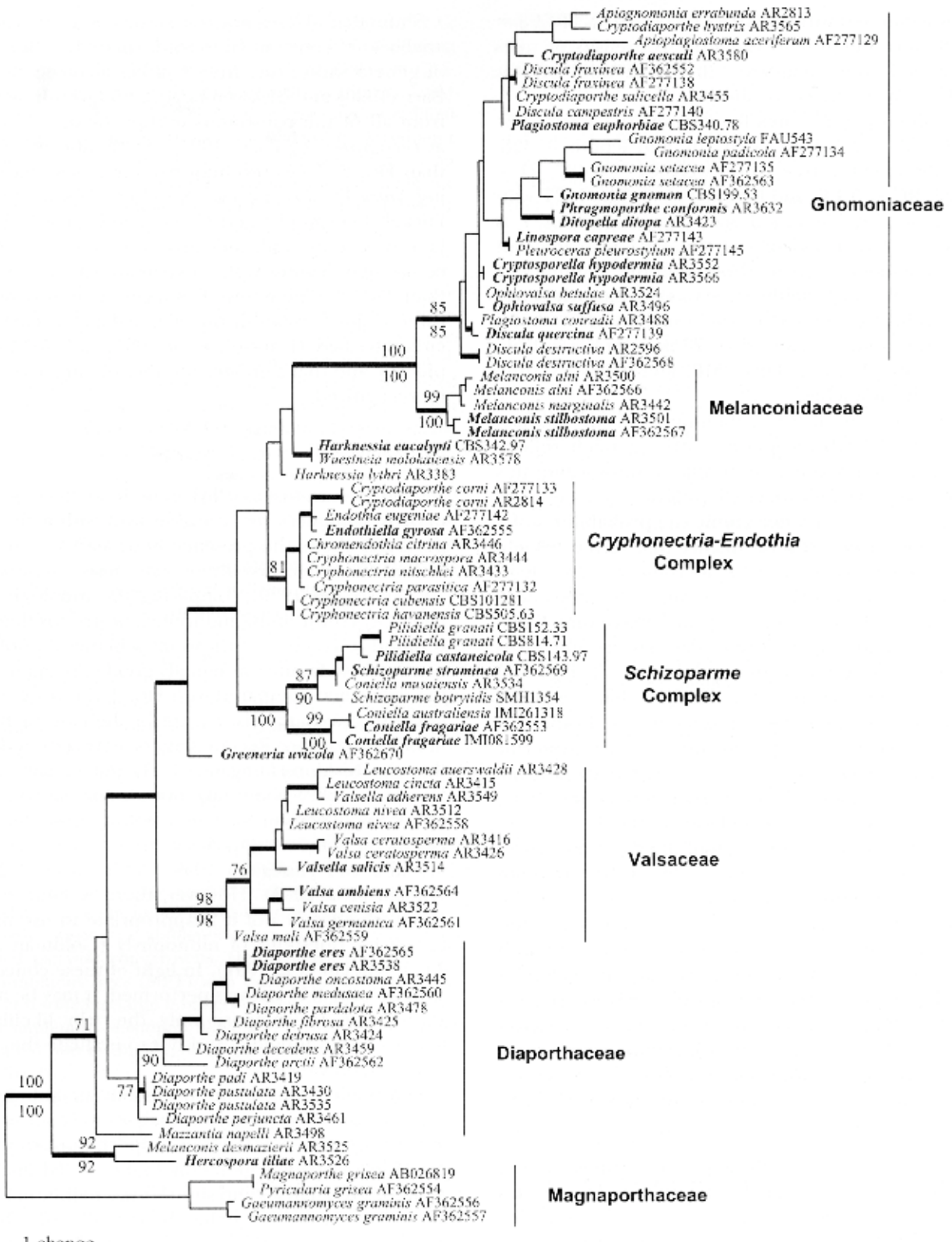

FIG. 1. One of 210 equally parsimonious trees based on analysis of 630 bp of the 5′ end of the LSU nrDNA (−ln likelihood = 3328.6773, CI = 0.428, RI = 0.879, RC = 0.376, length = 428 steps) for 82 diaporthalean sequences. Bootstrap values greater than 70% are shown above (MP) and below (NJ) each branch. Taxa in bold represent type species of their respective genera. Thickened lines indicate that branch appeared in the strict consensus of the 210 trees.

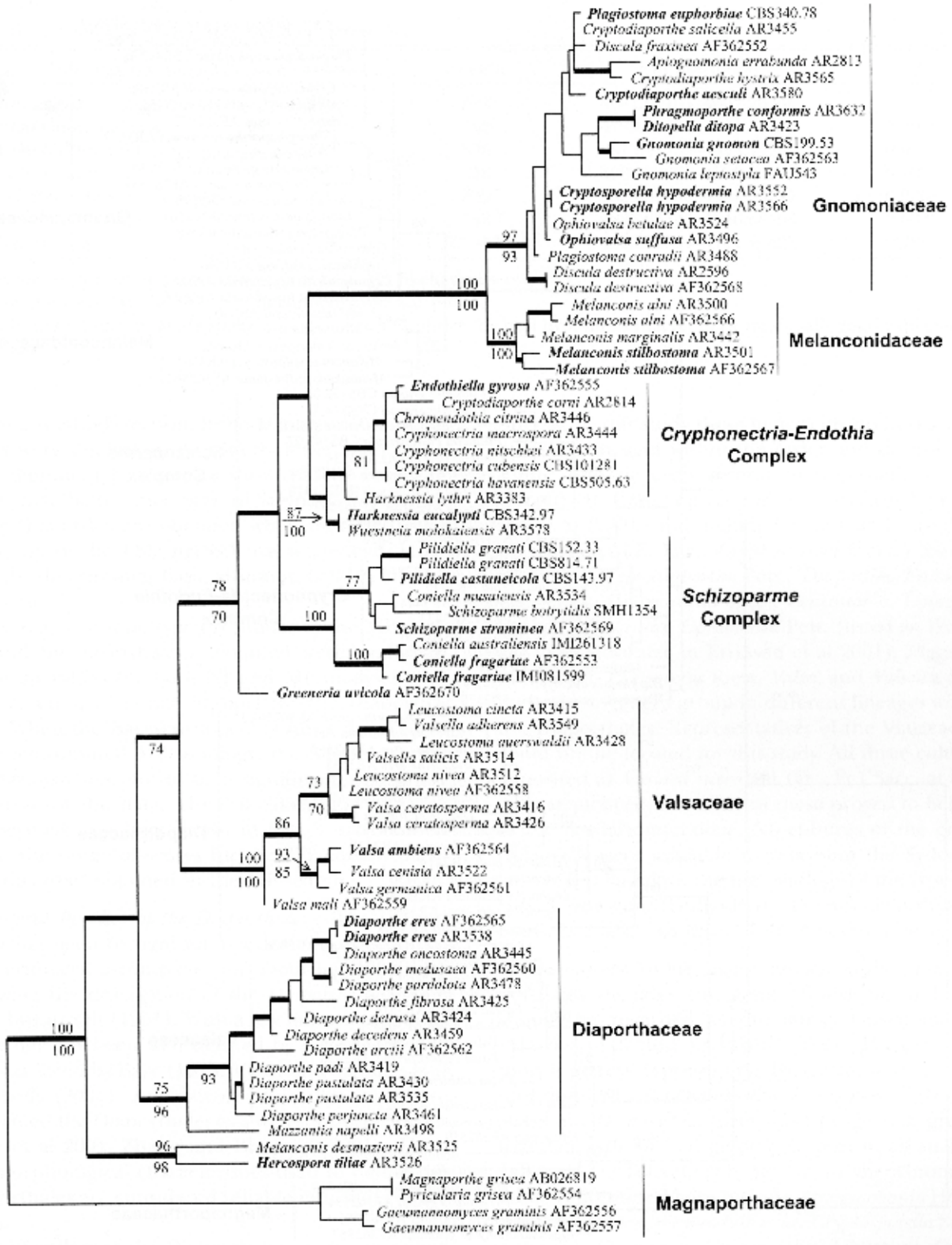

FIG. 2. One of two trees with the best −ln likelihood score of 372 equally parsimonious trees based on analysis of 1264 bp of the 5′ end of LSU mDNA (−ln likelihood = 4681.5015, CI = 0.487, RI = 0.803, RC = 0.435, length = 503 steps) for 71 diaporthalean taxa. Bootstrap values greater than 70% are shown above (MP) and below (NJ) each branch. Taxa in bold represent type species of their respective genera. Thickened lines indicate that branch appeared in the strict consensus of the 372 trees.

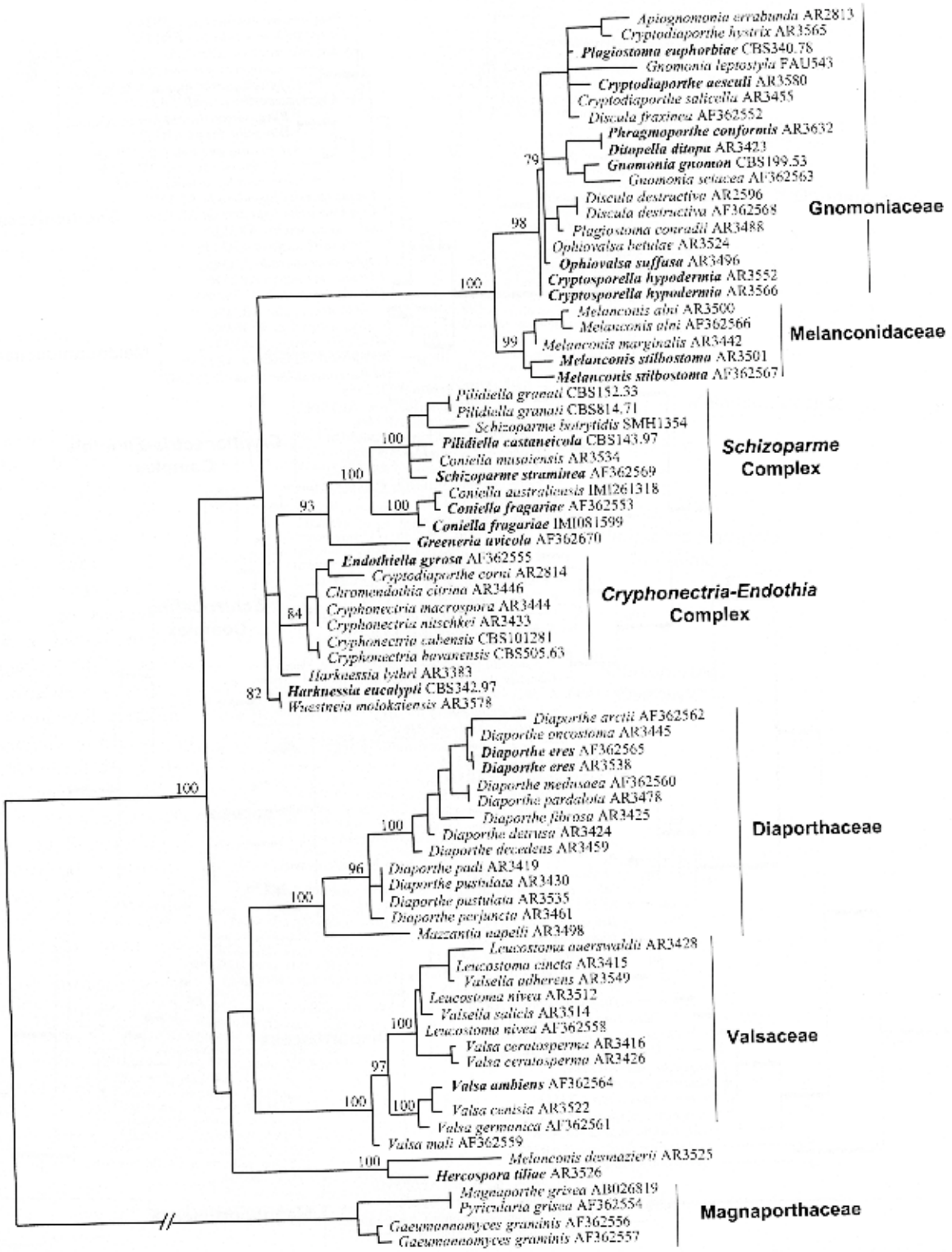

FIG. 3. Phylogenetic tree resulting from Bayesian analysis of 1264 bp of the LSU nrDNA (−ln likelihood = 4693.39844) for 71 diaporthalean taxa. Numbers on branches indicate the posterior probability given the observed data that the group exists, expressed as a percentage. Taxa in bold represent type species of their respective genera.

TABLE II. Shimodaira-Hasegawa likelihood test results

Topology	Trees	Length	−ln likelihood	P[a]
Unconstrained	372	503	4681.50151–4697.26700	—
Barr 1990 Gnomoniaceae	193[b]	558	4783.96828–4801.5197	0.000*
Barr 1990 Melanconidaceae	118[b]	549	4815.91753–4834.40237	0.000*
Barr 1990 Valsaceae	216[b]	518	4709.20630–4728.20071	0.409
Eriksson 2001 Melanconidaceae	117[b]	549	4825.80000–4837.33077	0.000*
Eriksson 2001 Valsaceae	674[b]	548	4825.65341–4846.68496	0.000*
Bayesian analysis	4[b]	523–531	4698.39844–4711.49428	0.784

[a] P-values only reported for the tree with best −ln likelihood score.

[b] Only the tree with the best −ln likelihood score was tested.

* Indicates significant at $P < 0.05$ in a one-tailed test under the null hypothesis that all trees are equally good explanations of the data.

rials and methods section. In both analyses, bootstrap values were equivalent or slightly lower than those from alignment 1 (trees not shown). This would indicate that better measures of support for groups within this order are obtained when more than the 5′ 650 bp of the LSU nrDNA are sequenced. However, for determining basic affinities, 650 bp may be sufficient.

The Bayesian topology was similar to the MP analysis and the probabilities obtained were similar to bootstrap values for both NJ and MP analyses with the exception of better support for the Diaporthaceae. When the Bayesian topology was compared to the unconstrained MP topology, the S-H test found the Bayesian tree not to be a significantly worse explanation of the data. The −ln likelihood score of the Bayesian topology shown in FIG. 3 is contained within the range of scores for the 372 equally parsimonious trees obtained in the MP search.

Order and families of the Diaporthales.—The Diaporthales has been recognized as a distinct order within the perithecial ascomycetes for about half a century following the description of the *Diaporthe*-type centrum by Luttrell (1951). With a few minor exceptions the order has been well defined by previous mycologists as listed by Barr (1978, 1990) and Samuels and Blackwell (2001). Analyses of molecular data have confirmed the Diaporthales as a well-supported order (Farr et al 2001, Zhang and Blackwell 2001). Thus, the morphological characteristics used to define the Diaporthales are considered reliable indicators of the order.

The families of the Diaporthales as circumscribed by Eriksson et al (2001) were not supported by this study. Genera placed in the Melanconidaceae by Eriksson et al (2001) and included in this study are *Allantoporthe* Petr. as *Diaporthe decedens*, *Ditopella* De Not., *Herrospora* Fr., *Melanconis* Tul. & C. Tul., *Phragmoporthe* Petr., *Schizoparme* Shear, and *Wuestneia* Auersw. In this study these genera group into several different lineages within the order and do not constitute one or even several monophyletic groups (FIGS. 1–3). Genera placed in the Valsaceae by Eriksson et al (2001) and included in this study are *Apioplagiostoma* M.E. Barr, *Cryphonectria* (Sacc.) Sacc. & D. Sacc., *Cryptodiaporthe* Petr., *Diaporthe*, *Endothia*, *Gnomonia*, *Gnomoniella* Sacc., *Leucostoma*, *Linospora* Fuckel, *Mazzantia*, *Ophiovalsa* Petr. (listed as *Winterella* (Sacc.) Kuntze in Eriksson et al 2001), *Plagiostoma* Fuckel, *Pleuroceras* Riess, *Valsa*, and *Valsella*. Similarly, these genera group in different lineages within the Diaporthales. Representatives of the Vialaeaceae could not be located for this study. All three cultures deposited as *Vialaea insculpta* (Fr. : Fr.) Sacc. at CBS were sequenced, but none of these proved to belong in the Sordariomycetidae. No cultures of the genus *Sydowiella* were available to represent the Sydowiellaceae, but based on the morphology of the type species, *S. fenestrans* (Duby) Petr., it seems likely that this family is a later synonym of the Gnomoniaceae.

Gnomoniaceae.—One major lineage within the Diaporthales includes the genus *Gnomonia* and thus should be regarded as the family Gnomoniaceae (Hawksworth and Eriksson 1988). Twelve teleomorph genera representing 18 species and the anamorph taxa *Discula campestris*, *D. destructiva*, *D. fraxinea*, and *D. quercina* form this lineage that groups together with 85% or greater support in all analyses (FIGS. 1–3). Teleomorph genera in the Gnomoniaceae represented here include *Apiognomonia* Höhn, *Apioplagiostoma*, *Cryptodiaporthe*, *Cryptosporella* Sacc., *Ditopella*, *Gnomonia*, *Gnomoniella*, *Linospora*, *Ophiovalsa*, *Phragmoporthe* Petr., *Plagiostoma*, and *Pleuroceras*. No well-supported subdivisions were found in this group, although a number of taxa consistently grouped together in the analyses.

These results generally agree with the concept of the Gnomoniaceae as recognized by Monod (1983)

and include taxa characterized by ascomata that are immersed, solitary, without stromata or aggregated in reduced, prosenchymatous stromata in herbaceous plant material, especially in leaves or stems, but also in wood. The ascomata are generally soft-textured, thin-walled, and prosenchymatous with either central or lateral beaks. The asci may or may not have a distinct ring and the ascospores are generally small, less than 25 µm long, and range in septation from nonseptate, one-septate (median or eccentric) to multiseptate. The anamorphs of members of the Gnomoniaceae are acervular or pycnidial, often with a broad opening, and phialidic, with pallid, non- or one-septate conidia.

One genus, *Mazzantia*, placed in the Gnomoniaceae by Monod (1983) and included in this study, does not belong in the Gnomoniaceae, rather it falls within the Diaporthaceae (see below). Another genus placed in the Gnomoniaceae by Monod (1983), *Gaeumannomyces* Arx & D.L. Olivier, has been shown to belong in the Magnaporthaceae outside of the Diaporthales (Berbee 2001, Farr et al 2001, Zhang and Blackwell 2001).

Melanconidaceae.—Unlike the Melanconidaceae *sensu* Eriksson et al (2001) the results of this study suggest that all genera except the type genus *Melanconis* should be excluded from the family. The type species of *Melanconis*, *M. stilbostoma*, and two additional species, *M. alni* and *M. marginalis*, form a well-supported group with greater than 98% support in all analyses (FIGS. 1–3). These three species of *Melanconis* produce well-developed stromata having a light-colored ectostromatic disc and a concolorous central column with circinately arranged, immersed ascomata; hyaline, one-septate ascospores; and anamorphic states placed in *Melanconium* Link. Pycnidia develop in the stromata prior to formation of the ascomata and produce unicellular, dark-brown conidia. One species, *Melanconis desmazierii*, falls outside the Melanconidaceae. This species is a distantly related diaporthalean taxon that is allied with *Herrospora tiliae* in this study (>90%) in all analyses (FIGS. 1–3). The Melanconidaceae, herein restricted to *Melanconis sensu stricto*, groups with the Gnomoniaceae with support of 99% or greater in all analyses (FIGS. 1–3) and these two families could be combined and regarded as the Gnomoniaceae. This result is somewhat surprising and suggests that morphological characteristics like thickness of ascospore wall and stromatal development are of less importance than suggested previously (Barr 1978, 1990).

Cryphonectria-Endothia *complex.*—Representatives of three genera, namely *Chromendothia*, *Cryphonectria*, and *Endothia*, and one additional species, *Cryptodiaporthe cornii*, grouped together at greater than 80% in all analyses except MP analysis of alignment 1 (FIGS. 1–3). Morphologically these taxa are united by ascomata immersed in well-developed, yellow to orange or orange-red stromata. The pigments within the stromatal wall turn purple in 3% KOH (KOH−) and yellow in lactic acid and are produced in culture. The ascomatal wall of members of the *Cryphonectria-Endothia* complex is dark brown to black, often evident as darkened ostiolar papillae extending beyond the stromata. Although a similar KOH reaction is also characteristic of the Nectriaceae, Hypocreales, the pigments occur in cell walls of the ascomatal wall and are not often produced in culture. Anamorphs in the *Cryphonectria-Endothia* complex produce small, hyaline, one-celled conidia enteroblastically in multiloculate pycnidia in well-developed stromata similar in appearance to those producing ascomata. The close relationship of these three genera was recognized by Vasilyeva (1998) who placed them in tribe Endothiae M.E. Barr.

Cryptodiaporthe cornii, a species having the typical KOH+ purple color reaction characteristic of this group and occurring on the temperate host, *Cornus alternifolia* (Redlin and Rossman 1991) falls in this group rather than in the Gnomoniaceae with the type species of *Cryptodiaporthe*, *C. aesculi* (FIGS. 1–3). This species undoubtedly belongs in either *Cryphonectria* or *Endothia*. Three of the five species of *Cryphonectria*, namely *C. macrospora*, *C. nitschkei*, and *C. parasitica*, and *Chromendothia citrina* and *Endothiella gyrosa* occur primarily on temperate hardwood trees. Two additional species of *Cryphonectria*, *C. cubensis* and *C. havanensis*, appear to be more closely related to each other than to the other three species of *Cryphonectria*. Venter et al (2001) have recently suggested that the latter two species belong in a separate genus.

Schizoparme *complex.*—Two species of *Schizoparme* including the type species, *S. straminea*, and *S. botrytidis* grouped with seven strains of *Coniella* Höhn. and *Pilidiella* Petr. & Syd. at 97% or greater in all analyses (FIGS. 1–3). Although placed by Samuels et al (1993) in the Melanconidaceae, the genus *Schizoparme* and its allied taxa do not fall within any established family in the Diaporthales and may eventually be recognized as its own family. Unlike most members of the Diaporthales, species of *Schizoparme* are often erumpent through the host epidermis, becoming superficial. The ascomatal wall often includes "an epistromatic region of small, pale-colored cells around the ostiolar opening" (Samuels et al 1993). A similar, thickened outer wall was observed on species of *Coniella* and *Pilidiella* in culture. The ana-

morph of *S. straminea* is *P. castaneicola* (Samuels et al 1993 as *C. castaneicola*) while that of *S. botrytidis* also belongs in *Pilidiella* (pers obs). Although *Coniella* is generally recognized in the broad sense to include the genus *Pilidiella* (Sutton 1980, Nag Raj 1993), the well-supported separation of these taxa in all analyses suggests that *Pilidiella* may be distinct from *Coniella*. These genera can be distinguished by conidial pigmentation which in *Coniella fragariae*, type of *Coniella*, and *C. australiensis* are dark brown while in *Pilidiella castaneicola*, type of *Pilidiella*, and related taxa including *C. musaiensis*, the conidia tend to be pale brown.

Valsaceae.—The genus *Valsa* and representatives of two related genera, *Leucostoma* and *Valsella*, group together with 98% or greater bootstrap values (FIGS. 1–3) in what is considered the family Valsaceae *sensu stricto*. The close relationship of species of *Valsa* to *Leucostoma* and *Valsella* has been recognized by a number of authors (Spielman 1985, Vasilyeva 1993) and is confirmed here.

Members of the Valsaceae occur on woody angiosperms in temperate regions throughout the world (Barr 1978, Spielman 1985). The ascomata are aggregated in well-developed entostromata with beaks emerging centrally through a white to black stromatic disc. In *Leucostoma* and *Valsella* the entostromata are delimited basally by a black stromatic zone, while in *Valsa* such a zone is lacking. The genus *Leucostoma* has eight-spored asci while *Valsella* is characterized by multisporous asci. Although the type species of *Leucostoma*, *L. massariana* Höhn., was not available for this study, three species of *Leucostoma* were included, namely *L. nivea*, *L. cincta* and *L. auerswaldii*. The type of the genus *Valsella*, *V. salicis*, was included along with a second species, *V. adherens*. Neither the species of *Leucostoma* nor those of *Valsella* grouped together, exclusive of other taxa. Rather they were interspersed with each other and *Valsa ceratosperma* suggesting that neither the black stromatic disc nor polysporous asci unite related species.

Within the Valsaceae, there were three well-supported subgroups in all analyses. The three species of *Leucostoma* with *Valsella salicis* and *Valsa ceratosperma* represent a subdivision although there is not obvious correlation with any morphological or biological features. The genus *Valsa* is represented in this study by the type species, *V. ambiens*, which grouped closely with *Valsa germanica* and *V. cenisia*. *Valsa mali* was basal to the *V. ambiens* group and the *Leucostoma/Valsella* group in all analyses.

Diaporthaceae.—This lineage consists of eleven species of *Diaporthe* including representatives of the type species, *D. eres*, and *Mazzantia napelli* which grouped together at 75% or greater bootstrap support in all analyses except MP analysis of alignment 1 (FIGS. 1–3). The family Diaporthaceae is delimited here in a much more restricted sense than by previous authors. This family was established by Höhnel (1917) who recognized this and only one other family, the Gnomoniaceae, in the Diaporthales. Wehmeyer (1975) had a somewhat narrower concept of the Diaporthaceae, including *Diaporthe* and *Mazzantia* as well as many more genera, some of which are included in this study and excluded from this lineage. The Diaporthaceae was considered a synonym of the Valsaceae by Barr (1978) and other workers since then. Based on the results presented here, the Diaporthaceae *sensu stricto* includes only *Diaporthe* and *Mazzantia*.

All species of *Diaporthe* included in this study formed a well-supported group. *Diaporthe* is a large genus that is well-defined morphologically and includes several hundred described species, many of which have anamorphs belonging to the genus *Phomopsis*. Species occur on a wide range of substrates ranging from woody dicotyledonous plants to herbaceous monocots (Wehmeyer 1933). In *Diaporthe* each stroma covers and subtends several ascomata usually forming a black line in the hardened host tissue. The ascospores of species of *Diaporthe* are one-septate, hyaline, and usually ellipsoidal. The *Phomopsis* anamorphic states are even more ubiquitous forming uni- or multiloculate, pycnidial stromata in which are produced hyaline, usually non-septate, primary conidia on elongate, phialidic conidiogenous cells, and often producing filiform beta conidia.

The other member of the Diaporthaceae in this study is the genus *Mazzantia*. Although not the type species of the genus *Mazzantia*, *M. napelli* is similar to the type, *M. galii* (Fr.) Mont., in stromatal and ascomatal morphology, anamorph, and occurrence on dicotyledonous herbaceous hosts. *Diaporthe* and *Mazzantia* are similar in producing well-developed stromata immersed in relatively newly killed wood or stems. In *Mazzantia* the stromata are well-developed covering only one or a few, immersed ascomata. Although placed in the genus *Mazzantiella* Höhn., the anamorph of *Mazzantia* is similar to *Phomopsis* in producing hyaline, one-celled, elongate conidia on filiform, phialidic conidiogenous cells in a pycnidial locule (Wehmeyer 1975).

Other taxa.—The anamorph species, *Greeneria uvicola*, the cause of bitter rot of grapes, was recently determined to belong in the Diaporthales (Farr et al 2001). Despite its inclusion in this expanded account of the Diaporthales, *G. uvicola* is not closely affiliated with any of the taxa included in this study (FIGS. 1–

3) and thus it is not possible to determine what its teleomorph may be, if one exists.

One species of the genus *Wuestneia* having a *Harknessia* Cooke anamorph and the type species of *Harknessia*, *H. eucalypti*, grouped together in all analyses (FIGS. 1–3). The connection between *Wuestneia* and its anamorph *Harknessia* was established by Reid and Booth (1989) and is confirmed by these data. *Harknessia lythri* did not group with *Harknessia* and *Wuestneia*. This species is unusual in having longitudinal striations on the conidia (Farr and Rossman 2001) and may not belong in that genus. Many additional species of *Harknessia* have been described and most of them lack a known sexual state.

Two species, *Hercospora tiliae* and *Melanconis desmazierii*, grouped together and may represent the Pseudovalsaceae (FIGS. 1–3). *Melanconis desmazierii* forms rudimentary stromata and produces a typical *Melanconium* anamorph. The well-developed stromata of *Hercospora tiliae* developed in culture producing a distinctive *Rabenhorstia* anamoph (Sutton 1980). Both species occur on species of *Tilia* in temperate regions.

Our study suggests that four of the five major families previously established for members of the Diaporthales should be recognized, albeit circumscribed differently, and that two additional major lineages exist within the order. The lineages discussed in this paper were defined using a relatively high number of diaporthalean taxa, certainly the greatest number to date. Emphasis was placed on obtaining type species of key genera including those of anamorph genera where possible. Additionally, an attempt was made to locate fresh, accurately identified material from which single ascospore cultures could be isolated. Despite considerable effort, less than half of the type species of the 98 genera in the Diaporthales were obtained. With an increased number of taxa, especially those representing type species, it is likely that additional lineages will be defined. It is also expected that sequencing additional genes for taxa in this order will make it possible to identify relationships of genera within families and to determine morphological characters for reliable generic identifications.

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