Hydrophobin genes and their expression in conidial and aconidial *Neurospora* species

Robert D. Winefield a,b, Elena Hilario a, Ross E. Beever c, Richard G. Haverkamp b, Matthew D. Templeton a,*

a The Horticulture and Food Research Institute of New Zealand Ltd., Private Bag 92-169, Auckland, New Zealand
b Institute of Technology and Engineering, Massey University, Private Bag 11-222, Palmerston North, New Zealand
c Landcare Research, Private Bag 92-170, Auckland, New Zealand

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Abstract

Homologs of the gene encoding the hydrophobin EAS from *Neurospora crassa* have been identified both in the other conidial species of *Neurospora* (*N. discreta, N. intermedia, N. sitophila,* and *N. tetrasperma*) and selected aconidial species (*N. africana, N. dodgei, N. lineolata, N. pannonica,* and *N. terricola*). Southern blot analysis indicated the presence of a single gene in all species examined. EAS-like proteins were purified from the conidial species and each was shown to be the proteolytically processed gene-product of the corresponding *eas* homolog. While EAS-like proteins were not detected in the aconidial species, putative *eas* transcripts were detected in some isolates following RT-PCR and the aerial hyphae of these species were hydrophobic. DNA sequences of the coding region of the *eas* homologs were amplified by PCR and cloned and sequenced from all species except *N. pannonica*. Phylogenetic analysis of these sequences produced two clusters, the first comprising the conidiating species *N. crassa, N. intermedia, N. sitophila,* and *N. tetrasperma* forming a closely related group with *N. discreta* more distant, and the second comprising the aconidial species *N. africana, N. dodgei, N. lineolata* forming another closely related group with *N. terricola* more distant.

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1. Introduction

The genus *Neurospora*, which includes a range of conidial and aconidial species, has been widely used for studies of genetics and, more recently, population biology and species recognition (Perkins and Turner, 1988; Davis, 2000; Dettman et al., 2003a,b). Traditionally, *Neurospora* has been separated from the genus *Gelasinospora* on the basis of ascospore ornamentation, but phylogenetic analyses using ITS rDNA sequence data from species of *Neurospora* and a selection of *Gelasinospora* species clearly demonstrated that ascospore morphology did not accurately reflect phylogenetic relationships (Dettman et al., 2001). However a proposal to synonymise *Gelasinospora* with *Neurospora* based primarily on 28S rDNA sequence data (Garcia et al., 2004) is not supported by a multiple gene study including a wider sampling of related genera (Cai et al., 2006). Nevertheless, the five conidial species, including the model species *Neurospora crassa*, form a monophyletic clade distinct from the aconidial species which are dispersed through a number of clades (Dettman et al., 2001; Garcia et al., 2004; Cai et al., 2006).

*Neurospora crassa* macroconidia (hereafter referred to as conidia), which are typically produced in abundance on recently burnt substrates, are strongly hydrophobic and aerially dispersed (Perkins and Turner, 1988). Hydrophobicity is conferred by an amphipathic membrane of fascicles of individual fibers or rodlets, which are absent from ‘easily wettable’ mutants (*eas = ccg-2*) (Beever and Dempsey,
1978). Rodlets are comprised primarily of hydrophobins, a class of small hydrophobic proteins that are secreted by diverse filamentous fungi onto air-exposed cell-wall surfaces (Wessels, 1997; Wösten, 2001). Here they spontaneously polymerize into amphipathic membranes, with the hydrophilic surface facing the hydrophilic cell wall and the hydrophobic surface facing the environment. Hydrophobins are typically about 100 amino acids in length with characteristic hydrophathy patterns, and contain eight cysteine residues arranged in a conserved pattern (Schuren and Wessels, 1990). Based on physical properties and sequence similarity, hydrophobins are classified into two classes (Wessels, 1994): Class I hydrophobins form highly insoluble membranes that can resist boiling in 2% (w/v) sodium dodecyl sulfate (SDS), while membranes formed by Class II hydrophobins are soluble in this reagent and do not have the characteristic rodlet morphology. The eas gene hydrophobin from N. crassa, which belongs to Class I, has been cloned and sequenced (Bell-Pedersen et al., 1992; Lauter et al., 1992).

Rodlets from N. crassa, which have been shown to be formed from the post-translationally cleaved product of eas, have been purified, characterized and used to study the structure and assembly of hydrophobins (Templeton et al., 1995; Mackay et al., 2001; Kwan et al., 2006).

Previous studies have demonstrated the presence of rodlets, indistinguishable from those of N. crassa, on conidia of other conidiating Neurospora species (Hallett and Beever, 1981). The present study examined a range of conidial and aconidial species of Neurospora for the presence and expression of eas homologs, which were found to be present in all conidial and aconidial species examined. Phylogenetic analysis of the hydrophobin genes confirms and supports published proposals of the relationships between conidial and aconidial species (Dettman et al., 2001; García et al., 2004; Cai et al., 2006).

2. Materials and methods

2.1. Fungal strains and cultivation

The identification number and designated species of the strains used in this study are listed in Table 1. Isolates were maintained in the short term as agar slopes of Vogel's medium N (Vogel, 1964) supplemented with 2% (w/v) sucrose and 1.2% (w/v) agar or permanently as silica gel stocks at 4°C (Perkins, 1977). Cultures were grown with a 12-h on 12-h off cycle of either fluorescent or long wave UV light.

For DNA extraction, liquid cultures (50 mL) were grown in 250 mL flasks of Vogel's medium N supplemented with 2% (w/v) sucrose and 1.2% (w/v) agar or permanently as silica gel stocks at 4°C (Perkins, 1977). Cultures were grown with a 12-h on 12-h off cycle of either fluorescent or long wave UV light.

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probes were radioactively labeled with (α-<sup>32</sup>P) dCTP (GE-Healthcare) using the Rediprime kit (GE-Healthcare) as instructed by the manufacturer. Prehybridization (30 min) and hybridization (overnight) were carried out in tubes using Church buffer at 65 °C (Church and Gilbert, 1984). The membranes were washed with SSC/SDS buffers of increasing stringency at 65 °C with the final wash in 0.1 × SSC, 0.1% (w/v) SDS (Sambrook and Russell, 2001). Autoradiography was carried out using a Storm 840 Phosphor Imaging System (GE-Healthcare).

2.6. PCR and RT-PCR

Homologs of <i>eas</i> were amplified from genomic DNA using PCR (5 min, 94 °C, 35 cycles of: 94 °C, 30 s, 60 °C, 30 s, and 72 °C, 1 min, with a final extension step of 7 min at 72 °C). The sequences of the <i>eas</i> 5' primer (ATG CAG TTC ACC AGC GTC TTC ACC ATC) and <i>eas</i> 3' primer (TTA GGC AAC GCA GTT GGC AGC GTT GAT) were based on the respective regions of the coding sequence of <i>eas</i> from <i>N. crassa</i> <i>bda</i> (Bell-Pedersen et al., 1992). Sequencing of ITS/5.8S rDNA regions was performed using primers ITS5 (GGG AGT AAA AGT CGT AAC AAG G) and ITS4 (TCC TCC GGT TAT TGA TAT GC) (White et al., 1990). RT-PCR analysis of total mRNA was carried out using a SuperScript Kit (Invitrogen, Carlsbad, CA). RNA was quantitated using a ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). First strand synthesis was performed with DNase I treated total RNA samples as described by the manufacturer. These samples were diluted 100-fold with DNase I treated total RNA samples as described by Wilmington, DE). First strand synthesis was performed with DNase I treated total RNA samples as described by Wilmington, DE). First strand synthesis was performed with DNase I treated total RNA samples as described by Wilmington, DE). First strand synthesis was performed with DNase I treated total RNA samples as described by Wilmington, DE). First strand synthesis was performed with DNase I treated total RNA samples as described by Wilmington, DE). First strand synthesis was performed with DNase I treated total RNA samples as described by Wilmington, DE).

2.7. DNA sequencing

Homologues of <i>eas</i> and their cDNAs were cloned into pGEM-T or pGEM-T Easy vectors (Promega, Madison, WI). Sequence reactions were performed using either an ABI Prism BigDye Terminator Cycle Sequencing Kit (Applera, Norwalk, CT) or a DYEEnamic ET terminator cycle sequencing pre-mix kit (GE-Healthcare). DNA sequencing was performed on an ABI 377XL automated DNA Sequencer at the Waikato DNA Sequencing Facility (University of Waikato, Hamilton, New Zealand), or on a LI-COR 4000LD IR2 automated sequencer (LI-COR, Lincoln, NE) at the Department of Molecular Medicine and Pathology, Auckland University. The sequences have been deposited in GenBank (AY577545, AY577546, and AY577548 to AY577557).

2.8. Sequence analysis

The nucleotide multiple sequence alignments were produced with default settings in ClustalX 1.83: gap opening penalty: 15; gap extension: 6.66; delay divergent sequences at 30%; DNA transition weight: 0.5; DNA weight matrix: IUB (Thompson et al., 1997). The alignment was prepared with Multalin (version 5.4.1) using the Dayhoff-8-0 settings (Dayhoff et al., 1979; Corpet, 1988). The optimal model of evolution that describes each data set was selected with the program ModelTest 3.06 (Posada and Crandall, 1998). The maximum likelihood tree was constructed with PAUP 4.0b10 (Swoford, 2003) using the transition/transversion value, base frequencies, and substitution matrices specified by Modeltest 3.06. The <i>Neurospora</i> spp. <i>eas</i> maximum likelihood phylogenetic tree was constructed assuming the HKY85 model: a transition/transversion ratio equal 1.4903 (kappa = 2.7390839); nucleotide frequencies:

Table 1
<i>Neurospora</i> strains used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Sporulation class (breeding system)</th>
<th>Strain</th>
<th>Origin (original designation)</th>
<th>GenBank # for &lt;i&gt;eas&lt;/i&gt; homolog&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;i&gt;N. africana&lt;/i&gt;</td>
<td>Aconidial (homothallic)</td>
<td>Sta</td>
<td>FGSC° 1740 (Africana N200)</td>
<td>AY577552</td>
</tr>
<tr>
<td>&lt;i&gt;N. crassa&lt;/i&gt;</td>
<td>Conidial (heterothallic)</td>
<td>StaA</td>
<td>J.R.S. Fincham (St. Lawrence Sta)</td>
<td>AY577545</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;i&gt;eas&lt;/i&gt; mutant</td>
<td>FGSC 2961 (UCLA191)</td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;N. discreta&lt;/i&gt;</td>
<td>Conidial (heterothallic)</td>
<td>FGSC 3228 (Kirbyville-6)</td>
<td>AY577551</td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;N. dodgei&lt;/i&gt;</td>
<td>Aconidial (homothallic)</td>
<td>FGSC 1692 (PR300)</td>
<td>AY577553</td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;N. intermedia&lt;/i&gt;</td>
<td>Conidial (heterothallic)</td>
<td>FGSC 1766 (Taipei-1c)</td>
<td>AY577546</td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;N. lineolata&lt;/i&gt;</td>
<td>Aconidial (homothallic)</td>
<td>FGSC 1910 (A-236)</td>
<td>AY577554</td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;N. pannonica&lt;/i&gt;</td>
<td>Aconidial (homothallic)</td>
<td>FGSC 7221 (TRTC 5137)</td>
<td>AY577550</td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;N. pannonica&lt;/i&gt;</td>
<td>Conidial (heterothallic)</td>
<td>FGSC 2216 (P8085)</td>
<td>AY577550</td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;N. sittphila&lt;/i&gt;</td>
<td>Conidial (heterothallic)</td>
<td>FGSC 1889 (WFS 5000)</td>
<td>AY577555</td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;N. terricola&lt;/i&gt;</td>
<td>Aconidial (homothallic)</td>
<td>FGSC 1720 (85A)</td>
<td>AY577548</td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;N. tetrasperma&lt;/i&gt;</td>
<td>Conidial (pseudohomothallic)</td>
<td>FGSC 2541 (259.35)</td>
<td>AY577549</td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;N. torui&lt;/i&gt;</td>
<td>Conidial (pseudohomothallic)</td>
<td>FGSC 1889 (WFS 5000)</td>
<td>AY577555</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, KS.
<sup>b</sup> This study and Benson et al. (2002).
$A = 0.17510$, $C = 0.38370$, $G = 0.20010$, $T = 0.24110$; and equal distribution of rates at variable sites. The majority-rule consensus tree was obtained by bootstrap analysis with PAUP: 100 replicates, stepwise addition of starting trees, branch-swapping by tree-bisection-reconnection. Branch lengths were obtained from one tree with the neighbor joining algorithm.

### 3. Results

#### 3.1. EAS gene survey

The eas probe prepared from N. crassa hybridized to discrete bands of digested genomic DNA from all of the species examined (Fig. 1). The probe hybridized at high stringency to only one band in Xho I- and Hind III-digested DNA in most instances. Where the probe hybridized to two bands subsequent nucleotide sequence analysis revealed a Hind III-restriction site in the fragment. These results indicate the presence of a single-copy of eas in each genome.

#### 3.2. Sequencing and phylogenetic analysis

A putative eas gene homolog was obtained and sequenced from the genomic DNA of all species except N. pannonica (Table 1). We attribute the inability to amplify a putative homolog from N. pannonica, despite the presence of a band in the Southern analysis (Fig. 1B), to a polymorphism at one of the primer binding sites. In order to confirm the identity of a number of the isolates, the ITS/5.8S sequence was obtained from the same DNA preparation and compared with published sequences (Dettman et al., 2001). Our ITS sequence of N. toroi (561 bp = AY577556) matched exactly the authentic sequence of N. tetrasperma (AF388929); our sequence of N. lineolata (560 bp) and N. pannonica (560 bp) matched exactly the authentic sequence for those species (AF388924, AF388925).

The two N. crassa eas gene sequences (Table 1) from this study were identical to each other and >99% similar to the eas/ccg-2 sequence (S50953) reported by Bell-Pedersen et al. (1992), the occasional nucleotide differences presumably reflects differences between isolates. The N. crassa eas gene contains a single intron (Eberle and Russo, 1992; Bell-Pedersen et al., 1992), which appears to be conserved in all Neurospora eas homologs.

The homologs are very similar at both the DNA and predicted protein level (Fig. 2). The insertion and deletion differences present in the first exon of the homologs from the aconidial species (N. africana, N. dodgei, N. lineolata, N. terricola) occur in multiples of three, and thus do not introduce potential frame-shifts. Alignments predicted that protein sequences from aconidial species would differ from the N. crassa EAS sequence at positions 23–25 (Fig. 2). None of these differences introduces significant changes to the hydropathy profiles for EAS (data not shown). Neither is there a significant increase in the distance between disulfide-linked cysteines, nor do any of the changes fall in a region considered to be critical to either the monomeric or polymeric EAS structure (Kwan et al., 2006).

A gene tree constructed by maximum likelihood analysis (Fig. 3) indicates that the eas genes of the conidiating species N. crassa, N. intermedia, N. tetrasperma, N. toroi, and N. sitophila form a closely related group, with the conidiating N. discreta more distant. Likewise, the aconidial species N. africana, N. dodgei, N. lineolata form a second closely related group with the aconidial N. terricola more distant.

#### 3.3. Analysis of hydrophobins in conidial species

As observed for N. crassa, the rodlet extracts from each of the conidial species were resolved into three separate isoforms of EAS (Mackay et al., 2001). The isoform variation is due to differential processing of the N-terminus and oxidation of the methionine residue (Mackay et al., 2001; Kwan et al., 2006). ESI/MS of each EAS isoform isolated...
demonstrated that the pattern of post-translational modification of EAS is preserved in the other conidiating species (Table 2). However, in the case of N. sitophila additional isoforms were observed that indicate additional processing of the leader sequence at position Ala6-Asn7.

### 3.4. Expression of eas in aconidial species

Aerial hyphae of the aconidial species and N. toroi were able to support intact water droplets indicating that, like wild-type N. crassa, they are hydrophobic. However TFA-treated mycelial extracts of the aconidial strains obtained using the hydrophobin extraction method for N. crassa did not reveal any protein with an RP-HPLC elution profile characteristic of that of EAS (data not shown). We conclude that an EAS-like protein was not present in a form or in a concentration similar to that seen in the conidiating species or, alternatively, is present but is so strongly attached to the cell wall, that it cannot be solubilised even by TFA.

To further explore the transcription of eas homologs in aconidial strains, we analyzed mRNA expression using total RNA extracted from cultures grown under conditions...
that support conidiogenesis and the expression of EAS in *N. crassa*. RT-PCR using conserved primers to the housekeeping gene *gapdh* amplified a product of the expected size (440 bp) from all species examined (Fig. 4A). None of the negative controls amplified any product, indicating that the samples were free of contaminating genomic DNA.

For the amplification of *eas* homologs, primers were redesigned to sequences strongly conserved in all isolates to ensure that amplification was not biased by a lack of homology to the first-strand template. RT-PCR using these primers amplified products of the expected size (250 bp) from three of the five aconidial isolates. The *eas* cDNA was, as expected, strongly expressed in *N. crassa* StA4 and *N. toroi* (Fig. 4B), the latter conidiating vigorously under the higher temperature used in this experiment. We conclude that *eas* is expressed in at least some of the aconidial species when they are cultured in conditions that stimulate both conidiation and the production of EAS in *N. crassa*. The expression of *eas* mRNA by *N. toroi*, is consistent with this species status as a conidiating species.

4. Discussion

The finding of an *eas* homolog in each of the conidiating species of *Neurospora* complements the observation of rodlets on the surface of macroconidia of these species (Hallett and Beever, 1981). In each species the EAS protein is produced by a single-copy of a gene with an almost identical coding region to that of *N. crassa*, a finding consistent with the proposed monophyly of this ‘macroconidial’ group, which now includes the relatively recently recognized *N. discreta* as well as three more as yet un-named species recognized by phylogenetic analysis (Dettman et al., 2001, 2003a,b).

The hydrophobin rodlet layer was initially associated with conidial dispersal of dry-spored fungi (Beever and Dempsey, 1978) and the expression of *eas* in *N. crassa* is closely correlated with conidiation (Lauter et al., 1992). Hydrophobins have subsequently been implicated in diverse aspects of fungal physiology and morphology typically involving air–water interfaces (Wo¨sten and de Vocht, 2000; Wo¨sten, 2001; Whiteford and Spanu, 2002) including

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**Table 2**  
ESI/MS mass measurements of *Neurospora* EAS extracts

<table>
<thead>
<tr>
<th>Species</th>
<th>Isoform Ia (Observed mass)</th>
<th>Isoform IIa (Predicted mass)</th>
<th>Isoform IIIa (Predicted mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. crassa</em> StA4</td>
<td>8180.4 (8178.26)</td>
<td>7908.0 (7904.98)</td>
<td>—</td>
</tr>
<tr>
<td><em>N. crassa</em> Sta</td>
<td>8179.0 (8178.26)</td>
<td>7906.0 (7904.98)</td>
<td>—</td>
</tr>
<tr>
<td><em>N. discreta</em></td>
<td>8207.0 (8205.29)</td>
<td>7906.0 (7904.98)</td>
<td>—</td>
</tr>
<tr>
<td><em>N. intermedia</em></td>
<td>8180.0 (8178.26)</td>
<td>7907.0 (7904.98)</td>
<td>—</td>
</tr>
<tr>
<td><em>N. tetrasperma</em></td>
<td>8177.0 (8178.26)</td>
<td>7904.0 (7904.98)</td>
<td>—</td>
</tr>
<tr>
<td><em>N. sitophila</em></td>
<td>8209.0 (8210.25)</td>
<td>7934.0 (7936.97)</td>
<td>7632.0 (7637.85)</td>
</tr>
</tbody>
</table>

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**Fig. 4.** RT-PCR analysis of *eas* transcript homologs from aconidial *Neurospora* spp. Amplification products of the second strand RT-PCR using *gapdh*-specific (A) and *eas*-specific (B) primers are shown. Lanes: 1, *N. africana*; 2, *N. dodgei*; 3, *N. lineolata*; 4, *N. pannonica*; 5, *N. terricola*; 6, *N. toroi* (= *N. tetrasperma*); 7, *N. crassa*; 8, water control; 9, positive control (*N. crassa* genomic DNA).
the formation of aerial hyphae (Wessels, 1997). While the aerial mycelium of the aconidial species apparently lacks extractable EAS protein, it is nevertheless hydrophobic, consistent with the presence of hydrophobin rodlets. The transcription, albeit it weak, of the conidial hydrophobin in some of the aconidial species supports the proposal that rodlets may be formed.

In N. crassa, eas transcription is under a variety of cis-acting controls including a negative developmental element that suppresses eas expression in germinating conidia and vegetative mycelium but can be over-ridden independently by circadian clock and light-responsive elements (Lauter et al., 1992; Arpaia et al., 1993; Bell-Pedersen et al., 1996; Correa and Bell-Pedersen, 2002). Analysis of the promoter regions of the various eas homologs would provide insight into possible expression of these genes. While we failed to amplify an eas homolog in N. pannonica, the presence of one in the relatively closely related N. terribicola and the distinctive restriction pattern following Southern hybridization (Fig. 1), makes us suspect that there is a homolog in this species but that it could not be amplified using the primers used in this study.

As well as being characterized by the presence of conidia, the conidial species are all outbreeding with heterothallic or pseudohomothallic breeding systems, whereas the aconidial species are inbreeding and homothallic (Perkins and Turner, 1988). The breeding systems of the outbreeding species are controlled by the idiomorphic mating type genes mat A and mat a, with ascospores of the heterothallic species containing one or other idiomorph and those of the single pseudohomothallic species (N. tetrasperma) typically containing both idiomorphs albeit in separate nuclei (Poggeler, 1999; Perkins and Turner, 1988). It is generally considered likely that the homothallic species have evolved from heterothallic ancestors (Glass et al., 1990), but it is probable that homothallism is polyphyletic in origin as in two clades at least some species retain both mat A and mat a idiomorphs, whereas in the third clade, some species retain only the mat A idiomorph (Glass et al., 1990; Dettman et al., 2001; Garcia et al., 2004). The presence of some as yet unidentified heterothallic isolates in some aconidial Gelasinospora species (Dettman et al., 2001), and the presence of heterothallic and homothallic species in outgroup such as Sordaria (Glass et al., 1990), support the hypothesis of a heterothallic ancestor for Neurospora. In contrast, it is not apparent that this ancestor was conidial, as the ability to produce conidia is apparently restricted to the outbreeding species (Garcia et al., 2004) and is not present in outgroups such as Sordaria. Nevertheless, the presence of eas homologs in species of at least two of the three aconidial clades recognized by Dettman et al. (2001) and Garcia et al. (2004), suggests that the ancestor possessed an eas gene homolog.

Our sequence studies allow commentary on delineation and phylogenetic relationships of some of the species studied. The similarity of both the ITS and eas gene of our isolates of N. toroi to N. tetrasperma (they differed only by one nucleotide) support the proposal of Perkins that these taxa are conspecific (Perkins et al., 1976; Perkins and Turner, 1988), a conclusion formalized in the placement of N. toroi as a synonym of N. tetrasperma by Garcia et al. (2004). The good conidiation of our isolate of N. toroi at 34 °C is consistent with this conclusion as strains of N. tetrasperma often conidiate much better at this temperature than at 25 °C (Perkins and Turner, 1988). Identical eas sequences were recovered from N. africana and N. dodgei, consistent with the proposed close relationship between these homothallic species based on morphological, cytological, and sequence data (Glass et al., 1990; Dettman et al., 2001). However, Garcia et al. (2004) differentiate N. dodgei from N. africana by differences in ascospore morphology, although they do place N. africana in synonymy with N. galapagosenis (not examined in the present study). The branching pattern of our eas gene tree (Fig. 2) is consistent with the multiple gene tree species phylogenies proposed by Dettman et al. (2001, 2003a) and by Cai et al. (2006), with the species studied here grouped into two clades: one aconidial including N. africana (= N. galapagensis) and N. dodgei with N. lineolata slightly more distant; and the second comprising the conidial species with N. discreta separated on a basal branch from the other species, which are themselves poorly resolved.

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