Heterogeneity in NTS of rDNA in localized populations of *Neurospora*

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***Neurospora*, a filamentous ascomycete, occurs in tropical climate. We have examined the relationships that exist among wild isolates in local populations of *Neurospora* by using PCR–RFLP to analyse the Non Transcribed Spacer (NTS) of the rDNA. The size of the NTS was found to be polymorphic. Phylogenetic trees constructed using this data support the hypothesis that mating groups represent monophyletic lineages. Specifically, the results suggest monophyly for *N. tetrasperma* and *N. sitophila*, a close relationship between the pseudohomothallic species *N. tetrasperma* and true heterothallics, and the placement of *N. discreta* as the most distant among all species. The yellow ecotype of *N. intermedia* has been assigned a putative phylogenetic position for the first time. Surprisingly, these yellow isolates, considered to be an ecotype of *N. intermedia*, appear to represent a separate lineage, distinct from a larger *N. crassa*/*N. intermedia* clade.**

SHEAR and Dodge[1] identified two heterothallic species of *Neurospora*, *N. crassa* and *N. sitophila*, each having two stable mating types, *mat A* and *mat a*. They also described a four-spored, self-fertile pseudohomothallic species, *N. tetrasperma*. In this species, nuclei of both mating types, *mat A* and *mat a*, are enclosed in each ascospore. Subsequently two more heterothallic species, *N. intermedia*[2] and *N. discreta*[3] were identified. There are two ecotypes of *N. intermedia*, orange and yellow. The orange ecotype is pinkish or 'salmon' orange coloured[4]. The 'yellow' ecotype is saffron yellow[5,6]. Both heterothallic and pseudohomothallic species have been isolated from pustules on burnt plant material[4,7]. In addition, a number of non-conidiating homothallic species, having a dull gray mycelium, were also isolated from soil. These included *N. africana* and *N. galapagosensis*[8], *N. dodgei*[9], *N. lineolata*[10], *N. terricola*[11], and *N. pannonica*[12]. In *N. terricola* and *N. pannonica*, sequences related to both *mat A* and the *mat a* idiomorph were found[13]. In others, only the sequence related to the *mat A* idiomorph has been detected.

The process of assigning a wild isolate of *Neurospora* to a species involves crossing it to tester strains and looking for the production of black ascospores[4,7,14]. In mating of two strains belonging to the same species, the fertility was very high, as observed from the rapid production of large numbers of black ascospores (50 to 90%). In contrast, inter-species crosses were either sterile or the fertility was highly reduced.

Since at a lower taxonomic level highly conserved regions of the genome like the 18S rRNA genes do not differ significantly so as to reveal differences, evolution within the genus *Neurospora* has been traced in studies involving less conserved regions. These include mitochondrial plasmids[15], mitochondrial DNA[16,17], random fragments of the nuclear DNA[18], sequence of the *frq* gene[19,20], sequences upstream of the *al-1* and *frq* genes[21], sequence of *gpd* gene[22,23], sequences of the mating type idiomorphs[22–24], and sequence of ITS/5.8S (ref. 24).

Though the concept of species based on fertility in crosses with tester strains of opposite mating type has been validated in these studies, different approaches have given different phylogenies[21–23]. This has been attributed to ancestral polymorphism or a horizontal flow of genes or both[21]. Another reason for observed differences in phylogeny could be that different genes are evolving at different rates and thus give different gene trees. It has been suggested that the mating type genes evolve faster compared to housekeeping genes[22].

Neurospora grows in warm and tropical climates on burnt sugary or starchy substrates. We had an opportunity to collect wild *Neurospora*, use them for phylogenetic analysis and to examine the relationships that exist between wild isolates in local populations. Wild isolates of *Neurospora* were collected from bright orange pustules on burnt sugarcane from agricultural fields in the vicinity of Bangalore (Karnataka) or yellow growth on roasted corncobs in Ujjain (Madhya Pradesh). Strains were also isolated from soil by plating methods. The recovery of the four heterothallic species and one pseudohomothallic species from close-by areas indicates the existence of a high degree of species diversity in this region. The Non Transcribed Spacer (NTS) of the rDNA was amplified for the first time from the different species testers and these wild isolates of *Neurospora*. The PCR products were subjected to RFLP analyses. Variation in the NTS was used to trace the phylogeny in the genus *Neurospora*.

The strains used in this study are listed in Table 1. The strains were isolated from burnt substrates[4], or soil[25]. The mating types were determined by spot crosses on lawns of *fluffy* (*fl*) tester strains[26]. The genetic crossing was performed as described by Davis and de Serres[27]. Species were identified based on fertility in crosses with the tester strains[4]. Cultures were stored as silica-gel stocks or on agar at – 20ºC.

For DNA isolation, mycelia were grown in liquid Vogel's Medium N[27] supplemented with 1.5% sucrose using seven-day-old conidia as inoculum. After 18–24 h

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Table 1. List of *Neurospora* strains used for phylogenetic analysis

Species	Strain	Mating type	Colour	Isolation		Source
				Place	Substrate	
N. crassa	OR23-IV (FGSC 2489)[a]	A	Orange	–	–	FGSC
N. crassa	Bangalore 1991-1	A	Orange	Bangalore	Soil	This study
N. sitophila	*fl*^{p}; *Sk-1*^{K} (FGSC 4763)[a]	*a*	Orange	–	–	FGSC
N. sitophila	Mercara	*a*	Orange	Madikeri	Soil	This study
N. sitophila	RM 1989-10	*A*	Orange	Bangalore	Soil	This study
N. discreta	P851(FGSC 3228)[a]	*A*	Orange	–	–	FGSC
N. discreta	Bandipur	*a*	Orange	Bandipur	Soil	This study
N. discreta	P1859	*A*	Orange	–	–	D. D. Perkins
N. discreta	P1913	*A*	Orange	–	–	D. D. Perkins
N. tetrasperma	85 (FGSC 1270)[a]	*A*	Orange	–	–	FGSC
N. tetrasperma	Maddur 1993-24	(*A* + *a*)	Orange	Maddur	Sugarcane	This study
N. tetrasperma	Maddur 1993-38	(*A* + *a*)	Orange	Maddur	Sugarcane	This study
N. tertasperma	Maddur 1993-42	(*A* + *a*)	Orange	Maddur	Sugarcane	This study
N. intermedia	Shp-1 (FGSC 3416)[a]	*A*	Orange	–	–	FGSC
N. intermedia	RM 125-2[b]	*A*	Orange	–	–	This study
N. intermedia	Maddur 1991-1	*a*	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1991-3	*A*	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1991-4	*A*	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1991-21	*a*	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1991-60	*A*	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1991-76	*a*	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1991-78	*a*	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1991-85	*A*	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1991-101	*a*	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1991-107	*a*	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1993-10	n.d.	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1993-21	n.d.	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1999-44	*A*	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1999-46	*A*	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1999-52	n.d.	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1999-56	*a*.	Orange	Maddur	Sugarcane	This study
N. intermedia	Maddur 1991-100	*A*	Albino	Maddur	Sugarcane	This study
N. intermedia	Maddur 1993-19	*a*	Albino	Maddur	Sugarcane	This study
N. intermedia	RM 190-1[b]	*a*	Yellow	–	–	This study
N. intermedia	Ujjain 12	*a*	Yellow	Ujjain	Corncob	A. Pandit
N. intermedia	Ujjain 16	*a*	Yellow	Ujjain	Corncob	A. Pandit
N. intermedia	Ujjain 18	*a*	Yellow	Ujjain	Corncob	A. Pandit
N. intermedia	Ujjain 25	*a*	Yellow	Ujjain	Corncob	A. Pandit
N. intermedia	Ujjain 26	*A*	Yellow	Ujjain	Corncob	A. Pandit
N. intermedia	Ujjain 27	*A*	Yellow	Ujjain	Corncob	A. Pandit
N. intermedia	Ujjain 28	*a*	Yellow	Ujjain	Corncob	A. Pandit
N. intermedia	Ujjain 30	*a*	Yellow	Ujjain	Corncob	A. Pandit
N. intermedia	Ujjain 41	*a*	Yellow	Ujjain	Corncob	A. Pandit

[a]Species tester; [b]Species tester developed for local strains; n.d., not determined.

of growth at 34ºC on a rotary shaker, the mycelium was harvested, washed and lyophilized. DNA was isolated with minor modifications of the method of Zolan and Pukkila[28].

The NTS region of the ribosomal DNA was amplified using the primers CNL12 (5′CTGAACGCCTCT-AAGTCAG3′) and CNS 1 (5′GAGACAAGCATATGA-CTACTG3′) (ref. 29; Figure 1 *a*). Fifty microlitre reactions were carried out in 0.5 ml PCR tubes (Bangalore Genei Pvt Ltd). The reaction mixture contained, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 3.0 mM $MgCl_2$, 0.4 mM of dNTPs (Amersham Pharmacia Biotech Asia Pacific Ltd), 1.5 units of *Taq* DNA polymerase (Amersham Pharmacia Biotech Asia Pacific Ltd) and 0.5 µM of each of the primers (Bangalore Genei Pvt Ltd.). The reactions were carried out in a Techne Progene Thermal Cycler, with maximum ramp rate set for temperature transitions. An initial denaturation step for 5 min at 94°C was followed by 35 cycles of amplification. Each cycle had denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 4 min. After 35 cycles, the samples were further incubated for 10 min at 72°C (final extension step). Negative controls (lacking DNA) were included in each set of experiments

to test for the presence of contaminating DNA in the reagents and reaction mixtures. Aliquots (5–10 µl) of the reaction mixture were separated on 1% agarose gels in 10 mM Tris-acetate (pH 8.0) buffer containing 0.5 µg/ml ethidium bromide and then visualized by UV illumination. For restriction analysis aliquots (5–10 µl) of the PCR products were digested for 3 h with 2.5–5 units of restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Xho*I, *Mbo*I) in 20 µl reaction volume as per the manufacturers instructions (Bangalore Genei Pvt Ltd and Amersham Pharmacia Biotech Asia Pacific Ltd). Reaction mixtures were separated on 1% agarose gels and visualized. Photographic images of all gels were recorded using a Kodak Digital Science 1D system. The images were stored as .TIFF files and the molecular mass of the bands were determined using the same program.

The RESTSITE package[30,31] was used to analyse the data generated from the restriction analysis. Each restriction site was given a unique number 'label' (1, 2, 3, etc.). Null sites were represented by a zero. In this program, site data were analysed by Jukes-Cantor and Nei & Li methods to generate genetic distances[32–35]. Standard errors were derived via jackknifing. A distance matrix was generated from these genetic distances. This distance matrix was used to construct trees by the clustering methods, namely UPGMA[36] and NJTREE[37]. The distance matrix was also used to generate phylogenies using the FITCH and KITSCH programs of the PHYLIP[38] package. The TreeView version 1.6.1[39] was used to view the trees.

In all strains, amplification of NTS region gave a single PCR product. The amplified NTS from *Neuro-*

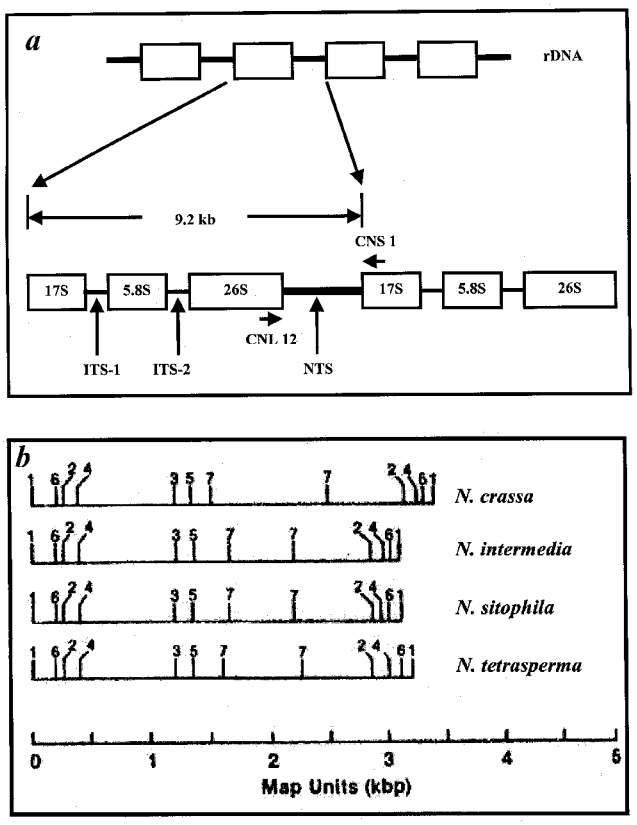

Figure 1 *a*. The rDNA repeat unit in *Neurospora crassa*. The primers used to amplify the NTS, CNL 12 and CNS 1 are indicated by small arrows. ITS-1, Intervening transcribed spacer – 1; ITS-2, Intervening transcribed spacer – 2; NTS, Non-transcribed spacer; ***b***, Restriction maps of NTS isolated from different species of *Neurospora*. Based on RFLP analysis of genomic DNA[41]. The number indicates restriction enzymes, 1, *Eco*RI; 2, *Pst*I; 3, *Sst*II; 4, *Hin*cII; 5, *Xho*I; 6, *Sma*I; 7, *Hin*dIII.

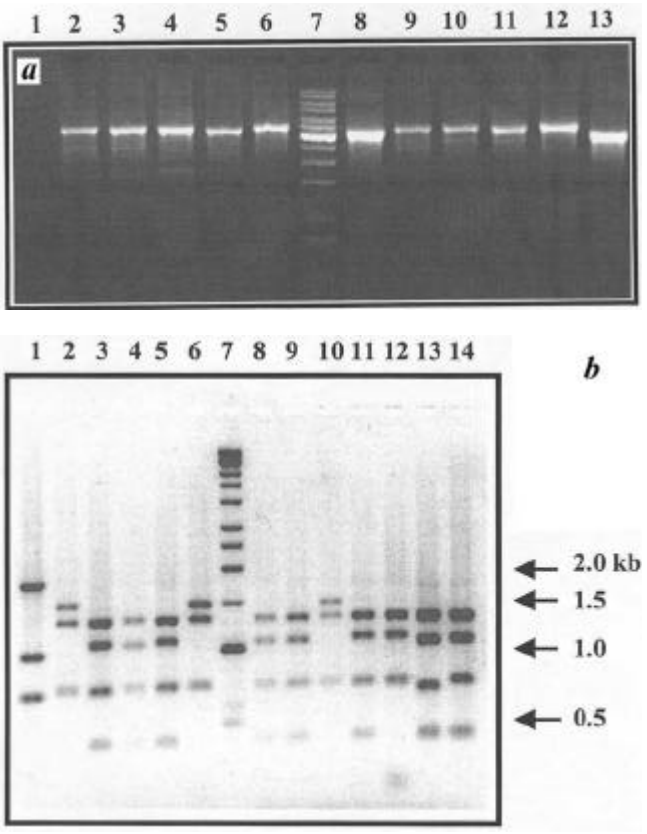

Figure 2 *a*. Amplified NTS of rDNA from wild isolates of *Neurospora*. Lane 1, Control (no DNA); 2, Shp 1 *A* (*N. intermedia*); 3, RM 125-2 (*N. intermedia*); 4, Bangalore 1991-1 (*N. crassa*); 5, RM 1989-10 (*N. sitophila*); 6, Mercara (*N. sitophila*); 8, Bandipur (*N. discreta*); 9, Maddur 1993-24 (*N. tetrasperma*); 10, Maddur 1993-38 (*N. tetrasperma*); 11, Maddur 1993-42 (*N. tetrasperma*); 12, Maddur 1991-101 (*N. intermedia*); 13, RM 190-1 (*N. intermedia* tester developed for yellow strains); Lane 7, Marker: GeneRuler DNA ladder (MBI Fermentas), size (kb) of fragments: 0.5, 1.0, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8 and 10; ***b***, RFLP in the amplified NTS of rDNA of orange strains of *N. intermedia*. The amplified products have been digested with *Hin*dIII. Lane 1, *N. crassa* OR23-IV *A* (Positive control); Lanes 2 through 14, Wild isolates of *N. intermedia* from the sugarcane fields in Maddur. Lane 2, 1991-1; 3, 1991-3; 4, 1991-4; 5, 1991-21; 6, 1991-60; 8, 1991-76; 9, 1991-78; 10, 1991-85; 11, 1991-107; 12, 1993-10; 13, 1993-19; 14, 1993-21. Lane 7, Marker: Kilobase DNA Marker (Amersham Pharmacia Biotech Asia Pacific Ltd), size (kb) of fragments: 0.5, 1.0, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 10. The image has been inverted to have a white background with the black bands for easy visualization of faint bands, which are highlighted by dots.

spora crassa (OR 23-IV *A*) was 3.5 kb. This corresponds to the size reported for genomic DNA based on Southern analysis[40–42]. The NTS was found to be similar in size in *N. sitophila*, *N. tetrasperma* and *N. intermedia* (Figure 2 *a*). It was shorter in *N. discreta* (3.1 kb). This observation was somewhat different from the report by Russell *et al.*[39]. Based on the size of the NTS, the strains of the yellow ecotype of *N. intermedia* could be classified into two distinct groups: one 2.8 and another 2.6 kb.

The restriction map of PCR-amplified NTS from *Neurospora crassa* (OR 23-IV *A*) was similar to the map previously obtained for genomic DNA by Southern analysis[40,41] (Figure 1 *b*). There were no sites for *Sac*I and *Bam*HI in the amplified NTS of any of the strains. Maddur 1991-3 was an exception in having a single *Bam*HI site. A single *Eco*RI site was conserved in all the strains. *N. tetrasperma* isolates could be distinguished because they had two sites for *Kpn*I, whereas the other species had a single site. Another character, which distinguished *N. tetrasperma* was the presence of a single site for *Hin*dIII. Similarly, the *N. discreta* isolates were different from the others since they lacked sites for *Hin*dIII, and contain a single *Pst*I site, whereas other strains had two sites. Unlike the other species, the yellow ecotype of *N. intermedia* lacked a site for *Xho*I.

The variability among strains in terms of the size of the NTS and recognition sites for different restriction enzymes probably originates from the observation that this region is rich in certain repeat sequences like TCTC, TTTT, and TTGC[41].

Different methods of phylogenetic tree construction resulted in trees of similar topology. At the species level (Figure 3), *N. discreta* was divergent from all other species. *N. tetrasperma*, a pseudohomothallic species, was placed separately from the heterothallic species. In case of *N. intermedia*, the orange ecotype seems to be more closely related to *N. crassa* than to the yellow ecotype. The two albino strains of *N. intermedia* (Maddur 1991-100 and Maddur 1993-19), which probably are mutants, differ from each other, but are related to the orange ecotype of *N. intermedia*. At the strain level (Figure 3) the grouping was quite distinct, wherein all the strains of the same species cluster together with the tester strains. However, *N. intermedia* Maddur 1991-3 occupied a distinct position because of its unique *Bam*HI site. Strains of *N. crassa* and orange ecotype of *N. intermedia* were more related to each other than to strains of *N. sitophila*. Strains of the yellow ecotype of *N. intermedia* formed a distinct monophyletic group.

The strains representing all four known heterothallic and one pseudohomothallic species of *Neurospora* recognized currently were isolated from the vicinity of Bangalore and Ujjain. The phylogeny that emerges based on the study of these strains is quite similar to what has been observed in other studies using globally collected strains[15–24].

All strains belonging to *N. tetrasperma*, *N. sitophila*, *N. discreta* and the yellow ecotype of *N. intermedia* cluster together forming respective monophyletic groups (Figure 3). This supports the concept of identifying species based on fertility in crosses with species testers[7].

N. discreta appears to have diverged from the other heterothallic species before the divergence of *N. tetrasperma* (Figure 3). Similar observation has been made in studies involving random nuclear-DNA sequences[18], *mat A-1* ORF[24], sequences of the *frq* gene[19,20], sequences upstream of *al-1* and *frq* genes[21], sequence of ITS/5.8S (ref. 23), sequences of *gpd* gene[22,23], and sequence of the *mat A*-1 and *mat a*-1 ORF's[22,23]. The smaller size of NTS in *N. discreta* (3.1 kb) compared to other heterothallics (3.5 kb) might have resulted from the loss of certain repeat sequences like TCTC, TTTT, and TTGC present in the NTS[41]. These observations support the stringent reproductive isolation of this species[3].

In the largest group of strains of a single species, the orange ecotype of *N. intermedia*, the extent of intra-species variability (in terms of genetic distance) was as high as the inter-species variability between them and strains of *N. crassa*. Consequently, strains of the orange ecotype and *N. crassa* appear as a group in phylogenetic trees (Figure 3), suggesting closeness between them. This closeness has also been observed in studies involving random nuclear-DNA fragments[18], mitochondrial DNA[17], sequences upstream of the *frq* gene[21], sequence of the *mat a-1* ORF[22,23], and sequence of ITS/5.8S (ref. 23). *N. crassa* has been found to be closer to *N. sitophila* only in two cases: based on *mat A-1* ORF[22–24] and partial sequence of *gpd*[22]. Considering all these studies together, *N. crassa* appears to be more close to the orange ecotype of *N. intermedia* than to *N. sitophila*.

Based on the trees constructed in this study (Figure 3), *N. tetrasperma* separated from the heterothallic species and evolved as a distinct monophyletic group. This has also been concluded in studies with mitochondrial DNA[17] and the *mat A-1* ORF[22–24]. Based on other regions of the genome, *N. tetrasperma* is placed with other heterothallic species. It has been placed together with *N. intermedia* based on *mat A-1* ORF and partial sequence of *gpd*[22]. In contrast, based on random nuclear-DNA fragments[17], sequences upstream of *al-1* and *frq* gene[21], sequence of ITS/5.8S[22], and sequence of *mat a-1* ORF[22,23], *N. tetrasperma* has been placed closer to *N. sitophila*. In view of its pseudohomothallic lifestyle, it seems plausible that *N. tetrasperma* diverged from the heterothallic species early.

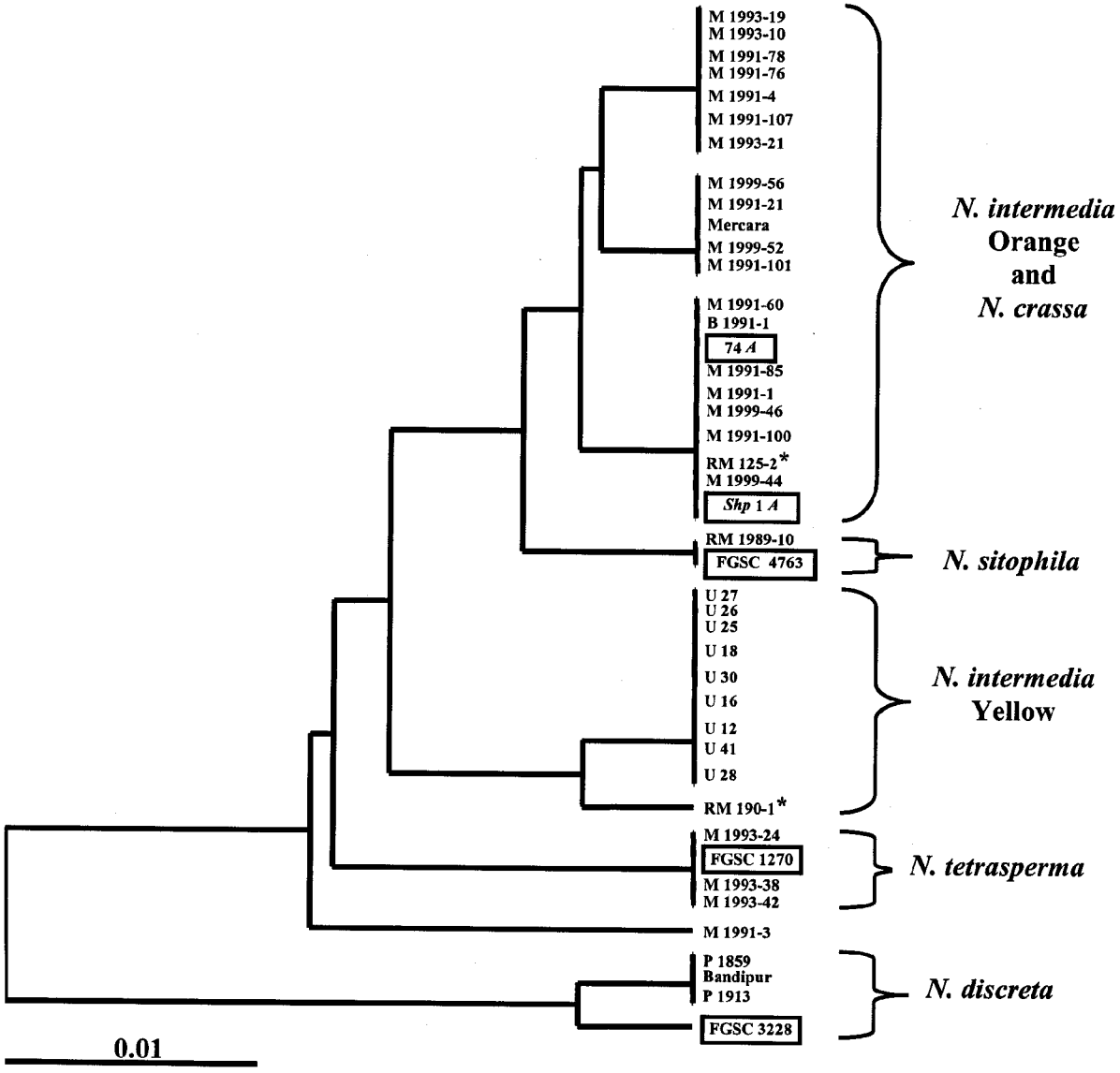

Figure 3. A phylogram illustrating the relationship between strains. This phylogram was constructed using KITSCH program of the PHYLIP package. FGSC species testers are indicated in bold. Local species testers, developed in this study are indicated by asterisks. The scale indicates the genetic distance.

An interesting observation was the placement of strains of the yellow ecotype of *N. intermedia.* The yellow strains formed a monophyletic group, which seems to have diverged from the other heterothallic species just after *N. tetrasperma* (Figure 3). The orange ecotype of *N. intermedia* was found to be closer to *N. crassa*. This suggests that, though identified as *N. intermedia* on the basis of its fertility in crosses with standard orange *N. intermedia* species testers, the yellow ecotype may be evolving independently due to a spacio-temporal separation from the orange ecotype. The sexual reproduction of the orange ecotype was found only on burnt sugarcane stem[43], whereas that of the yellow ecotype was found only on corncobs[44]. The presence of both the mating types (Table 1) and NTS of two different sizes (2.8 and 2.6 kb), in the sample of yellow strains suggests that possibly the population of yellow strains is sexual rather than clonal. We have just begun to understand the diverse lifestyles of different species of *Neurospora*. Further work will help understand how factors such as the nature of the substrate influence the evolution of a species.

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Moonlight inhibits and lunar eclipse enhances foraging activity of fruit bats in an orchard

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We studied the effect of the lunar eclipse that occurred on 9 January 2001, on the foraging activity pattern of fruit bats in an orchard under natural conditions. In addition, we observed bats when foraging on ripe black grapes *Vitis vinifera* for three days before and three days after the eclipse. The number of bat-visits was continuously recorded every hour between 1800 and 0600 h. A bat-visit indicates an individual bat either flying towards or away from the orchard. The total number of bat-visits during the night of the lunar eclipse was significantly higher compared to both the pre- and post-lunar eclipse days. There was no significant difference in the intensity of foraging between the pre- and post-lunar eclipse days. We captured short-nosed fruit bats *Cynopterus sphinx* using mist nets that were set in the vicinity of the orchard.

MOONLIGHT is an environmental factor that inhibits the nocturnal activity pattern of a few species of bats, both under natural[1–5] and laboratory[6] conditions. Apart from bats, moonlight also influences the activity pattern of other nocturnal mammals such as North American desert rodents[7–9], temperate rodents[10,11], desert and arctic lagomorphs[12,13], the marsupial *Caluromys philander*[14] and primates[15,16]. All these studies show that when there is an increase in the intensity of moonlight, animals reduce the use of open space and restrict their foraging activity to the periods of darkness. Our recent

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