Evolution of nematode-trapping cells of predatory fungi of the Orbiliaceae based on evidence from rRNA-encoding DNA and multiprotein sequences

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Among fungi, the basic life strategies are saprophytism, parasitism, and predation. Fungi in Orbiliaceae (Ascomycota) prey on animals by means of specialized trapping structures. Five types of trapping devices are recognized, but their evolutionary origins and divergence are not well understood. Based on comprehensive phylogenetic analysis of nucleotide sequences of three protein-coding genes (RNA polymerase II subunit gene, *rpb2*; elongation factor 1-α gene, *ef1-α*; and ß tubulin gene, *bt*) and ribosomal DNA in the internal transcribed spacer region, we have demonstrated that the initial trapping structure evolved along two lineages yielding two distinct trapping mechanisms: one developed into constricting rings and the other developed into adhesive traps. Among adhesive trapping devices, the adhesive network separated from the others early and evolved at a steady and gentle speed. The adhesive knob evolved through stalk elongation, with a final development of nonconstricting rings. Our data suggest that the derived adhesive traps are at a highly differentiated stage. The development of trapping devices is felicitous proof of adaptive evolution.

Ascomycetes | protein-coding genes | molecular phylogeny | fossil

Predation plays a major role in energy and nutrient flow in the biological food chain. Carnivorism is best known from the animal kingdom, but the fungal kingdom has flesh eaters as well (1). Over 200 species of fungi (distributed in Zygomycota, Basidiomycota, and Ascomycota) use special structures to capture free-living nematodes in the soil (2). The most widespread predatory fungi are in the family of Orbiliaceae, Ascomycota (3, 4). Within a few hours of close contact with nematodes, the sparse mycelia of these fungi will differentiate spontaneously into functional structures (traps). The mycelial traps then adhere to, penetrate, kill, and digest the nematodes' contents (5). To understand the origin and evolution of these fascinating trapping devices, it is essential to gain insights on how those novel devices are differentiated, how nematode trapping fungi are related to other organisms, and their reactions to the environments.

Five kinds of trapping devices have been recognized and studied in predatory fungi of the orbiliaceous ascomycete family (5–7). The adhesive network (AN), the most widely distributed trap, is formed by an erect lateral branch growing from a vegetative hypha, curving to fuse with the parent hypha and developing more loops exterior to the original loop or on the parent hypha (Fig. 1*A*). The adhesive knob (AK) is a morphologically distinct globose or subglobose cell that is either sessile on the hypha or with an erect stalk. AK are normally closely spaced along the hyphae (Fig. 1*B*). Nonconstricting rings (NCR) always occur alongside AK, and are produced when erect lateral branches from vegetative hypha thicken and curve to form a generally three-celled ring that then fuses to the supporting stalk (Fig. 1*B*). The adhesive column (AC) is a short erect branch consisting of a few swollen cells produced on a hypha (Fig. 1*C*). These trapping devices all capture nematodes by means of an adhesive layer covering part or all of the device surfaces. The constricting ring (CR), the fifth and most sophisticated trapping device (Fig. 1*D*) captures prey in a different way. When a nematode enters a CR, the three ring cells are triggered to swell rapidly inwards and firmly lasso the victim within 1–2 sec. Phylogenetic analysis of ribosomal RNA gene sequences indicates that fungi possessing the same trapping device are in the same clade (3, 8–11). Trapping devices are more informative than asexual reproductive structures for grouping the nematode-trapping fungi (4). Trapping devices remain inducible after many years of culture on artificial media, suggesting that these highly differentiated structures are significant for the survival of these fungi. Various hypotheses on the evolution of trapping devices based on either morphological features or molecular characters have been proposed (2, 4, 9, 10), but conflicts exist between molecular and phenotypic phylogenies.

Although the generic classification of predatory fungi and their evolutionary lineage have been proposed based on phylogenetic analyses of rRNA-encoding DNA (rDNA) sequences, the relationship among fungi with adhesive trapping devices were not well resolved (3, 8–11). Because rDNA sequences usually evolve slower than that of protein-coding genes (12), the rDNA gene sequence was unable to answer all of the questions on the relationships among this group of fungi (13–16). The protein-encoding genes such as RNA polymerase II subunit gene (*rpb2*), elongation factor-1α gene (*ef-1α*), and ß-tubulin gene (*bt*) are involved in transcription, translation, and cytoskeleton, respectively (17, 18), and they have been widely used in phylogenetic studies to resolve evolutionary questions that cannot be answered by rDNA genes. The combined use of internal transcribed spacer (ITS) rDNA and protein-coding genes allows improved understanding of the evolutionary events in different life forms (19). The maximum likelihood (ML) method has been successfully applied to reveal molecular evolution across diverse taxa (20, 21). In this study, rDNA ITS region, protein-encoding genes (*rpb2*, *ef-1α*, and *bt*), and ML were used to trace the evolution of trapping devices in predatory fungi.

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Abbreviations: NCR, nonconstricting ring; CR, constricting ring; AC, adhesive column; AN, adhesive network; AK, adhesive knob; ML, maximum likelihood; ME, minimal evolution; rDNA, rRNA-encoding DNA; BSV, bootstrap support value; ITS, internal transcribed spacer.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. are available in Table 1).

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EVOLUTION

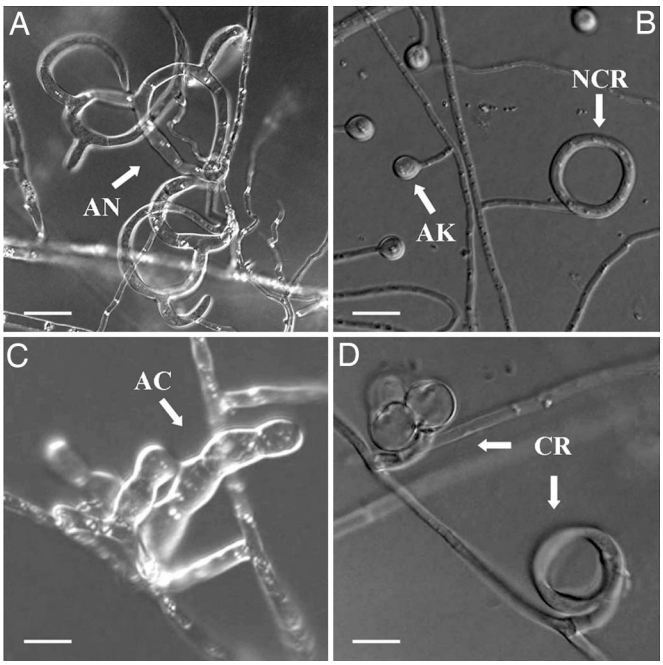

Fig. 1. Trapping devices of the Orbiliaceae. (*A*) AN. (*B*) AK with NCR. (*C*) AC. (*D*) CR. (Scale bar, 10 μm.)

Results

Phylogenetic Relationship of Trapping Devices. Cladograms based on parsimony analyses of nucleotide sequences of rDNA ITS regions (Fig. 2*A*) and the combined data set of four genes (ITS, *bt*, *rpb*, and *ef1-α*) (Fig. 2*B*) revealed similar topological structures [details of Fig. 2 can be obtained from TreeBASE (S1762)]. The ML tree (Fig. 3) based on the combined data set of 2,706 bp provided more detailed information [high bootstrap values as assessed by 1,000 minimal evolution (ME) bootstrap replications] than the trees based on rDNA in the ITS region (Fig. 2*A*) and revealed distinctive signatures that were diagnostic for different trapping devices. The data resulted in two main clades representing two different trapping mechanisms (adhesive and nonadhesive). The nonadhesive clade [98% bootstrap support value (BSV)] consists of species with CR and was paraphyletically evolved with the adhesive clade, including trapping of knob, stalked knob, hyphal column, NCR, and network. Evolution of the adhesive trapping structures with the same trapping mechanism was resolved with the combined data-set tree. Two subclades corresponding to the AN (100% BSV) and other adhesive structures (63% BSV) were strongly supported. AC, AK, and AK associated with NCR grouped in the same subclade, suggesting their close phylogenetic relationship (Fig. 3).

Phylogenetic Relationship of Adhesive Trapping Devices. In the subclade of AK and column-trapping devices (Fig. 3), eight strains forming AC clustered into one group with a 98% BSV and diverged from the other adhesive trapping devices. The species forming sessile or short-stalked knobs (*Dactylellina parvicollis*, *Dactylellina phymatopaga*, *Dactylellina querci*, *Dactylellina haptospora*, and *Dactylellina tibetensis*), representing the primitive character states, were separated early from other species (Fig. 3). The species forming adhesive short-stalked (*Dactylellina drechsleri*, *Dactylellina entomopaga*, *Dactylellina mammillata*, and *Dactylellina ellipsospora*) comprised a subgroup with a 78% BSV. Species with long stalked knobs (*Dactylellina copepodii*, *Dactylellina haptotyla*, and *Dactylellina leptospora*) were associated with NCR and are clustered into the other subgroup with a 70% BSV (Fig. 3).

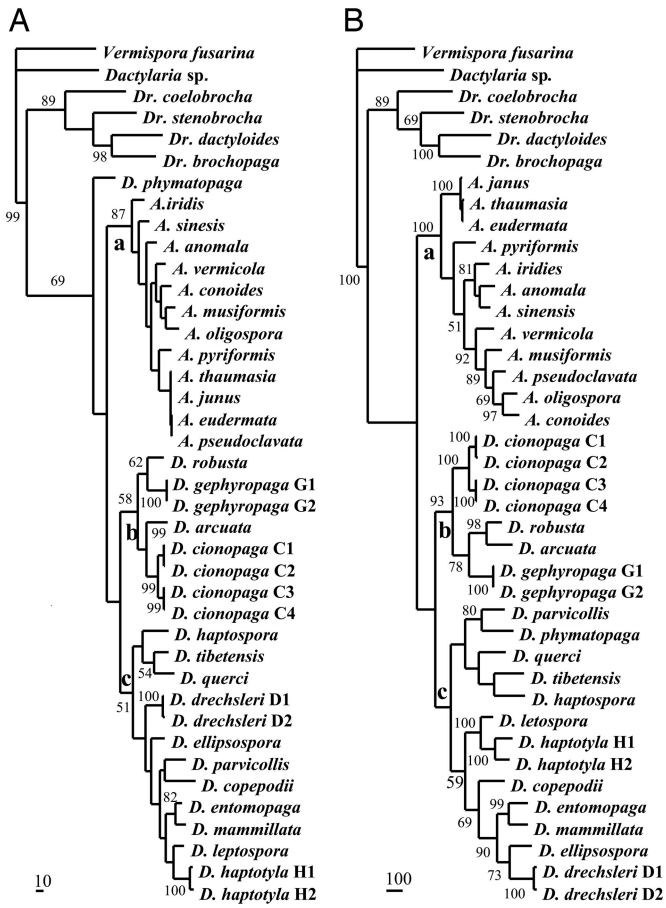

Fig. 2. Parsimony analyses of ITS regions (*A*) and combined data sets (*B*). Bootstrap values were obtained from 1,000 replications, and only >50% are shown.

Ancestral State Reconstruction. Six characters (five trapping device types and no traps), each with two states (present, absent), were calculated by tracing all changes, and a tree with tree length of 8 was generated (Fig. 3). Evolution of the CR went through two stages. One was the formation of the stalks, and the other was the formation of the rings. During evolution of the adhesive traps, each trap got one change from its ancestor. The primogenitor of the trapping device first obtained adhesive strategy and formed AN. Afterward, the evolution focused on covering one specialized cell (sessile knob or protuberance) with adhesive materials. The protuberance proliferated to form the AC. The sessile knob developed an extended stalk to form stalked knob, and some species reproduced several adhesive cells, which might be the origination of NCR (Fig. 3).

Discussion

Evolution of Trapping Cells. Trapping devices in predatory fungi provide an important function for obtaining nutrients and may confer competitive advantages over nonpredatory fungi (4, 22). Based on these morphological and some biological characters such as growth rate and trapping efficiency, Rubner (4) (Fig. 4) suggested that predatory fungi evolved from nonpredatory ancestors and proposed that the least-differentiated trapping device was the sessile AK, which may have evolved in three lineages: (*i*) adhesive hyphal column, then scalariform or 2D

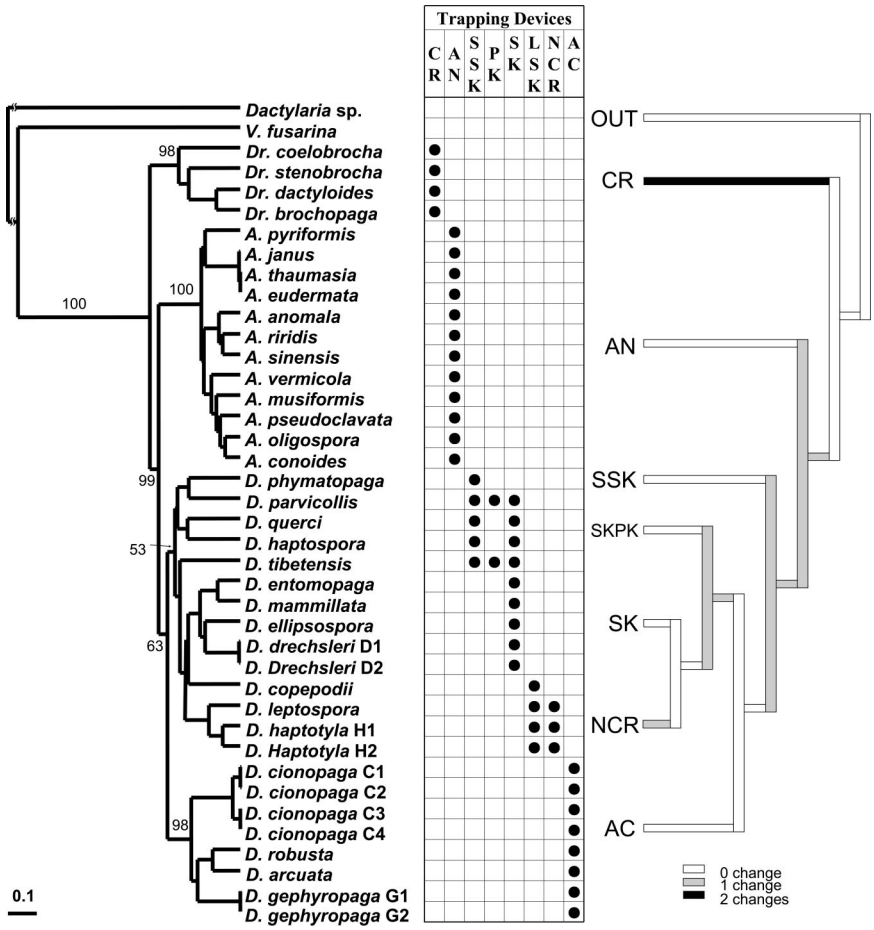

Fig. 3. ML tree of combined sequences with GTR+Γ+I model and character evolution reconstructed using parsimony. The characters of trapping devices are associated with each taxon. The numbers above each branch show the ME BSV after 1,000 replications. *Dr.*, *Drechslerella*; *A.*, *Arthrobotrys*; *D.*, *Dactylellina*; *V.*, *Vermispora*; SSK, simple sessile knobs; SK, stalked knobs; SK and proliferating knob (PK), stalked knobs with PK; SK and NCR, stalked knobs with NCR; OUT, outgroups without traps.

networks and finally 3D networks; (*ii*) adhesive stalked knobs, NCR, and CR; and (*iii*) hyphal column with globose terminal cell (AC*) and proliferating knob. CR and 3D networks were hypothesized to be the most advanced types of trapping organs, because they are the most widely spread (4). However, Li *et al.* (2) considered that the AN was primitive, because it has lower trapping efficiency. Further, some network-forming fungi such as *Arthrobotrys anomala* and *Arthrobotrys botryospora* form adhesive hypha, similar to traps of species of *Cystopaga* and *Stylopaga* in the Zygomycotina (23, 24). The AN might have been simplified and reduced to an AC, which, in turn, may develop into NCR and CR or into the adhesive hyphal protuberance and stalked knob (Fig. 4) (2). Both Rubner (4) and Li *et al.* (2) proposed that the CR and the NCR are closely related because of their morphological similarity. However, the CR and the NCR not only possess different trapping mechanisms, but they also differ in ontogeny development. A CR is formed by a bud at the basal portion that curves the hyphal column to fuse with its advancing tip (25). There is no bud formation during development of a NCR (26). The cells of CR before inflation contain some unusual, oblong, electron-dense inclusions, which are absent after inflation, whereas trapping cells of adhesive devices exhibit numerous globose electron-dense bodies (27, 28). Our analyses demonstrated that NCR were phylogenetically distant from the CR but closely related to stalk knobs. Furthermore, all early hypotheses assumed that that AC was the interim stage between a simple knob and the highly differentiated network. In contrast, our sequence analyses indicate that the AC was one of the most recent descendants of primitive adhesive cells.

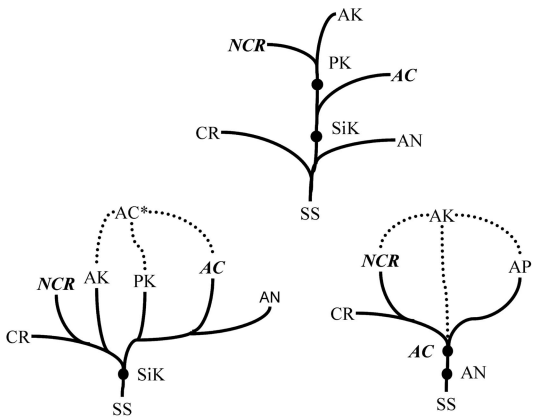

Fig. 4. Comparison of three hypotheses on the evolution of fungal nematode-trapping devices in the Orbiliaceae. SS, specialized structure; SiK, simple knob; SK, stalked knob; PK, chain of proliferating knob; *, adhesive column with globose terminal cell. *Lower Left*, see ref. 4; *Lower Right*, see ref. 2.

Evolution within Adhesive Trapping Devices. Adhesive structures are the most common traps in predatory fungi. Among them, the

EVOLUTION

Table 1. Biological materials used in phylogenetic analysis

Species name	Trapping organ	Strain number	GenBank accession no.			
			ITS	*Bt*	*rpb2*	*ef1-α*
A. anomala	AN	AS 3.6760	AY773451	AY773363	AY773422	AY773393
Arthrobotrys conoides	AN	AS 3.6764	AY773455	AY773367	AY773426	AY773397
Arthrobotrys eudermata	AN	CBS 113357	AY773465	AY773378	AY773436	AY773407
Arthrobotrys janus	AN	AS 3.6626	AY773459	AY773371	AY773430	AY773401
Arthrobotrys iridis	AN	AS 3.6761	AY773452	AY773364	AY773423	AY773394
Arthrobotrys musiformis	AN	AS 3.6778	AY773469	AY773382	AY773440	AY773411
Arthrobotrys oligospora	AN	AS 3.6770	AY773462	AY773374	AY773433	AY773404
Arthrobotrys pseudoclavata	AN	AS 3.6756	AY773446	AY773359	AY773417	AY773388
Arthrobotrys pyriformis	AN	AS 3.6759	AY773450	AY773362	AY773421	AY773392
Arthrobotrys sinensis	AN	AS 3.6755	AY773445	AY773358	AY773416	AY773387
Arthrobotrys thaumasia	AN	AS 3.6769	AY773461	AY773373	AY773432	AY773403
Arthrobotrys vermicola	AN	AS 3.6763	AY773454	AY773366	AY773425	AY773396
D. haptotyla H1	SK and NCR	CBS 113354	AY773470	AY773383	AY773441	AY773412
D. haptotyla H2	SK and NCR	XJ03–96–1	DQ999827	DQ999855	DQ999804	DQ999849
D. leptospora	SK and NCR	CBS 113356	AY773466	AY773379	AY773437	AY773408
D. drechsleri D1	SK	AS 3.6767	AY773458	AY773370	AY773429	AY773400
D. drechsleri D2	SK	CBS 549.63	DQ999819	DQ999861	DQ999810	DQ999840
D. entomopaga	SK	CBS 642.80	AY965758	AY965831	DQ358230	DQ358228
D. mammillata	SK	CBS 229.54	AY902794	AY965824	DQ999817	DQ999843
D. haptospora	SK	CBS 100520	DQ999820	DQ999869	DQ999814	DQ999850
D. copepodii	SK	CBS 487.90	U51964	AY965828	DQ999816	DQ999835
D. ellipsospora	SK	AS 3.6758	AY773449	AY773361	AY773420	AY773391
D. querci	SK	AS 3.6762	AY773453	AY773365	AY773424	AY773395
D. parvicollis	SSK	AS 3.6781	AY773472	AY773385	AY773443	AY773414
D. phymatopaga	SSK	XSBN22–1	AY804215	DQ999870	DQ999798	DQ999854
D. tibetensis	SSK	XZ04–92–1	DQ999833	DQ999856	DQ999803	DQ999848
Dactylellina cionopaga C1	AC	AS 3.6777	AY773468	AY773381	AY773439	AY773410
D. cionopaga C2	AC	CBS 113355	AY773467	AY773380	AY773438	AY773409
D. cionopaga C3	AC	AS 3.6782	AY773473	AY773386	AY773444	AY773415
D. cionopaga C4	AC	AS 3.6780	AY773471	AY773384	AY773442	AY773413
Dactylellina gephyropaga G2	AC	CBS 178.37	U51974	AY965821	DQ999802	DQ999847
D. gephyropaga G1	AC	CBS 585.91	AY965756	AY965829	DQ999801	DQ999846
Dactylellina robusta	AC	CBS 110125	DQ999821	DQ999867	DQ999800	DQ999851
Dactylellina arcuata	AC	CBS 174.89	AF106527	DQ999868	DQ999799	DQ999852
Drechslerella brochopaga	CR	AS 3.6765	AY773456	AY773368	AY773427	AY773398
Drechslerella stenobrocha	CR	AS 3.6768	AY773460	AY773372	AY773431	AY773402
Drechslerella dactyloides	CR	AS 3.6771	AY773463	AY773375	AY773434	AY773405
Drechslerella coelobrocha	CR	AS 3.6772	AY773464	AY773376	AY773435	AY773406
Dactylaria sp.	Outgroup	AS 3.6766	AY773457	AY773369	AY773428	AY773399
V. fusarina	Outgroup	AS 3.6757	AY773447	AY773360	AY773418	AY773389

network was considered the most evolved by Rubner (4) and the most primitive by Li *et al.* (2). The analysis of our combined data set suggests that the AN differentiated early and represents an ancient type, thereby supporting Li *et al.* (2). AN are 3D and more like vegetative hyphae covered with sticky materials. Although some AC can also develop into 2D networks (29), these scalariform networks constrict significantly at septae, resembling AC. AC and NCR possess a large area of attachment to nematodes compared with that of simple protuberances (sessile knobs), which are probably more primitive than all other adhesive devices except AN. NCR-forming species also produce stalk AK. When nematodes struggle to escape after capture, both the knob and the NCR may detach and break at their points of attachment to the stalk (30, 31). The detachable knob and the ring provide a distinct advantage for the fungus, because the detached knob or ring can travel with the swimming nematode. They incapacitate the nematode by firmly attaching to the nematode's cuticle, subsequently penetrating and allowing the fungus to feed on the nematode (5).

Evolution of Predation. Carbon and nitrogen are essential nutrients for fungal growth and reproduction. It has been proposed that the nematode-trapping phenotype is an evolutionary response by cellulolytic or lignin-degrading fungi to nutrient deficiencies in nitrogen-limiting habitats (32–34). Because nitrogen is essential to fungal growth and not freely available either in dead wood or in soil where carbon is abundant, direct capture of nitrogen compounds from other living life forms is an advantage (35). Many network-forming species do not form a network spontaneously; they are more saprophytic than other nematode-trapping fungi. Formation of network-trapping devices is induced by the presence of nematodes or substances of animal origin known as *nemin* (36). The AN is a primitive character induced only by covering the hypha with a thin film of stick fibrils. Fungal species with other types of trapping devices, such as AK (sessile or stalked), AC, and CR, produce trapping devices spontaneously (4). The spontaneous trap formers are more effective to prey nematodes than nonspontaneously formers, such as AN-forming species, which have the flexibility to become more predacious by induction of more traps (37). Carnivorous plants also exhibit evolution toward the development of predatory organs and increased capacity for predation under low-nutrient environment (38). Carnivorous plants have

adhesive traps and snap traps. Predatory fungi have adhesive traps and constricting traps. Both snap and constricting traps have developed a highly specialized sensory organ for trap triggering and closure (5, 38). Like carnivorous plants, predatory fungi have the ability to capture and to absorb nutrients from their prey, a fascinating example of convergent evolution.

A fossil of nematodes parasitized by nematophagous fungi has been dated to ≈22.5–26 million years ago (39) and can be used as a reference to estimate the divergence time of trapping devices. If the fossil is used in time calibration, the predatory fungi would be well established in the Tertiary period. However, *Orbilia fimicola*, a predatory fungus, was estimated to be first derived from its ascomycete ancestor (nonpredatory) at about >900 million years ago (40), and the time scale is much older than the fossil record.

Materials and Methods

Biological Materials. Forty fungi were used in this study, including 38 predatory fungal species from three genera and representing five trapping device types (Table 1). One strain each of *Dactylaria* sp. and *Vermispora fusarina*, which are morphologically similar to nematode-trapping fungi but not nematode trappers, were included as outgroup taxa (Table 1). Thirteen strains were obtained from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands), and the other strains were isolated from soil samples in China by using a soil-sprinkling technique (5, 41) and identified following the system of Yang and Liu (42).

PCR Amplification and Sequence Collection. The methods for fungal culture, genomic DNA extraction, PCR product purification, and sequencing have been described (43). The four gene segments selected for phylogenetic analysis were the ribosomal RNA genes in the ITS regions (ITS1–ITS4) (44), β-tubulin gene (*bt*) (Bt2a–Bt2b) (45), the second subunit of RNA polymerase II gene (*rpb2*) between exons 6 and 7 (6F-7R and 5F-7CR) (17), and elongation factor 1-α gene (*ef1-α*) (526F-1567R) (46). Primers 247F (5′-ggagcccttgcccattt-3′) and 609R (5′-tcacgatgtc-cgggagc-3′) were designed for *ef-1α* sequencing. To give specific PCR products, primers 5F and 7cR (17) were used to amplify the *rpb2* gene of *Dactylaria* sp. PCR amplification was conducted as follows: 3 min at 95°C followed by 35 cycles of 95°C for 1 min, 54°C for 40 s (56°C for *bt* gene), and 72°C for 90 s (40 s for rDNA in the ITS regions), then a final extension at 72°C for 10 min.

Sequence Alignment. Nucleotide sequences were aligned by using Clustal X 1.81 (47) under the default settings (multiple alignment parameters: gap opening 10.00 and gap extension 0.20) to produce an initial alignment. This process was followed by manual adjustments by using BioEdit version 5.0.6 (Tom Hall, North Carolina State University, Raleigh, NC). A large intron of 610 bp within *ef1-α* region was eliminated. After confirming that individual gene regions gave similar phylogenies, the four segments were combined into one alignment of 2,706 nucleotide sites (including gaps), which consisted of 575 bp from the 5′ end of *bt* gene, 775 bp between exon 6 and 7 of *rpb2* gene, 799 bp from the 5′ end of *ef1-α* gene, and 557 bp of rDNA in the ITS regions. There were seven noncoding regions in this alignment, including three in the *bt* gene, one in the *rpb2* gene, one in the *ef1-α* gene and two in the ITS regions. The outgroup *Dactylaria* sp. was a distant relative to the other species, so the noncoding regions at different sites were eliminated from the alignment. DNA sequences have been deposited in the GenBank database (Table 1).

Phylogenetic Analyses. Parsimony and ML analyses of both ITS regions and combined data sets were performed by using PAUP* 4.0b 10 (48). The procedure outlined by Huelsenbeck and Crandall (49) was followed for substitution model selection. All model parameters were estimated by the ML procedure as implemented in PAUP* through an iterative process (50). From each model, the likelihood scores were compared by using likelihood ratio test (51) as implemented in Modeltest 3.6 (52). The model of GTR+Γ+I was selected as the best-fit model for ML analysis of combined data sets, and the subsequent ML analysis was conducted under this model with enforcement of a molecular clock. The frequencies of each nucleotide base were 0.24175 for A, 0.27739 for C, 0.21334 for G, and 0.26752 for T. When assumed GT substitution rate [R(GT)] was 1, then the relative substitution rate of R(AC) was 1.371604, R(AG) was 4.033722, R(AT) was 1.427957, R(CG) was 1.026423, and R(CT) was 5.676098. The proportion of invariable sites was 0.295111, and the gamma shape was 0.650314.

Nodal support was estimated under a ME criterion based on distance and sequence evolution parameters obtained above by ML under the GTR+Γ+I model and the final ML tree. Support values were based on the full heuristic ME search on 1,000 bootstrap replications. Starting trees were obtained by stepwise addition with one tree held at each step.

Ancestral Character States Reconstruction. Evolution of the morphology and mechanism of trapping cells was simulated by parsimony reconstruction carried out by using MacClade 4.0 (53) and PAUP* 4.0b 10 (48). Hypothetical ancestral species were presented by the internal nodes of the cladogram, and inference of the ancestral character states was in accordance with parsimony.

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