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Convergent coevolution in the domestication of coral mushrooms by fungus-growing ants

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Comparisons of phylogenetic patterns between coevolving symbionts can reveal rich details about the evolutionary history of symbioses. The ancient symbiosis between fungus-growing ants, their fungal cultivars, antibiotic-producing bacteria and cultivar-infecting parasites is dominated by a pattern of parallel coevolution, where the symbionts of each functional group are members of monophyletic groups. However, there is one outstanding exception in the fungus-growing ant system, the unidentified cultivar grown only by ants in the *Apterostigma pilosum* group. We classify this cultivar in the coral-mushroom family Pterulaceae using phylogenetic reconstructions based on broad taxon sampling, including the first mushroom collected from the garden of an ant species in the *A. pilosum* group. The domestication of the pterulaceous cultivar is independent from the domestication of the gilled mushrooms cultivated by all other fungus-growing ants. Yet it has the same overall assemblage of coevolved ant–cultivar–parasite–bacterium interactions as the other ant-grown fungal cultivars. This indicates a pattern of convergent coevolution in the fungus-growing ant system, where symbionts with both similar and very different evolutionary histories converge to functionally identical interactions.

Keywords: convergent coevolution; symbiosis; fungus-growing ants; species assembly; phylogeny

1. INTRODUCTION

Phylogenetic approaches are commonly used to elucidate the various dynamics of coevolutionary interactions (reviewed in Page 2003). Phylogenies of symbionts have been compared to infer the processes that underlie coevolutionary interactions. Many coevolutionary interactions exist between monophyletic groups of symbionts, such as the gopher–gopher lice, beetle–ambrosia fungi and fig–fig wasp systems (Hafner & Nadler 1988; Farrell *et al.* 2001; Cook & Rasplus 2003), suggesting a single origin for these interactions. These systems are examples of parallel coevolution, where functionally equivalent interactions exist between specific clades of symbionts. However, the assembly of functionally equivalent interactions is not limited to parallel coevolution; in rare instances, one or more of the symbionts within a functional group can come from distantly related clades. We refer to such phylogenetic incongruence, i.e. polyphyletic origins of symbionts in a functional group, within systems dominated by parallel coevolution as convergent coevolution. By identifying and determining the phylogenetic position of a poorly studied fungal cultivar, we show that there is convergent coevolution in the fungus-growing ant system.

The ancient quadripartite symbiosis between fungus-growing ants, their fungal cultivars, the fungal parasites that infect the fungal cultivars and the bacteria used by the ants to control the growth of the parasites is a model example of coevolution (Currie *et al.* 1999, 2003; Mueller 2002). The ants eat the fungi as their main food source, while the fungi benefit through propagation by the ants. Ants inoculate new gardens with fungi from older gardens, such that specific fungal cultivars are passed from garden to garden within an ant lineage. Nearly pure cultures of vegetative mycelia are maintained with the help of antibiotic-producing bacteria, which inhibit the growth of fungal parasites that infect the fungal cultivars (Currie *et al.* 1999).

Four main lineages of fungal cultivars are grown by the 210 species of fungus-growing ant. Three out of the four main cultivar lineages have the same ancient evolutionary history as the ants that grow them and the parasites that infect the cultivars (Currie *et al.* 2003). The fourth cultivar, grown only by ants in the *Apterostigma pilosum* group, has not been identified, but it has been predicted to have an evolutionary history that is distinct from those of the other cultivars (Chapela *et al.* 1994; Currie *et al.* 2003). To determine the pattern of coevolution of the *A. pilosum* cultivar and its symbionts and to study the fungus-growing ant system as a whole, the identity and origin of the cultivar grown by the *A. pilosum* group must first be known.

The identification of the four main lineages of fungal cultivars has been hindered by the absence of taxonomically informative morphological characters in the nest mycelium and the inhibition of mushroom production by the ants. Phylogenetic analyses of vegetative mycelia (Chapela *et al.* 1994; Mueller *et al.* 1998; Johnson 1999) and identification of ant-garden-borne white-spored gilled mushrooms (reviewed in Muller 2002) led to the classification of three

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fungal-cultivar lineages in the family Lepiotaceae. Twelve out of the thirteen genera of fungus-growing ants grow one of the three lepiotaceous cultivars.

Previous phylogenetic analyses have suggested that the fourth cultivar, grown by the *A. pilosum* group, was also domesticated from a gilled mushroom (Chapela *et al.* 1994; Villesen *et al.* 2004). However, mushroom production in nature or in the laboratory has never been reported for this cultivar. In this study, we use phylogenetic analyses to identify the *A. pilosum* cultivar. We show that it is phylogenetically and morphologically distinct from the other cultivars and discuss how this cultivar converged to the same coevolutionary interactions as the other cultivars, despite its distinct origin.

2. MATERIAL AND METHODS

(a) *Isolation and voucher of specimens*

Pterula and *Deflexula* species were collected from Costa Rica, Trinidad, Puerto Rico, New Zealand, Taiwan and Louisiana (USA). All but two *Pterula* and *Deflexula* species were found growing on either decomposing leaves or wood. *Pterula* cf. *tenuissima* was isolated from within photosynthetic leaves of *Magnolia grandiflora* using a standard protocol for the isolation of endophytic fungi (Schulz *et al.* 1993). The mushroom collection identified as UGM011206-01 was found growing on vegetative mycelia grown by *Apterostigma dentigerum* ants.

Vouchers for *Pterula* and *Deflexula* collections (except *D. subsimplex*) are in the University of Minnesota Herbarium, James Ford Bell Museum of Natural History, and cultures are in the Mycological Culture Collection, Department of Plant Biology, University of Minnesota. The substrates and geographical origins of the isolates of the ant-grown fungal cultivars and other taxa have been described elsewhere (Moncalvo *et al.* 2002; Villesen *et al.* 2004).

(b) *DNA sequencing*

Genomic DNA was extracted from lyophilized mushrooms or vegetative mycelia using CTAB (hexadecyltrimethyl-ammonium bromide) in a standardized DNA extraction protocol (Zolan & Pukkila 1986). Genomic DNA was used as the template for PCR amplification of the first 900 base pairs of the large subunit of the nuclear ribosomal DNA gene. The PCR product was generated using the LR0R and LR5 primers, and forward and reverse sequences were obtained with the LR0R, LR3R, LR16 and LR5 primers (Moncalvo *et al.* 2002). Sequence data for UGM011206-01 and the *Pterula* and *Deflexula* isolates (except *D. subsimplex*, which was collected by other researchers and deposited in GenBank) were generated using dye-labelled dideoxy terminator cycle sequencing (Applied BioSystems, Inc.) and an ABI 377 automated DNA sequencer. Contiguous sequences were edited with SEQUENCHER 3.0 (GeneCorps, Inc.) and are available at GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html; accession numbers AY458121–AY458133).

In addition to the sequence data collected for this study, sequences for the following taxa were obtained from GenBank (accession numbers): *D. subsimplex* (AJ406572); fungal cultivars grown by ants in the *A. pilosum* group (AY367613–AY367633); *Gerronema strombodes* (AF261365); *G. subclavatum* (U66434); *Megacollybia platyphylla* (AF261366); *Clitocybula oculus* (AF261367); *Hydropus fuliginarius* (AF261368); *Porotheleum fimbriatum* (AF261370); *Marasmius fulvoferrugineus* (AF261584); *Chaetocalathus liliputianus* (AF261346); *Crinipellis maxima* (AF042630); *Tetrapyrgos nigripes* (AF261337); and *Campanella* sp. (AF261339).

(c) *Phylogenetic analyses*

Sequences for *Pterula* and *Deflexula* species were aligned by eye within the euagarics dataset (Moncalvo *et al.* 2002) using PAUP* (Swofford 2002). A neighbour-joining (NJ) analysis of 902 sequences identified their close relatives. Sequences for *Pterula* and *Deflexula* species and their closely related taxa were then aligned to generate a trimmed dataset for maximum-parsimony (MP) and Bayesian analyses. The tetrapyrgoid taxa were designated as the outgroup in analyses of the final dataset, which consisted of 901 nucleotide sites. The data matrix of the final dataset is available on request from A.B.M.

MP analysis was conducted with character-state changes weighted equally and gaps scored as 'missing'. Branch-support values in the MP phylogenies were obtained with 1000 replicates of non-parametric bootstrap analyses in PAUP*. Bayesian analyses were conducted using MRBAYES 2.01 (Huelsenbeck & Ronquist 2001) with the general time-reversible model of nucleotide substitution; some sites were assumed to be invariable and variable sites were assumed to follow a discrete gamma distribution ($GTR + \Gamma + I$ model); this was the best-fit model according to MODELTEST v. 3.04 (Posada & Crandall 1998). Starting with a random tree, four incrementally heated Markov chains in a Markov chain Monte Carlo sampling method were used for 1×10^6 generations, sampling a tree every tenth generation to ensure that successive samples were independent. Out of the 100 000 sampled trees, the trees that preceded convergence of the Markov chain were discarded. From the remaining trees a 50% majority-rule consensus tree was generated in PAUP*. Statistical support for branches is represented by the percentage in which the divergence is observed in the sampled trees (the posterior probability). Three independent Bayesian analyses were conducted to identify congruent results of individual analyses.

3. RESULTS

Based on a NJ phylogenetic analysis, the cultivar grown by the *A. pilosum* group was classified in the phylum Basidiomycota in the euagarics clade, along with the lepiotaceous cultivars grown by all other fungus-growing ants (figure 1). However, there was a great genetic distance between the *A. pilosum* cultivar and the lepiotaceous cultivars (figure 1). The euagarics clade comprises primarily gilled mushrooms together with several non-gilled forms not traditionally classified with gilled mushrooms (Moncalvo *et al.* 2002). The NJ phylogeny of the euagarics dataset ($n = 912$ taxa; figure 1) and the MP and Bayesian analyses of the trimmed dataset ($n = 46$ taxa; figure 2) strongly indicated that 21 isolates of the *A. pilosum* cultivar were sister to a monophyletic group of coral mushrooms in the genera *Pterula* and *Deflexula*. Independent Bayesian analyses produced the same majority-rule consensus tree, which was topologically identical to the two equally parsimonious trees derived from the MP analysis (164 parsimony-informative characters, length of 461, consistency index of 0.579, retention index of 0.85).

In addition, examinations of over 300 gardens maintained by ants in the *A. pilosum* group led to the collection of clusters of 1×0.25 mm pale-yellow coral mushrooms (collection number UGM011206-01; figure 2*a*) growing on a cultivar in an *A. dentigerum* nest in Panama. The dimitic skeletal hyphae of these coral mushrooms were characteristic of *Pterula* and *Deflexula* (Corner 1950) and confirmed the taxonomic association as either *Pterula* or *Deflexula*. However, the absence of spores prevented

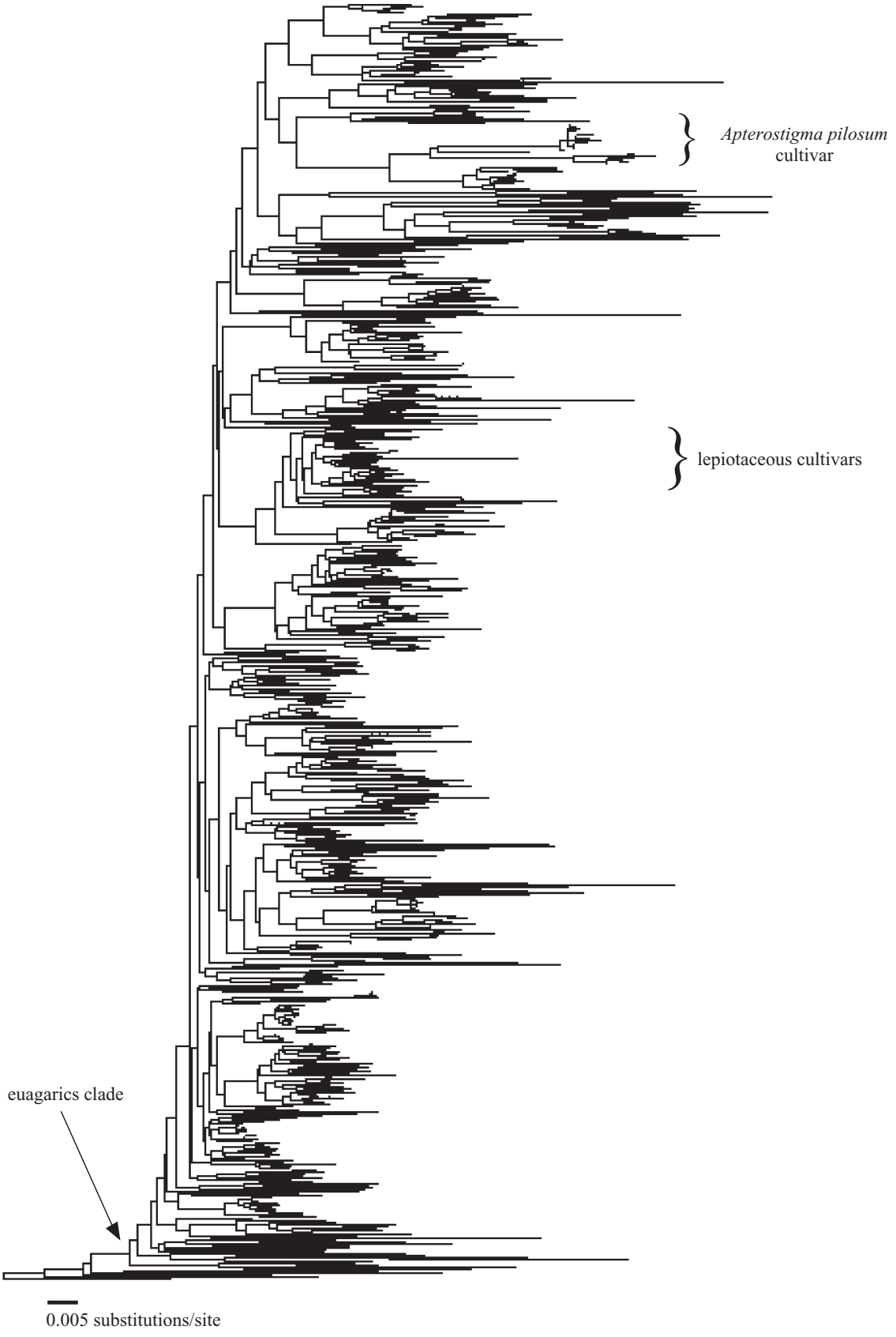

Figure 1. A NJ phylogeny illustrating the evolutionary relationships of ant-cultivated fungi within the euagarics clade. All fungus-growing ants, except those in the *Apterostigma pilosum* group, grow one of three lepiotaceous cultivars. The *A. pilosum* cultivar is clearly paraphyletic to the lepiotaceous cultivars and thus the only ancient phylogenetic divergence in the coevolutionary interactions between the fungus-growing ants, their fungal cultivars and cultivar-infecting parasites (Currie *et al.* 2003).

identification to genus and species level. Phylogenetic analyses placed these coral mushrooms within the pterulaceous cultivar clade (figure 2).

4. DISCUSSION

Using broad taxon sampling, including the first mushroom collected in a nest maintained by a species in the *A. pilosum* group, we present molecular and morphological evidence that showed the cultivar grown by the *A. pilosum* group was domesticated from fungi in the coral-mushroom family Pterulaceae. *Pterula* and *Deflexula* are the most speciose genera in the enigmatic primarily tropical Pterulaceae (Corner 1950, 1970; Kirk *et al.* 2001).

Prior to this study, only four genera of coral mushrooms were classified in the euagarics clade, and none of these was related to the cultivar grown by the *A. pilosum* group (Hibbett & Binder 2002; Moncalvo *et al.* 2002). The sister group of the pterulaceous cultivar + *Pterula* + *Deflexula* clade includes the gilled mushrooms that were previously identified as the closest relatives of the cultivar (figure 2; Chapela *et al.* 1994; Moncalvo *et al.* 2002; Villesen *et al.* 2004). Chapela *et al.* (1994) predicted that the cultivar was a member of the gilled-mushroom family Tricholomataceae. However, they included only gilled mushrooms in their study, since they were unaware that some non-gilled morphologies were derived from the gilled morphology (Hibbett *et al.* 1997). Moncalvo *et al.* (2002) sampled every group previously reported to be in the euagarics as well as taxa not predicted to be in the euagarics. Villesen *et al.* (2004) sampled taxa previously reported to be related to the *A. pilosum* cultivar. In all cases, gilled mushrooms in Tricholomataceae were predicted to be the free-living relatives of the cultivar.

In addition to finding the sister relationship between the pterulaceous clade and the *A. pilosum* cultivar, we classified a *Pterula* or *Deflexula* mushroom in the cultivar clade (figure 2). This mushroom was found growing on a cultivar farmed by *A. dentigerum* ants. Although it cannot be identified unambiguously as the mushroom of the cultivar domesticated by the *A. dentigerum* ants, several observations of that *A. dentigerum* nest suggest that it was the mushroom of the pterulaceous cultivar. Several mushrooms were chewed on by the *A. dentigerum* ants, paralleling the suppression of cultivar fruiting seen in nests of the lepiotaceous cultivars (Mueller 2002). In addition, the nest was queenless and had fewer than the average number of workers (11 workers and five alates). Queenlessness has been suggested to be a precondition for fruiting in the lepiotaceous cultivar, indicating that the cultivar in the *A. dentigerum* colony may have switched from an asexual existence in a formerly queenright colony to sexuality upon queen loss (Mueller 2002). Such a switch to sexuality is predicted because only queenright nests produce new foundress queens that can disperse the fungus to new nest locations, and queen loss thus terminates this avenue of reproduction for the cultivar.

(a) *Convergent coevolution*

The phylogenetic divergences that resulted in the three major lineages of fungus-growing ants are reflected in the phylogenies of the three lepiotaceous cultivars and the parasites associated with the lepiotaceous cultivars, illustrating a strong pattern of parallel coevolution (Currie *et al.* 2003). However, parallel coevolution is not the only mode of coevolution in the fungus-growing ant system.

Figure 2. (*Opposite.*) The evolutionary relationships of the *Apterostigma pilosum* cultivar and coral mushrooms in *Pterula* and *Deflexula*. This phylogeny is one of two equally parsimonious trees derived from a MP analysis and is topologically identical to the majority-rule consensus tree derived from Bayesian analyses. (*a–e*) Photographs of the mushrooms connected to the corresponding taxa by dashed lines. (*a*) UGM011206-01 is a Pterulaceae species mushroom collected in a garden maintained by ants in the *A. pilosum* group. (*e*) *Megacollybia platyphylla* is a representative of the gilled mushrooms that were previously predicted to be the free-living relatives of the *A. pilosum* cultivar. Bootstrap values (> 70) and posterior probability values (> 95) are indicated above and below nodes, respectively. Coloured boxes indicate the country of origin for isolates of selected taxa (GU, Guyana; PA, Panama; NZ, New Zealand; TR, Trinidad; CR, Costa Rica; PR, Puerto Rico; USA, United States of America; TA, Taiwan). Scale bars, 1 mm.

From our study, it is clear that the pterulaceous cultivar has an independent origin and is distantly related to the three lepiotaceous cultivars. However, the overall assembly of the four symbionts and their functional interactions are the same for the pterulaceous and lepiotaceous cultivars, suggesting a pattern of convergent coevolution in the fungus-growing ant system. Each cultivar is grown by particular ant species that use particular bacteria (*Pseudonocardia* spp.) to minimize the growth of particular garden parasites (*Escovopsis* spp.) on the cultivars (Currie *et al.* 2003; M. J. Cafaro and C. R. Currie, personal communication). To determine the processes leading to this convergence, we must first understand the origin of the interaction between the pterulaceous cultivar and the *Apterostigma* ants. Several hypotheses for the origin of the lepiotaceous cultivars have been proposed and tested (Mueller *et al.* 1998; reviewed by Mueller *et al.* 2001). There is no support for any of these hypotheses regarding the origin of the pterulaceous cultivar.

We propose that the *A. pilosum* group inadvertently domesticated the pterulaceous cultivar, which then displaced the cultivar originally grown by these ants. The new cultivar originated from either: (i) mycelia within material used to fertilize the previous cultivar; (ii) mycelia within or adjacent to the substrate of the ants' nests; or (iii) spores. The *A. pilosum* group constructs their gardens under or inside decomposing logs and fertilizes their gardens with wood fragments, dead plant debris and insect faeces (Mueller *et al.* 2001; Villesen *et al.* 2004). The ants in the *A. pilosum* group use wood to grow their fungal cultivar much more frequently than do any of the other fungus-growing ants (Villesen *et al.* 2004). Many *Pterula* and all *Deflexula* species are wood inhabiting (Corner 1950, 1970). Therefore, encounters between the ancestral pterulaceous-cultivating ant species and Pterulaceae species would be probable. The rare use of wood to grow lepiotaceous cultivars is consistent with the non-wood substrates of the free-living relatives of the lepiotaceous cultivars and the poor ability of the lepiotaceous cultivars to degrade cellulose (Gomes de Siqueira *et al.* 1998; Abril & Bucher 2002). Although the enzymatic potential for wood decay by the pterulaceous cultivar has yet to be investigated, a closely related species, *Pterula echo*, has been grown in axenic

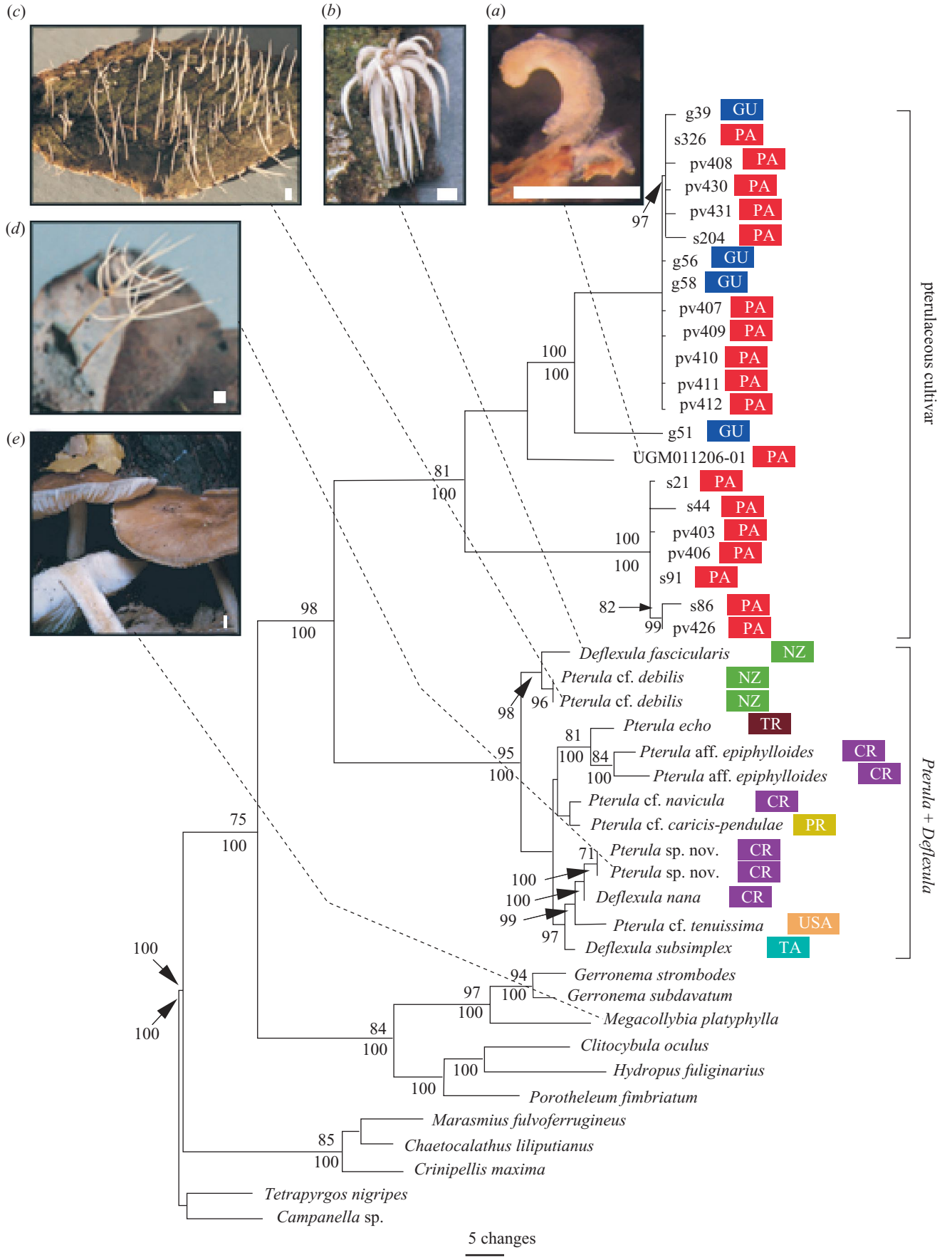

Figure 2. (*Caption opposite.*)

culture on wood (McLaughlin & McLaughlin 1980). The maintenance of this trait would enhance the growth rate of the pterulaceous cultivar in gardens fertilized with wood fragments. The pterulaceous cultivar may also have originated as a saprobe or an endophyte, as suggested by our isolation of the first endophytic coral mushroom, *Pterula* cf. *tenuissima*. It is also possible that spores borne on *Pterula* or *Deflexula* mushrooms were introduced to the garden via wind or on material used to fertilize the garden. Whether the pterulaceous cultivar originated within or adjacent to the original garden, the ancestral pterulaceous fungus survived the extensive physical and chemical processing of garden fodder by the ants (Currie & Stuart 2001) and possibly displaced the original cultivar.

The quadripartite symbiosis between *A. pilosum* ants, their pterulaceous cultivar, the antibiotic-producing *Pseudonocardia* species and the *Escovopsis* species that parasitize the pterulaceous cultivar is as highly coevolved as the interactions of the other ant–cultivar–parasite–bacterium symbioses, despite having a different evolutionary history. The evolutionary histories of the *A. pilosum* ants and the *Escovopsis* species are more similar than the evolutionary histories of the *A. pilosum* ants and the pterulaceous cultivar (Currie *et al.* 2003). This suggests that the ants may have introduced the parasite and bacteria to the novel cultivar. However, this phylogenetic pattern does not rule out the possibility that the pterulaceous cultivar originated at the same time as fungiculture in the *A. pilosum* group, suggesting that all the interactions in the quadripartite symbiosis could have arisen independently. Additional collections of Pterulaceae species and the pterulaceous cultivar from their common habitats, ranging from Argentina to Mexico, will help determine the phylogenetic, geographical and ecological origins of the pterulaceous cultivar. These data will allow a reconstruction of the symbiosis and serve as a foundation for understanding the convergence of coevolutionary interactions.

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