Endophytic fungal communities in leaves of tropical forest trees: Diversity and distribution patterns

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Endophytic fungi cause symptomless infections in healthy tissues of plants. This cryptic guild of fungi is regarded as a benchmark for estimating fungal biodiversity. We studied endophyte distribution, diversity and host recurrence in 24 tree hosts (belonging to 17 plant families) of two dry tropical forests of the Nilgiri Biosphere Reserve. A total of 81 endophyte taxa were isolated from 3600 tissue segments. Fifty-six species were isolated from more than one host. We discerned two groups of fungi in both forests, one group consisting of the ubiquitous forms that dominated the endophyte assemblage of many hosts and the second represented by the less frequent forms. Host density influenced the composition and distribution of endophytes in one of the forests. The existence of ubiquitous forms reduced the diversity of the endophytes in the plant communities. Our results suggest that dry tropical forests are not hyperdiverse with reference to endophytes and that the generalists among endophytes be identified before extrapolating data to calculate global fungal diversity.

**Fungi** are one of the most diverse life forms on this planet and predicting the number of fungal species is considered important among mycologists. Hawkins\(^3\) predicted that there are 1.5 million species of fungi; of these, about 74,000 are currently known. Recent studies from tropical forests\(^4\) suggest that fungal diversity is greater in the tropics than in the temperate regions, and many tropical mycologists view this 1.5 million as a conservative figure. Some researchers, however, feel that the figure of 1.5 million is too high\(^5\). Endophytes of tropical plants are among the groups of fungi that have been studied to arrive at the predicted figure of 1.5 million. Based on their studies on nine neotropical trees, Arnold et al.\(^1\) concluded that fungal endophytes are hyperdiverse in the tropics and that the figure of 1.5 million may markedly underestimate fungal diversity. More recently, studies on a forest in Guyana\(^1\) and four forests in Madumalai Wildlife Sanctuary, southern India\(^1\) revealed that certain tropical forests are not hyperdiverse with reference to fungal endophytes. In order to get a clear picture of the diversity of endophytes in tropical forest communities, we studied hosts of different abundance classes.

We collected leaves from 24 tree hosts found in the dry thorn and dry deciduous forests of Madumalai Wildlife Sanctuary (11°32' and 11°43'N lat and 76°22' and 76°43'E long; area 312 km\(^2\)), which is situated to the northwest of the Nilgiri Mountains in Tamil Nadu, and forms a part of the Nilgiri Biosphere Reserve. The tropical dry thorn forest is in the rain shadow of the Nilgiri Mountains and the mean annual rainfall is 800 ± 65 mm (ref. 13). The dry deciduous forest occupies the major part of the sanctuary and receives an annual rainfall of 1200 mm (ref. 13).

For each forest type, four hosts with higher abundance were grouped in abundance class I, four with intermediate abundance in class II and four with lower abundance in class III (Table 1). For each host, three individuals were selected and 20 healthy leaves were collected from each individual.

Leaves from each individual were processed separately within 48 h of collection. The leaves were washed thoroughly in running water and three segments of 0.5 cm\(^2\) were cut from the midrib portion of each leaf and surface sterilized by immersing in 70% ethanol for 5 s, followed by 4% NaOCl for 90 s, and finally washed in sterile water for 10 s (ref. 14).

Fifty segments from each individual (150 segments for each host species) were randomly chosen and placed in Petri dishes containing potato dextrose agar (with chloramphenicol 150 mg L\(^{-1}\)). Ten leaf segments were plated in each Petri dish, the dishes were sealed with parafilm and incubated in a light chamber at 26 ± 1°C for 21 days. The light regimen provided was 12 h light: 12 h darkness from cool white, daylight fluorescent lamps. The efficacy of the sterilization procedure was ascertained with the method of Schulz et al.\(^1\).

The fungi that grew out from the segments were periodically isolated and identified. Those fungi that failed to
Table 1. Host plants studied with their abundance classes and number of species and isolates of endophytes obtained from their leaves

<table>
<thead>
<tr>
<th>Host</th>
<th>Code</th>
<th>Abundance class</th>
<th>Species</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry thorn</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythroxylon monogynum Roxb.</td>
<td>T1</td>
<td>I</td>
<td>22</td>
<td>194</td>
</tr>
<tr>
<td>Zizyphus xilopurpis (Reez.) Willd.</td>
<td>T2</td>
<td>I</td>
<td>16</td>
<td>264</td>
</tr>
<tr>
<td>Anogeissus latifolia (DC) Wall. ex Bedd.</td>
<td>T3</td>
<td>I</td>
<td>17</td>
<td>170</td>
</tr>
<tr>
<td>Givottio rotteriformis Griff.</td>
<td>T4</td>
<td>I</td>
<td>14</td>
<td>227</td>
</tr>
<tr>
<td>Strachnos patiaturum L.f.</td>
<td>T5</td>
<td>II</td>
<td>22</td>
<td>243</td>
</tr>
<tr>
<td>Premna tomentosa Willd.</td>
<td>T6</td>
<td>II</td>
<td>14</td>
<td>237</td>
</tr>
<tr>
<td>Naringi crenulata (Roxb.) Nicolson.</td>
<td>T7</td>
<td>II</td>
<td>12</td>
<td>306</td>
</tr>
<tr>
<td>Cassia fistula L.</td>
<td>T8</td>
<td>II</td>
<td>14</td>
<td>257</td>
</tr>
<tr>
<td>Dalbergia lanceolaria L.f.</td>
<td>T9</td>
<td>III</td>
<td>12</td>
<td>242</td>
</tr>
<tr>
<td>Izora nigricans R.Br.</td>
<td>T10</td>
<td>III</td>
<td>21</td>
<td>176</td>
</tr>
<tr>
<td>Butea monosperma (Lam.) Taubert.</td>
<td>T11</td>
<td>III</td>
<td>12</td>
<td>242</td>
</tr>
<tr>
<td>Stereospermum sp.</td>
<td>T12</td>
<td>III</td>
<td>11</td>
<td>204</td>
</tr>
</tbody>
</table>

Dry deciduous

<table>
<thead>
<tr>
<th>Species</th>
<th>Code</th>
<th>Abundance class</th>
<th>Species</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syzygium cumini (L.) Skeels.</td>
<td>D1</td>
<td>I</td>
<td>24</td>
<td>229</td>
</tr>
<tr>
<td>Terminalia crenulata Roth.</td>
<td>D2</td>
<td>I</td>
<td>13</td>
<td>295</td>
</tr>
<tr>
<td>A. latifolia (DC.) Wall. ex Bedd.</td>
<td>D3</td>
<td>I</td>
<td>15</td>
<td>286</td>
</tr>
<tr>
<td>Grewia tiliifolia Valh.</td>
<td>D4</td>
<td>I</td>
<td>13</td>
<td>219</td>
</tr>
<tr>
<td>Oylea Eugenia (Roxb.) Hocher.</td>
<td>D5</td>
<td>II</td>
<td>13</td>
<td>239</td>
</tr>
<tr>
<td>Lagerstroemia parviflora Roxb.</td>
<td>D6</td>
<td>II</td>
<td>14</td>
<td>260</td>
</tr>
<tr>
<td>Shorea rothburghi G. Don.</td>
<td>D7</td>
<td>II</td>
<td>26</td>
<td>245</td>
</tr>
<tr>
<td>Stereospermum personatum (Hassk.) Chatterjee.</td>
<td>D8</td>
<td>II</td>
<td>20</td>
<td>285</td>
</tr>
<tr>
<td>Careya arborea Roxb.</td>
<td>D9</td>
<td>III</td>
<td>21</td>
<td>272</td>
</tr>
<tr>
<td>Gmelina arborea Roxb.</td>
<td>D10</td>
<td>III</td>
<td>10</td>
<td>301</td>
</tr>
<tr>
<td>Cordia wallichii G. Don.</td>
<td>D11</td>
<td>III</td>
<td>18</td>
<td>223</td>
</tr>
<tr>
<td>Schrebera sp.</td>
<td>D12</td>
<td>III</td>
<td>13</td>
<td>290</td>
</tr>
</tbody>
</table>

Colonization frequency (CF%) of an endophyte species was equal to the number of segments colonized by a single endophyte divided by the total number of segments observed x 100. Evenness index (E5) was computed using the method of Ludwig and Reynolds17. Diversity index (α) was calculated using the method of Fisher et al.18. For other analyses, Colwell's EstimateS software (version 6, http://viceroy.eeb.uconn.edu/estimates) was used.

Although there are several studies on the occurrence of endophytes in tropical plants, most of these are centred on single host species19,20. Sampling of different host species in a plant community and determining host recurrence and spatial heterogeneity among endophytes are needed to elucidate the contribution by these organisms to global fungal diversity10. In the present study, leaves of all the tree hosts harboured endophytes. From 1800 tissue segments, we isolated 2762 isolates belonging to 58 species for the dry thorn forest and 3142 isolates representing 57 species for the dry deciduous forest. Together, the two forests yielded 81 species, among which 56 were isolated from more than one host and 25 were unique forms (isolated only from one particular host). The total CF% ranged from 113 to 204 for the hosts of dry thorn forest and 146 to 200 for the hosts of dry deciduous forest. Each host harboured 10 to 26 endophyte taxa (Table 1). The endophytes of dry thorn forest were more diverse (α = 10.38) than those of the dry deciduous forest (α = 9.89). Chao 1 estimator21 of species richness revealed that we had recovered 93 and 86% of the endophytes present in the dry thorn and dry deciduous forests, respectively, indicating that our sampling procedure was adequate.

A species accumulation curve was plotted using the cumulative numbers of recorded endophyte species against individuals of host trees. The software EstimateS was used to randomize the data 100 times and to obtain a mean species accumulation curve (Figure 1). This showed, that unlike the progressive curve obtained for a moist tropical forest, the number of species of endophytes increased with the number of isolates initially, but the slope flattened with further addition of isolates. In addition, when uniques were plotted against the number of samples, the unique species increased only up to the fifteenth sample and then the curve regressed (Figure 2). This shows that as the sample size increased some of the uniques recurred, thus losing their 'unique' status. These results suggested that some tropical forests might not be hyperdiverse with reference to endophytes.
Figure 1. Species accumulation curve for endophytes isolated from trees of two dry tropical forests. Individuals were shuffled among samples and 100 randomizations were made.

Figure 2. Number of unique species isolated from different host trees. Data were randomized 100 times before plotting the graph. The curve represents a polynomial trendline.

We did not encounter any host specificity among the endophytes. Again, unlike plants of the temperate regions\(^2\), no distinct fungal communities were seen to be associated with any host. Cannon and Simmons\(^1\) obtained similar results for 12 tree species of Iwokrama Forest Reserve in Guyana. Further, we found that species of *Phyllosticta* or *Phomopsis* dominated the endophyte assemblages in 20 of the 24 hosts. The evenness of the endophyte assemblage increased from 0.45 to 0.59 for the dry thorn forest and from 0.58 to 0.61 for the dry deciduous forest, when the dominant fungus was excluded from the analysis. This index decreases when any assemblage is dominated by a single genus\(^3\).

There appears to be no study regarding the influence of host abundance on the occurrence of endophytes in tropical forests. In the present investigation, the tree hosts studied in each forest fell into three abundance classes (Table 1). In dry thorn forest, exclusion of abundance class I (most abundant hosts) from the analysis reduced the diversity index from 10.38 to 7.60. However, excluding the abundance class III (least abundant hosts) did not affect this index (Table 2). A calculation of similarity index showed that the endophyte assemblage without the host abundance class III was 97% similar to the endophyte assemblage of the entire forest, while the assemblage without the host abundance class I showed only 72% similarity with the endophyte assemblage of the entire forest (Table 2). Thus, the density of host population determined the composition and distribution of endophytes in this tropical forest. However, the distribution of hosts had little influence on the endophyte assemblage in the dry deciduous forest (Table 2). Factors such as leaf age\(^20,21\) and precipitation\(^14,24\) are known to influence the density of endophyte colonization in tropical forests. Further studies are needed to determine if the patchiness in host distribution influences endophyte distribution.

Petrini\(^25\) was of the opinion that host specificity among endophytes is expressed at the family level. However, some endophyte genera such as *Phyllosticta*, *Phomopsis* and *Colletotrichum* occur in a wide variety of distantly-related host plants\(^9,20,26\). Such a host recurrence\(^27\) was observed in the present study with reference to *Phyllosticta* and *Phomopsis*. We also found that these ubiquitous endophytes dominated the endophyte assemblages of different hosts. Host recurrence of a few dominant fungi indicated that these forests might not be hyperdiverse with reference to endophytes. This was substantiated when the diversity index was calculated after removing the dominant fungus. The endophyte species diversity increased when the dominant species was excluded from the analysis (\(\alpha = 11.16\) and 10.28 for dry thorn and dry deciduous respectively).

There were two groups of foliar endophytes in both the forests. One group comprised the ubiquitous forms that invariably dominated the assemblage; the less frequent and unique forms contributed the other. The ubiquitous forms are generalists and have attained extraordinary success in occupying a niche such as the internal tissues of a plant. The unique forms represent a higher degree of specialization among endophytes, as they could probably be host-specific. However, among the 25 unique species, 15 were singletons, probably indicating that they were rare rather than host-specific; the rest of the unique were ubiquitous fungi such as *Phomopsis* sp., *Phyllosticta* sp., *Cladosporium* sp. and *Curvularia* sp. Thus, apparently no host-specific forms could be isolated from trees of either forests, a condition akin to Iwokrama forest in Guyana\(^11\).

Host specificity of endophytes concomitant with high host diversity is expected to increase the diversity of endophytes. We did not encounter clear host specificity in our study. Even though plant diversity is high in tropical forests, diversity of the endophytes may not be high in all of them. This is attributed to the presence of dominant generalists and low frequency of occurrence of host-specific forms. Our results on the study of endophytes in the dry forests of Madumalai Sanctuary, South India, and those of Cannon and Simmons\(^11\) on endophytes of Iwokrama forest, Guyana, strengthen the view of May\(^28\) that
highly diverse tropical forests could select against host specificity due to the disjunct distribution of hosts. Thus, some tropical forests are expected to have less host-specific species.28

This study was aimed at comparing the endophyte status of dry tropical forests of South India with that of a moist tropical forest of Central Panama. Hence we followed essentially the methodology as described for the study of the moist forest27. This methodology favours quick-growing fungi29. Maybe some endophytes within plants cannot grow on the agar medium used, or grow slowly on the medium so that there is a bias towards isolating the quicker-growing endophytes. In addition, recent molecular sequencing studies have shown that morphologically similar fungi may, in fact, be different species30. Thus, it is possible that the diversity of endophytes is slightly underestimated here. However, ITS–RFLP analysis of a dominant Phyllosticta isolated from several trees in the present study, showed that they all belonged to the same species31.

We suggest that host abundance should be considered while studying a tropical plant community for endophytes. Moreover, while attempting to study tropical forests for their endophyte assemblages, it is suggested to identify the generalists first in order to rapidly calculate the diversity. Host specificity is critical to extrapolation on global biodiversity of fungi since this estimate heavily depends on fungus: plant ratios3. Hence, prediction of global fungal diversity based on studies of a particular geographic area or host assemblage may be erroneous, as it may not be representative in nature. This appears to be especially true for tropical endophytes.

Sequestration of ecdysteroid hormone into the ovary of the mole crab, *Emerita asiatica* (Milne Edwards)

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Ecdysteroids are the principal steroid hormones of arthropods, regulating different physiological functions such as growth, metamorphosis and reproduction. Radioimmunoassay indicates the accumulation of ecdysteroids into the ovary during maturation of a sand crab *Emerita asiatica*. These ecdysteroids are sequestered from the hemolymph during the entire period of intermolt stage. However, the ovariian ecdysteroids drastically declined during the premolt stage corresponding to a steep rise in the hemolymph ecdysteroid level. The possible role of ecdysteroids in vitellogenesis and embryonic development is discussed.

ECDYSTEROIDS, a group of polyhydroxy steroids, occur in all arthropod groups serving the main function of molting hormones. In the holometabolous insects, during larval stages, ecdysteroids produced by the prothoracic glands control the molting and metamorphic activities; in the reproductive adulthood, ovarian ecdysteroids are thought to play a role in vitellogenesis, as demonstrated in the higher dipteran flies. Embryonic ecdysteroids, derived both from maternal contribution during vitellogenesis as well as the *de novo* synthesis by larval prothoracic gland, function as morphogenetic hormones, controlling vital activities such as the secretion of embryonic envelopes and embryonic molting.

Crustaceans differ from insects that they continue to molt and grow even after attainment of sexual maturity. In the large decapod crustaceans such as crabs and lobsters, the female reproductive cycle is completed within the protracted intermolt period and the molting is initiated only after reproductive arrest. Conversely, in many soft-shelled shrimps and prawns, the molting is permitted to occur during the course of the oogenetic cycle, thus revealing a close synchronization between the molting and reproductive processes (see ref. 4 for a review). This kind of synchronized activity of these two rather energy-demanding physiological processes may suggest that molting and reproductive cycles are regulated in a co-ordinated manner by common endocrine means. The anomuran mole crab, *Emerita asiatica* not only molts and breeds throughout the year, but also exhibits synchronization of these two processes to bring about the continued body growth even in the egg-laying adults. In these crabs, the ovarian development, commencing in the early intermolt stage, continues well into the premolt stage, when the preparatory processes for molting occur. Such an overlapping of reproductive and molting activities found in *E. asiatica* is similar to that described in a freshwater prawn *Macrobrachium rosenbergii*. Furthermore, radioimmunoassay of the active molting hormone, 20-hydroxyecdysone (20E) revealed a premolt surge in the hemolymph of *E. asiatica*, as shown already in other crustaceans. In this communication, we report on the sequestration of hemolymph ecdysteroids into the ovary during a specific period in the molt cycle of *E. asiatica*. *E. asiatica* is a typical burrowing decapod, found on the wave-washed sandy beaches of Madras coast. *E. asiatica*, ranging in size from 18 to 33 mm carapace length (CL), were collected from the intertidal region of Elliot’s Beach, Besant Nagar, Chennai. The size was measured from the posterior margin of the carapace along the mid-dorsal line to the tip of the rostrum. At least 50 to 100

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