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Assemblages of Endophytic Fungi on *Bruguiera gymnorrhiza* in the Shiira River Basin, Iriomote Is.

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Summary

Assemblages of endophytic fungi were investigated on the leaves of *Bruguiera gymnorrhiza* from the upper reach to the estuary of the Shiira River, Iriomote Is., Okinawa Pref., Japan. Few species were detected in summer (July 1997 and September 1998; 2-5 spp.) than in winter (February 1998; 5-9 spp.). The colonization frequency of fungi was higher on leaves collected in the upper reach than in the lower reach in each of the three surveys, but the number of species isolated was higher from leaves collected in the lower reach. *Colletotorichum* sp. was isolated in high colonization frequency from leaves collected at two upper stations, 33% at P-1 and 40% at P-3; but in low frequency from leaves collected at three lower stations, 5% at P-5, 4% at P-7 and 6% at P-9. *Phyllosticta* sp. and *Pestalotiopsis* sp. 1 were isolated in low frequency from leaves collected at all stations, and no difference in colonization frequency between the upper and lower reach was found. It is considered that assemblages of endophytic fungi of *B. gymnorrhiza* vary according to the surrounding vegetation.

Keywords: *Bruguiera gymnorrhiza*, endophytic fungi, fungal diversity, mangrove.

Endophytic fungi within tissues of aerial parts of vascular plants have been extensively studied over the past 15 years and found in a wide range of plants (17). In Japan, endophytic fungi have been investigated in conifers, grasses and several broad-leaf plants (3, 4, 5, 8, 9, 12, 13), but further investigations are necessary to clarify their ecological and species diversity on various plants. Because of its hot, moist climate during the growing season and diverse vascular plant flora, Japan is expected to have a very rich flora of endophytic fungi (1).

Most study on endophytic fungi has been carried out on plants from the temperate regions (17, 18), and it is only comparatively recently that the endophytic mycobiota of tropical ecosystems have been investigated. Petrini and Dreyfuss (19) isolated fungal endophytes of *Araceae, Bromeliaceae* and *Orchidaceae* from French Guyana, and from *Piperaceae* and *Crassulaceae* collected in Brazil and Colombia (2). Rodrigues and

Samuels (21) reported on the endophytes of the fan palm *Licuala ramsayi* (Muell.) Domin. from a tropical rain forest in Australia. However, studies on assemblages of endophytes in mangrove plants are very few (7, 20, 22), while mangrove regions preserve a great amount of biological resources including mycobiota.

In this study, we surveyed the assemblages of endophytic fungi within the leaves of *Bruguiera gymnorrhiza* Lamk. collected at five research stations along the Shiira River, Iriomote Is., Okinawa Pref.

Materials and Methods

Sample collection and fungal isolation. Young (new) and mature leaves (older than the third leaf) of *B. gymnorrhiza* were collected from trees at five stations (P-1, P-3, P-5, P-7 and P-9) along the Shiira River in Iriomote Is. (24N, 124E), Okinawa Pref., Japan. P-1 is 2.2 km from the river mouth, and at this uppermost point a stand of *B. gymnorrhiza* grows. P-3 is 400 m downstream from P-1, and P-5 is 400 m downstream from P-3 on the middle reach of the river, where *B. gymnorrhiza* forms dominant forest. P-7 is 400 m downstream from P-5, and P-9 is 400 m downstream from P-7 at the river mouth. Samples were collected in July 1997, February 1998 and September 1998. In Iriomote Is., the highest monthly average temperature occurs in July, the lowest in February. To detect fungi, two disks of 8 mm diam were punched out with a cork borer from 10 leaves of each leaf stage. The surface of the leaf disks was sterilized by immersion in 70% ethanol for 1 min and sodium hypochlorite solution (1% available chlorine) for 2 min. The disks were rinsed in sterile distilled water and put into sterile paper towels for 3 hr to remove water from the surface. They were then placed on half-strength corn meal seawater agar (CMSWA) (half-strength Corn Meal Agar with compensatory agar was dissolved in 15 ppt seawater) in 90-mm plates and incubated at 17°C for more than 3 months. The fungi growing out of the leaf disks were isolated and identified. The colonization frequency (CF) of individual fungi was calculated by the following equation:

CF (%) = (number of disks from which the fungus was detected/total number of disks examined in each sample) × 100.

Data analysis on species richness and diversity of endophytic fungi among seasons, stations, and dominant species. In this analysis, data of each leaf stage (young and mature) in three trials (July 1997, February and September 1998) were applied as one sampling unit. For analysis by station, six sampling units were applied as a data set of each station.

Species richness (number of species) was estimated by the jackknife estimate (10) by use of the following equation:

$$S = s + [(n-1)/n]\ k$$

where S = jackknife estimate of species richness, s = observed total number of species present at a station, n = total number of sampling units: 6 in this study, k = number of unique species, defined as species occurring in only one sampling unit.

Species diversity was measured by using Simpson's reciprocal index and the Shannon- Wiener function (10), as expressed by the following equation:

Simpson's reciprocal index : $1/D=1/\Sigma p_i^2$

where D=Simpson's index, p_i=Proportion of species i at a station

Shannon-Wiener function: $H'=\sum_{i=1}^{S}(p_i)(\log_2 p_i)$

where H'=information content of sample=index of species diversity, s=number of species, p_i=proportion of total sample belonging to ith species.

To test a difference in colonization frequency of all fungi at each station, a statistic for test for the proportion, $T(m_1, m_2)$, was calculated by using the following equation:

$$T(m_1, m_2)=[(m_1/N_1)-(m_2/N_2)-d_0]/\sqrt{p(1-p)(1/N_1-1/N_2)}$$

where m=number of leaf disks from which fungi were detected, N=total number of leaf disks, $d_0=p_1-p_2=0$ (no difference between p_1 ($=m_1/N_1$) and p_2 ($=m_2/N_2$) in statistical hypothesis), $p=(m_1+m_2)/(N_1+N_2)$

Differences in colonization frequency of three dominant fungi, *Colletotrichum* sp., *Phyllosticta* sp. and *Pestalotiopsis* sp. 1 at each station were also tested by using the above equation.

Results and Discussion

Several fungi including sterile mycelia were detected from the leaves of *B. gymnorrhiza* in the Shiira River basin. In the three years of this study, we isolated fungi from 296 of 600 leaf disks examined (ca. 50% colonization frequency). No significant difference was found in the colonization frequency of fungi between the surveys: 46% in July 1997, 51% in February 1998 and 52% in September 1998. However, the number of species isolated was different in summer (July 1997 and September 1998) and winter (February 1998): 8 spp. in July 1997, 6 spp. in September 1998 and 14 spp. in February 1998 (Table

Table 1. Colonization frequency (CF) (%) of the endophytic fungi on leaves (Y and M) of *B. gymnorrhiza* in July 1997.

Station	P-1		P-3		P-5		P-7		P-9	
Fungus	Y[a]	M[a]	Y	M	Y	M	Y	M	Y	M
Colletotrichum sp.	10	35	35	60	0	10	15	0	25	0
Pestalotiopsis sp.1	0	0	15	0	0	0	0	0	0	5
Phyllosticta sp.	0	0	0	0	20	35	0	30	0	15
Phomopsis sp.	15	20	5	0	0	0	0	0	0	0
xylariaceous fungi[b]	0	0	0	0	0	5	0	0	0	0
Surculiseries rugispora[c]	0	0	5	0	0	0	0	0	0	0
white sterile mycelia	5	15	10	15	10	15	10	10	0	10
colored sterile mycelia	0	0	0	0	0	0	0	15	0	10
CF in each leaf stage	30	70	45	75	30	70	25	50	25	35
CF in station	50		60		50		38		30	
Number of fungi detected	3		5		4		4		5	

[a] Y, young leaves; M, mature leaves.

[b] including *Geniculosporium* sp.

[c] new species (14).

1-3). *Pestalotiopsis* sp. 2, *Phoma* sp., *Acremonium* sp., an unidentified ascomycete and a coelomycete were only found in the survey of February 1998. The jackknife estimate put the number of species at 18.5 (in the range of 14-23) according to the actual number of species detected in February 1998, but 9.8 (7-13) in July 1997 and 6.9 (5-9) in September 1998 (shown in Table 5). Simpson's reciprocal index (1/D) and Shannon-Wiener function (H′) showed that the diversity of species inhabiting leaves of *B. gymnorrhiza* is higher in winter (1/D=7.21, H′=3.16) than summer (1/D=4.02, H′=2.31 in July 1997; 1/D=3.45, H′=1.99 in September 1998) (Table 5). The high temperature, high humidity and strong ultraviolet irradiation in summer in the Ryukyu Islands including Iriomote Is. may affect sporulation of the fungi, spore germination and invasion of the

Table 2. Colonization frequency (CF) (%) of the endophytic fungi on leaves (Y and M) of *B. gymnorrhiza* in February 1998.

Station	P-1		P-3		P-5		P-7		P-9	
Fungus	Y[a]	M[a]	Y	M	Y	M	Y	M	Y	M
Colletotrichum sp.	25	35	20	40	5	5	0	10	5	5
Pestalotiopsis sp.1	0	0	0	0	5	30	0	10	5	10
Pestalotiopsis sp.2	0	0	0	0	0	5	0	0	0	0
Phyllosticta sp.	5	15	0	0	0	5	0	0	5	10
Phomopsis sp.	0	10	0	0	0	0	10	0	0	10
Phoma sp.	0	15	5	0	0	0	0	5	0	15
Acremonium sp.	0	0	0	0	0	0	0	0	0	5
xylariaceous fungi[b]	0	0	5	0	50	15	5	0	10	5
Surculiseries rugispora[c]	0	0	0	0	0	5	0	0	0	0
ascomycete sp.	0	0	0	0	5	0	0	0	0	0
white sterile mycelia	15	15	0	30	5	15	10	20	5	0
colored sterile mycelia	0	0	0	45	0	25	0	0	0	0
coelomycete sp.	0	0	0	0	0	0	0	5	0	0
CF in each leaf stage	45	70	25	75	60	80	25	45	30	50
CF in station	58		50		70		35		40	
Number of fungi detected	5		5		9		7		9[b]	

[a] Y, young leaves; M, mature leaves.
[b] including *Geniculosporium* sp.
[c] new species (14).

Table 3. Colonization frequency (CF) (%) of the endophytic fungi on leaves (Y and M) of *B. gymnorrhiza* in September 1998.

Station	P-1		P-3		P-5		P-7		P-9	
Fungus	Y[a]	M[a]	Y	M	Y	M	Y	M	Y	M
Colletotrichum sp.	45	50	45	40	10	0	0	0	0	0
Pestalotiopsis sp.1	0	5	0	0	5	0	0	0	0	0
Phyllosticta sp.	0	0	35	40	0	25	10	0	5	5
xylariaceous fungi[b]	10	20	0	0	0	15	0	0	0	0
Pithomyces sp.	0	0	0	0	0	0	0	0	0	5
white sterile mycelium	5	25	5	35	10	0	10	55	5	30
CF in each leaf stage	60	90	75	100	25	40	20	55	15	40
CF in station	75		88		33		38		28	
Number of fungi detected	4		3		5		2		3	

[a] Y, young leaves; M, mature leaves.
[b] including *Geniculosporium* sp.

leaves of the host plants.

The colonization frequency of fungi was higher on the leaves collected from the upper reach than from the lower reach in all three surveys (Table 1-3). The colonization frequency increased as the leaves aged, and the number of fungal species isolated also tended to increase in most cases. The colonization frequencies at stations P-1 and P-3 were above 60%, and that at P-5 was 50.8%, while those at P-7 and P-9 were 36.7% and 32.5%, respectively (Table 4). The statistical tests supported a significant difference in colonization frequency of all fungi detected between the upper and lower stations (Table 6, (a)). While the colonization frequency increased from the lower to the upper reach, however, the number of fungal species isolated from leaves was higher in the lower reach than the upper: 12 spp. at P-9 as compared to 7 spp. at P-1 (Table 4). The number of species at each station estimated by the jackknife estimate are as follows: 8.67 (range, 6-11) at P-1, 13.99 (7-21) at P-3, 13.33 (7-20) at P-5, 13.99 (7-21) at P-7, 18.66 (11-26) at P-9 (Table 5). Both indexes of species diversity showed a richer diversity of fungal species harbored in *B. gymnorrhiza* in the lower reach than the upper (Table 5).

Colletotorichum sp., *Pestalotiopsis* sp.1 and *Phyllosticta* sp. were isolated from leaves at every station. *Colletotrichum* sp. was isolated more frequently from the leaves collected at the upper stations (33% at P-1, 40% at P-3) than the lower stations (5% at P-5, 4% at P-7, 6% in P-9) (Table 4 and Fig. 1). The statistical tests supported a significant difference in colonization frequency of *Colletotrichum* sp. between the upper stations, P-1 and P-3, and the lower stations P-5, P-7 and P-9 (Table 6, (b)). No such difference was found for the other dominant fungi, *Pestalotiopsis* sp.1 and *Phylllosticta* sp. (Table 6, (c)

Table 4. Colonization frequency (CF) (%) of the endophytic fungi on leaves of *B. gymnorrhiza* at five research stations.

Station / Fungus	P-1	P-3	P-5	P-7	P-9	No. of stations at which fungus was detected
Colletotrichum sp.	33.3	40	5	4.2	5.8	5
Pestalotiopsis sp.1	0.8	2.5	6.7	1.7	3.3	5
Pestalotiopsis sp.2	0	0	0.8	0	2.5	2
Phyllosticta sp.	3.3	12.5	14.2	6.7	6.7	5
Phomopsis sp.	7.5	0.8	0	1.7	1.7	4
Phoma sp.	2.5	0.8	0	0.8	2.5	4
Acremonium sp.	0	0	0	0	0.8	2
xylariaceous fungi[a]	5	0.8	14.2	0.8	2.5	5
Pithomyces sp.	0	0	0	0	0.8	1
Surculiseries rugispora[b]	0	0.8	0.8	0	0	2
ascomycete sp.	0	0	0.8	0	0	1
white sterile mycelia	13.3	15.8	9.2	18.3	8.3	5
colored sterile mycelia	0	7.5	4.2	2.5	1.7	4
coelomycete sp.	0	0	0	0.8	0	1
CF in station	60.8	65.8	50.8	36.7	32.5	
No. of fungi detected	7	9	10[a]	9	12[a]	

[a] including *Geniculosporium* sp.

[b] new species (14).

and (d), respectively).

In the upper reach of the Shiira River, *Barringtonia racemosa* Blume, *Mallotus*

Table 5. Species richness and diversity of endophytic fungi on *B. gymnorrhiza* in three surveys and at five research stations.

	Jul. 1997	Feb. 1998	Sep. 1998	P-1	P-3	P-5	P-7	P-9
Number of species detected	8	14	6	7	9	10	9	12
No. of species by jackknife estimate (S)	9.8	18.5	6.9	8.67	13.99	13.33	13.99	18.66
(range)	7-13	14-23	5-9	6-11	7-21	7-20	7-21	11-26
Simpson's reciprocal index (1/D)	4.02	7.21	3.45	3.12	3.22	6.21	3.32	7.54
Shannon-Wiener Function (H')	2.31	3.16	1.99	2.08	2.12	2.86	2.3	3.21

Note: the equations used to analyze species richness and diversity were adapted from Krebs (10).

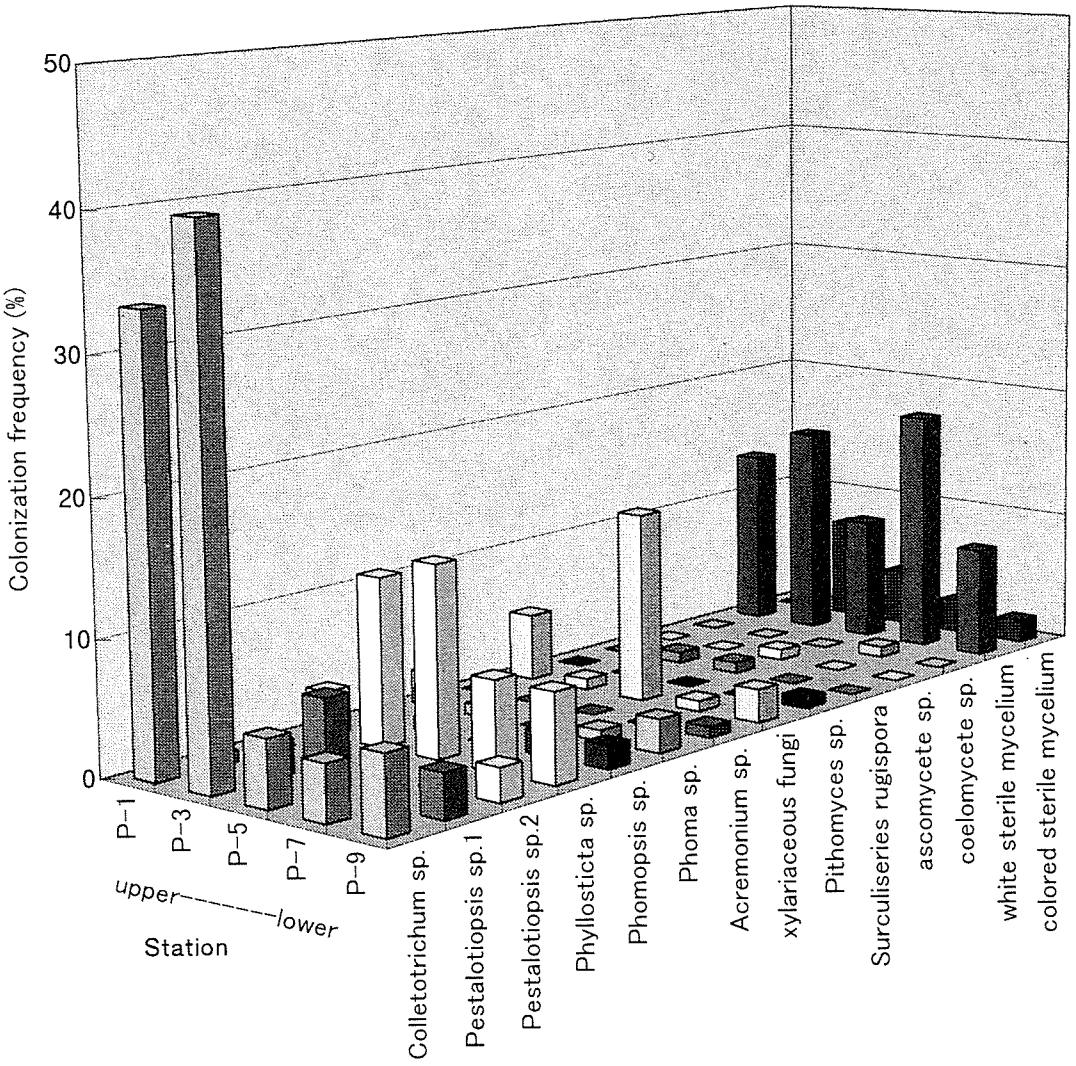

Fig. 1. Colonization frequency of endophytic fungi of *Bruguiera gymnorrhiza* at each station.

Table 6. Statistical tests for a difference in the colonization frequency of all fungi, and of three dominant fungi at each station.

(a) all fungi detected

	P-3	P-5	P-7	P-9
P-1	0.8	1.56	**3.73	**4.39
P-3		*2.36	**4.51	**5.16
P-5			*2.2	**2.88
P-7				0.68

(b) *Colletotrichum* sp.

	P-3	P-5	P-7	P-9
P-1	1.08	**5.57	**5.78	**5.37
P-3		**6.49	**6.69	**6.30
P-5			0.3	0.27
P-7				0.57

(c) *Pestalotiopsis* sp.1

	P-3	P-5	P-7	P-9
P-1	1.01	*2.38	0.58	1.36
P-3		1.54	0.46	0.39
P-5			0.39	0.95
P-7				0.41

(d) *Phyllosticta* sp.

	P-3	P-5	P-7	P-9
P-1	**2.64	**2.96	1.17	0.35
P-3		0.37	1.55	*2.34
P-5			1.9	*2.01
P-7				0.84

Note: *, significantly different at 0.05 level; **, significantly different at 0.01 level.

moluccanus Mueller-Arg. and *Trema orientalis* (L.) Blume are dominant plants around P-1, while *Helitiera littoralis* Ait., *Rhaphiolepis indica* (L.) Lindley and *Persea thunbergii* (Sieb. et Zucc.) Kostermans dominate the forest around P-3. Forest in the upper reach consists of many species of subtropical evergreen trees, which in places form a canopy over mangrove trees growing at the river's edge. In the lower reach, vegetation consisting of *B. gymnorrhiza, Kandelia candel* (L.) Druce and *Pandanus tectorius* Park. is dominant around P-5, while *B. gymnorrhiza* is dominant P-7. Around P-9, the lowest station in this study, *Rhizophora stylosa* Griff. predominates over *B. gymnorrhiza*. The richer vegetation in the upper reach suggests a more diverse fungal flora in the upper reach than the lower. However, fewer fungal species were found in leaves of *B. gymnorrhiza* collected in the upper reach, while the colonization frequency of fungi, especially *Colletotrichum* sp., was higher in the upper reach. In the case of apple bitter-rot and peach anthracnose caused by *Colletotrichum* spp., it has been reported that other trees growing in close proximity to these fruit trees appear to be major source of primary infection of these diseases (6, 11, 15). In the upper reach of the Shiira River, other trees growing near *B. gymnorrhiza* are likely to serve as an inoculum source of *Colletotrichum* sp., which is found in *B. gymnorrhiza* as an endophyte. This fungus may quickly infect the leaves and predominate over the other fungi, or prevent other fungi from invasion. In the lower basin, from around P-5 to the river mouth, *B. gymnorrhiza* and another mangrove trees form open forest, which is never covered by other trees throughout the year. This may be why the colonization frequency of *Colletotrichum* sp. decreased sharply in the lower forest of *B. gymnorrhiza*. It is considered that assemblages of endophytic fungi of *B. gymnorrhiza* vary according to the surrounding vegetation.

On the other hand, we should also consider the physiological nature of *B. gymnorrhiza*. Tsukamoto and Nakanishi (23, 24) reported that inorganic salt and chemical

element contents of the leaves of *B. gymnorrhiza* and *R. stylosa* at the Shiira River basin, which control osmotic pressure inside plant tissue to restore moisture and nutrition, change according to the concentration of salt in water. They also reported that a positive correlation between manganese and tannin contents is an important factor controlling oxidation-reduction potential in these mangrove plants. With the increase in manganese content that accompanies the decrease salt concentration in river water in upper reach, oxidative stress inside the leaves builds up. Conversely, reductive stress inside the leaves increases in the lower reach because of a sharp drop of manganese content. Further study on the influence on fungi of chemical elements and the internal environment of plant tissues is necessary to clarify the factors involved in establishment of assemblages of endophytes.

Finally, in the course of this study, an unknown imperfect fungus was isolated from the leaves collected at P-3 and P-5, which we accommodated in a new genus and named *Surculiseries rugispora* Okane *et al.* (14). This fungus is considered to belong to Xylariaceae based on sequence analysis of 18S ribosomal DNA. Xylariaceous fungi have been found from some plant families, especially in tropical regions, and they are considered to appear commonly on census lists of endophytes (16, 19). Discovery of this new fungus from *B. gymnorrhiza* indicates that mangrove forests preserved in the Ryukyu Islands harbor unknown fungi and encourages further investigation of fungal diversity in this region.

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