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Isolation of endophytic fungi from leaves of neem (*Azadirachta indica* A. Juss.)

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Occurrence of asymptomatic fungal endophytes in green and senescent leaves of neem is reported. Only five endophytes were isolated from the leaves. Of these, four were sterile forms and one was *Fusarium avenaceum*. The frequency of occurrence of endophytes was significantly higher in the basal leaflets than in the apical or middle leaflets and in the main vein of the leaflet than in the lamina tissue. The frequency of colonization of green leaves by endophytes increased during the rainy season although no new endophyte species could be recovered. The restricted number of endophytic fungal genera and the absence of common endophytic fungi in the neem leaves could be due to the antifungal metabolites present in the leaves. The results suggest that occurrence of foliar endophytes in tropical trees is influenced by environment, and type and chemistry of the host tissue.

ENDOPHYTIC fungi colonize living plant tissues without producing any apparent disease symptoms or obvious negative effects[1]. The endophytes are usually ascomycete and mitosporic fungi. Endophytes are being intensively studied since some of them may be sources of novel biochemicals of pharmaceutical importance[2,3]. The investigation of this relatively unexplored group of fungi may also reveal new forms[4]. Most work on fungal endophytes however, has been centred on plants from temperate regions and relatively few tropical plants have been studied for the presence of endophytes[5]. The tropical plants that have been studied for the presence of endophyte include members of Araceae, Bromeliaceae and Orchidaceae from French Guyana[6], Piperaceae and Crassulaceae from Brazil[7], Musaceae[8] and Poaceae[9] from Hong Kong and various palms[10–12]. In India, only two species of mangrove trees[13], some trees of tropical montane evergreen forest and dry deciduous forest[14], halophytes[15] and an angiosperm parasite[16] have been screened for the presence of endophytes. We report here the presence of endophytes in the leaves of neem, a tropical tree native to India.

The neem belongs to the family Meliaceae and is of considerable economic importance. The beneficial uses of the products of neem tree have been known to the people of India for centuries, but more recently their economic properties have been recognized by the developed countries[17]. Neem products are broad-spectrum pesticides and act on several insect species including phytophagous insects[18]. The medicinal importance of different parts of neem are well known to the practitioners of Indian Ayurveda and Unani systems of medicine[18].

Mature green leaves and senescent (yellow) leaves of neem were collected from five medium-sized trees growing in Chennai, South India, which is at sea level and experiences a dissymmetric tropical climate[19]. To determine the influence of seasons on the occurrence of endophytes, green leaves were collected on a monthly basis for twenty-four months (May 1996 to April 1998) and screened for the presence of endophytes.

Each leaf was thoroughly washed in running water before sampling. Lamina segments (approximately 0.5 cm^2) cut from the middle portion of the leaflets of green and senescent leaves were dipped in 70% ethanol for 5 sec immersed in 4% NaOCl for 1 min and rinsed in sterile water for 10 sec (ref. 20) and plated on potato dextrose agar medium amended with streptopenicillin (150 mg l^{-1}). To determine the distribution of endophytes in a single leaflet, the middle portion with the main vein, the leaf blade devoid of the main vein and the rachis were sampled.

The sterilized segments of the leaf were placed on 15 ml of the medium contained in a petri dish (9 cm diameter) and incubated in a light chamber for 21 days at 26°C (refs 21, 22). The source of light was three 4-feet Philips daylight fluorescent lamps. The leaf segments received approximately 2200 lux of light through the petri dish lid as measured by a Lutron LX-101 (Germany) lux meter. At each sampling period, twenty compound leaves were collected from each tree and three hundred segments were screened for the presence of endophytes.

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The colonization frequency (CF%) of each endophyte species was calculated by the method of Hata and Futai[23]. CF% = $(N_{col}/N_t) \times 100$; where N_{col} and N_t are the number of segments colonized by each endophyte and the total number of leaf segments examined respectively. Both two-way ANOVA and ranked ANOVA tests were performed in order to ensure that statistical differences between endophyte colonization and their location in the leaf existed[24]. These tests were also used to compare the CF% of the endophytes occurring in neem leaves during different seasons. Some endophyte isolates remained sterile even after prolonged incubation under light and hence could not be identified. They were grouped based on colony characters such as colony morphology and growth rate and were given code numbers[20].

The basal leaflets, middle leaflets and the apical leaflets of green and senescent leaves were screened for endophyte presence. *Fusarium avenaceum* and two sterile endophytes (VCC 51 and VCC 74) were present in both green and senescent leaves (Tables 1 and 2). Senescent leaves showed a higher CF% (46%) than the green leaves (30%) (Table 1). It has been established that the total infection frequency of endophytes increases with the age of the host tissue[12,25,26]. In both green and senescent compound leaves of neem, the basal leaflets showed significantly more endophyte colonization than the middle or apical leaflets (Table 1). This could be due to the availability of larger surface areas for endophyte colonization in the basal leaflets (mean surface area 13 cm^2) than in the middle (7.2 cm^2) or apical leaflets (4.2 cm^2). For further studies, the basal leaflets were used.

The distribution of endophytes varied with the position of the leaflet sampled. The CF% was significantly higher for the main vein than for the lamina (Table 2). This was similar to the results obtained by Wilson and Carroll[27] for *Quercus garryana* and Taylor, Hyde and Jones[12] for *Trachycarpus fortunei*. Again, the CF% of endophytes was significantly higher in rachis base of both senescent and green leaves when compared to that in the leaflets (Table 2). Such a within-leaf variation in the distribution of endophytes in leaf tissues has been reported for some temperate trees[28]; for example, petioles of Douglas-fir[29] and Oregon white oak[27] harbour more endophytes than their lamina tissues. Such within-leaf variation in the occurrence of endophytes is attributed to the differential leaf expansion[27]. During maturation, the lamina region of the leaf expands more than the petiole region; and hence infections by endophytes established before leaf expansion may become 'diluted' as the leaf expands[27].

The occurrence of foliar endophytes is influenced by seasonal changes[27,30]. Generally, leaves sampled during the wet season harbour more endophytes[13,31]. The occurrence of endophytes in the basal leaflets of green leaves of neem also showed this trend (Figure 1). The climate in Chennai may be broadly classified into three seasons based on rainfall pattern and moisture content of the environment[32]. The dry season (January to March) receives the least rainfall and dew is the main source of moisture. The summer season (April and May) is characterized by high temperature and erratic rainfall. The monsoon season (June to December) receives maximum precipitation from both the South-west (June to September) and the North-east (October to December) monsoons. The CF% of the endophytes was significantly higher in the monsoon season (49.6%) than during the dry season (24.3%). No endophytes could be isolated from leaves collected in the summer season (Figure 1).

Generally, a large number of endophytic fungal genera can be isolated from a single host species[33]. However, a prolonged sampling of the basal leaflet of green leaves

Table 1. Occurrence of fungal endophytes in different leaflets of green and senescent compound leaves of neem

Leaf part	Endophyte	CF%	Total CF%
Green leaf			
Basal leaflet	*Fusarium avenaceum*	12	
	VCC 51 (sterile)	4	16
Middle leaflet	*F. avenaceum*	7	
	VCC 51 (sterile)	2	9
Apical leaflet	*Fusarium avenaceum*	2	
	VCC 51 (sterile)	3	5
Senescent leaf			
Basal leaflet	*F. avenaceum*	13	
	VCC 51 (sterile)	6	
	VCC 74 (sterile)	2	21
Middle leaflet	*F. avenaceum*	10	
	VCC 74 (sterile)	6	16
Apical leaflet	*F. avenaceum*	3	
	VCC 74 (sterile)	6	9

300 segments of each category were sampled. At 5% level, there was significant difference between treatments. CF, Colonization frequency.

Table 2. CF% of endophytes in different parts of basal leaflet and rachis of neem

Leaf part	Endophyte	CF%	Total CF%
Green leaf			
Main vein	*Fusarium avenaceum*	17	
	VCC 74 (sterile)	1	18
Lamina	*F. avenaceum*	11	
	VCC 74 (sterile)	2	13
Rachis base	*F. avenaceum*	11	
	VCC 51 (sterile)	18	
	VCC 52 (sterile)	7	
	VCC 74 (sterile)	6	42
Senescent leaf			
Main vein	*F. avenaceum*	22	
	VCC 51 (sterile)	6	28
Lamina	VCC 51 (sterile)	3	3
Rachis base	*F. avenaceum*	22	
	VCC 51 (sterile)	19	
	VCC 52 (sterile)	1	
	VCC 74 (sterile)	7	
	VCC 75 (sterile)	5	54

300 segments of each category were sampled. At 1% level, there was significant difference between treatments. CF, Colonization frequency.

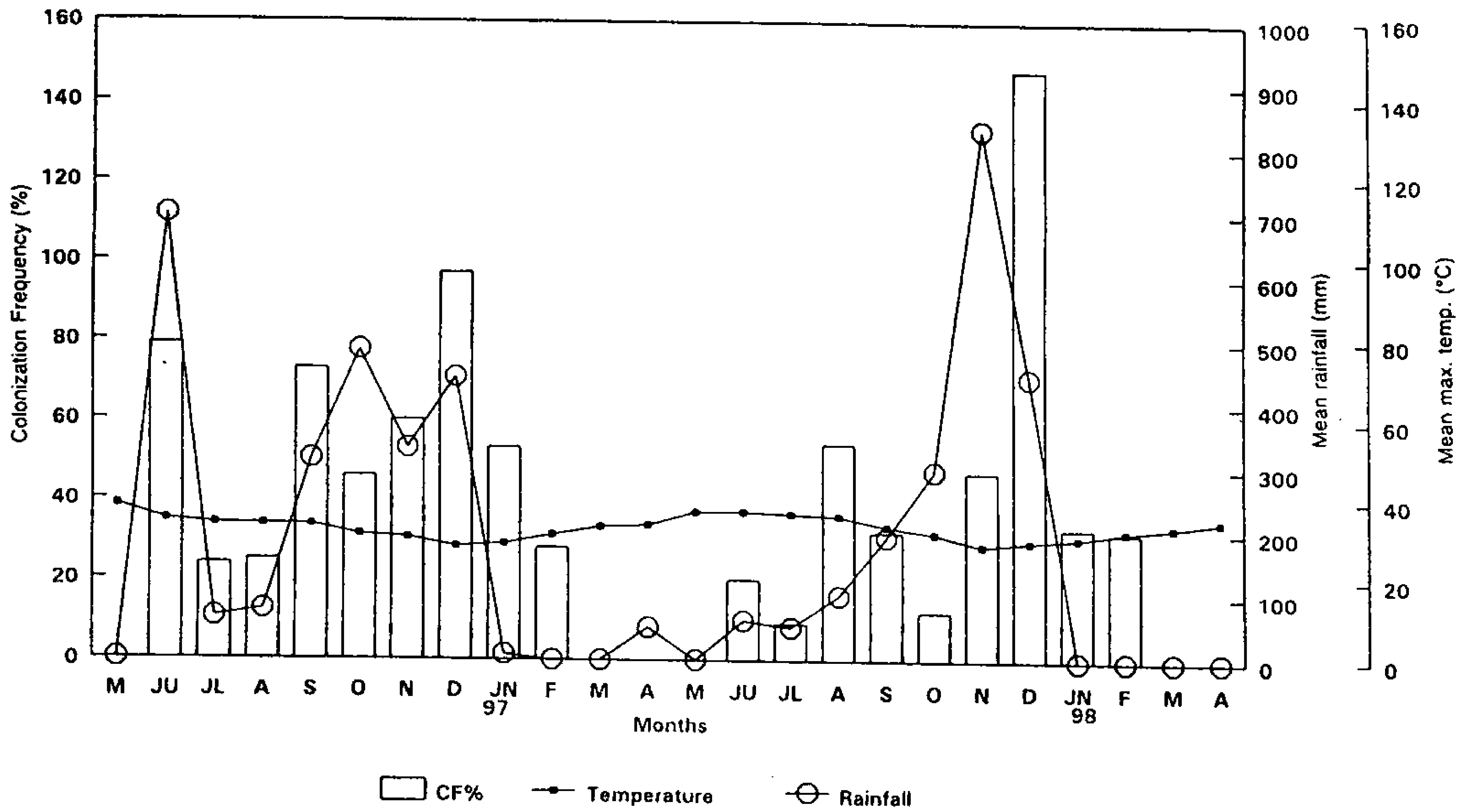

Figure 1. Colonization frequency of endophytes isolated from green leaf of neem over a period of 24 months.

of neem yielded only five different endophytes. Of these, *F. avenaceum* and the sterile form VCC51 were dominant with mean CF% of 15 and 12, respectively. The other three sterile forms (VCC52, VCC74 and VCC75) showed low CF%. The endophytic genera such as *Phomopsis*, *Phyllosticta* and *Xylaria* that are ubiquitous and are commonly isolated from many hosts (including tropical plants)[34] were absent from the leaves of neem. Suresh *et al.*[35] have shown that the limonoids present in neem leaves are antifungal in nature and perhaps this is the reason for the occurrence of restricted number of endophytes in neem. Okane *et al.*[36] attribute the presence fewer endophytes in the leaves of *Pieris japonica* to such antifungal compounds in the leaf tissue of the plant. Although the CF% of the endophytes in neem increased during the wet season, the diversity of the endophytes did not. This suggested that these endophytes are adapted to exploit such a niche as the neem leaf which is rich in antifungal compounds. Thus, it appears that the occurrence of fungal endophytes is influenced by the environment, type of host tissue and chemicals present in the host tissue.

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Analysis of a north-south magnetic profile over the Central Indian Ocean

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Marine magnetic anomalies provide valuable information on the nature and evolution of the oceanic crust. Magnetic profiles aligned along the direction of spreading enable us to calculate the spreading rates of oceanic crusts on which they pass. This can be done with computed profiles assuming the model and magnetic reversal time scale of the earth. In the present study the north-south magnetic profile of 1280 nautical miles extending from 15°30′S, 77°E to 6°N, 79°E in the Central Indian Ocean is analysed. The profile runs between the 76°30′E and 79°E Fracture Zones passing over part of the Australian plate, the Central Indian Ocean deformation zone and part of the Indian plate along the Comorin Ridge. The analysis shows that the profile has a continuous sequence of prominent age anomalies from 22 to 34 N. Australian plate and deformation zone segments have well preserved continuous age anomalies from 22 to 33 and thus their spreading rates are calculated. The results show that the profile is characterized by sets of anomalies with varied spreading rates, anomalies 23–30 having higher values compared to the rest. The spreading rate of anomalies 22–27 is 7.8 cm/yr whereas for anomalies 27–30 it is 10.3 cm/yr. Anomalies 30–33 have a moderate spreading rate of 4.6 cm/yr. Another interesting feature is that the profile has the characteristic edge-effect anomaly on the segment over the Indian plate indicating the oceanic–continental crust boundary.

MCKENZIE and Sclater[1], Sclater and Fisher[2] and Schlich[3] have studied broad regional tectonic features of the Indian Ocean. Detailed investigations by Royer and Schlich[4], Patriat and Segoulin[5], Royer *et al.*[6] and Munschy and Schlich[7] have contributed to the understanding of the evolution of the Indian Ocean. Magnetic anomalies of the Indian Ocean are well preserved due to the absence of active trenches except the Indonesian trench in eastern Indian Ocean. Earlier studies revealed that the major basins lying between spreading ridges, continental margins and submarine ridges evolved during three main phases since the break-up of the Gondwana in the Late Jurassic, the three phases being Late Jurassic to mid-Cretaceous, mid-Cretaceous to Middle Eocene and Middle Eocene to Present. The Central Indian Basin between Central Indian Ridge and Ninetyeast Ridge, bounded in the south by the south-east Indian Ridge and the Indian Ocean Triple Junction, has a complex evolutionary history. The complexity is caused mainly by the variations in spreading rates. Between anomalies 32 and 21, i.e. between 80 and 50 Ma, rapid spreading took place about two east-west ridges separated by a long north-south fracture zone (Chagos–Laccadive lineament) which was uninterrupted during this epoch[1]. The intense tectonic deformation of sediments and the basement in the equatorial region of the Central Indian Basin displays clear evidence of intraplate lithospheric deformation on long wavelength (100–300 km) and short wavelength (5–20 km) scales. The nature of deformation can be visualized from seismicity[8,9], geoid and gravity anomalies[10] and anomalous heat flow[11]. Seismic reflection studies in the Central Indian Ocean[12–17] revealed undulations of the basement in an E-W lineated pattern. The basement highs and lows have correlation with the geoid anomalies[18]. The characteristic edge-effect anomaly in the Comorin Ridge of the Indian plate was reported by Kahle *et al.*[19]. They delineated the oceanic–continental crust boundary by isostatic and free air anomaly studies. The present magnetic study, apart from providing spreading rates of a continuous sequence of age anomalies of the profile, supplements information on the oceanic–continental crust boundary with the edge-effect anomaly.

Figure 1 shows the M9M9 magnetic profile which extends from 15°30′S, 77°E to 6°N, 79°E covering a total distance of 1280 nautical miles in the Central Indian Ocean. The profile is aligned almost in the direction of spreading which took place during the second phase (mid-Cretaceous to the Middle Eocene) of the Indian Ocean evolution. The limits of the deformed zone within the study area are adopted from Gordon and DeMets[20]. In a recent work in this area, a new fracture zone was recognized at 76°30′E

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