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Molecular phylogeography of *Ficus benghalensis* Linnaeus using nrDNA ITS 1, cpDNA trnL and cpDNA rps16 from the Indian subcontinent

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***Ficus benghalensis* Linnaeus (Moraceae) is the national tree of India and is well known for its pharmacological properties. The present study was aimed to determine the genetic diversity of *F. benghalensis* from the Indian subcontinent using sequence-based multi-locus phylogeography. A total of 20 geographical isolates were collected from different regions, covering major parts of its species range within the country. Sequence data from nuclear-encoded internal transcribed spacer region (ITS1), plastid-encoded trnL-F spacer region (trnL) and ribosomal intron region (rps16) were generated. The trnL-based maximum likelihood phylogram revealed the existence of two haplotypes, whereas ITS1 and rps16-based maximum likelihood analysis did not reveal much variation for this species distributed in the Indian subcontinent. These results depict long-distance random gene flow across the subcontinent, and support the post-glacial population contraction events. To validate the impact of palaeo-historic climatic events on current geographic and genetic distribution, species distribution modelling-coupled phylogeography is suggested.**

Keywords: Banyan, genetic heterogeneity, haplotypes, maximum likelihood, phylogeography.

FICUS Linn. (Moraceae) is one of the largest genera amongst angiosperms with around 1000 tree species native mainly to the world tropics. Fig trees (*Ficus* spp.) are considered to be the foundation species with major influence on community structure and ecosystem function, as they are often abundant and fruit throughout the year in many tropical forests[1]. Figs are exclusively pollinated by female agaonine wasps that lay their eggs in fig flowers, where wasp larvae thrive on the developing seeds[2]. This obligate mutualism is considered to be the basis for co-evolution of fig-wasp species over millions of years[3]. The short-lived (1–2 days) fig wasps are vulnerable to climate change and global warming may lead to their extinction in the near future[4]. Fig plants are the centre for complex web animals, including specialists and generalists, but are at major risk owing to the declining population of fig wasps, which will further affect many trophic levels.

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Ficus benghalensis is an important species of the genus and is known for many variations in the species habits[5]. This fabled tree is well-documented in Ayurveda and holds a sacred place in Hinduism, Jainism and Buddhism[6]. The characteristic copious aerial roots of this tree on reaching the ground grow into a new indistinguishable trunk, forming a miniaturized forest. Being the national tree of India, it is widely grown and revered across the subcontinent. Bioactive molecules useful in wound healing, teeth disorders, diarrhoea, allergies, skin disorders, diabetes, immune dysfunctions and cancer have been reported from banyan tree[7].

Despite its long-known pharmacological worth, the tree has been ignored largely in terms of molecular assessment. *F. benghalensis* is facing problems of genetic erosion and mislabelling. All the previous studies on molecular phylogeny of figs were done to examine the co-evolutionary patterns with fig-wasps, and were conducted elsewhere in the world[3,8]. While lot of literature is available on the bioactive potentials of *F. benghalensis*[9,10], assessment on either its genetic heterogeneity or phylogeny has never been done for Indian isolates. This important tree species has never been subjected to sequence-based DNA barcoding, despite being the national tree of India. The present study evaluates phylogenetic relationships between various geographical isolates of *F. benghalensis* from India using Internal Transcribed Spacer-1 (ITS1), ribosomal protein s16 (rps16) and trnL markers.

Leaf samples of 20 geographical isolates of *F. benghalensis* were collected from different locations in the Indian subcontinent (Figure 1). Samples were transported in zip-lock polythene bags under cold conditions. Then they were cleaned thoroughly with tap water to remove the overlay dust and contaminant particles, and then cleaned with distilled water for further processing. Leaf morphology was studied, and pressed sample vouchers were prepared, and deposited in the Central National Herbarium, Botanical Survey of India, Kolkata (Index Herbarium code: CAL). Samples for molecular analysis were stored at –80°C.

Apical parts of leaf samples were excised and total genomic DNA was extracted using HiPurA™ plant genomic extraction kit (HiMedia Laboratories Pvt Ltd, Mumbai) according to the manufacturer's protocol. The DNA was quantified on a spectrophotometer at 260 nm and by electrophoresis on 0.8% agarose gel.

PCR amplifications were carried out using primers ITS1, rps16 and trnL (Table 1)[11–14]. The 20 µL reaction mix consisted of 10× PCR buffer, 10 µM of each primer, 3 mM $MgCl_2$, 10 mM dNTP and 1 unit of *Taq*® DNA polymerase. The reactions were carried out in programmable thermal cycler as optimized for each primer set. Amplified products were electrophoresed on 1.5% agarose gel and positive reactions were selected. These products were purified using ExoSAP-IT® PCR clean-up kit according to the manufacturer's protocol. Sequencing was performed using Applied Biosystems reagents and a DNA analyzer (ABI 3730 xl, Applied Biosystem, USA). The chimeras were removed and contig alignment was done using CodonCodeAligner (CodonCode Corporation, USA). The obtained DNA sequences were annotated with other pertinent information such as collection date and herbarium voucher accession, and submitted to GenBank with accession nos KT924351–KT924368 and KT884619–KT884657 (Table 2). The FASTA format sequences of all accessions generated in this study are provided under Supplementary Table 1.

Sequences were compared with non-redundant database available at the National Centre for Biotechnology Information (NCBI) using BLASTn. Sequences were first aligned by MUSCLE and CLUSTALW, and alignments were edited manually. Gaps were considered as missing data and indels were removed from the alignments. Step-by-step protocol for phylogenetic analysis, including alignment construction, model test, phylogeny reconstruction using maximum likelihood method and distance analysis was done according to Bast[15]. Best-fitting nucleotide substitution models were tested using maximum

Figure 1. Overlay of sampling locations on the map of India (map outline based on the Survey of India).

Table 1. Universal primers used for amplifying nuclear DNA

Primer	Sequence (5′–3′)
ITS1 (ref. 13)	TCCGTAGGTGAACCTGCGG
trnLF (ref. 12)	CGAAATCGGTAGACGCTACG
trnLR (ref. 11)	ATTTGAACTGGTGACACGAG
rps16 intronF (ref. 26)	GTGGTAGAAAGCAACGTGCGACTT
Rps16 intronR (ref. 14)	TCGGGATCGAACATCAATTGCAAC

Table 2. Details of geographical isolates analysed in this study

Voucher no.	Location	Collected by	Collection date	Latitude (N)	Longitude (E)	ITS 1 sequence generated (GenBank Accession no.)	trnL sequence generated (GenBank Accession no.)	rps16 sequence generated (GenBank Accession no.)
CUPVOUCHER-FB-2014-1	Madhya Pradesh (Bina)	Gajendra Singh Vishwakarma	02–07–2013	24°10′02.9000″N	078°11′00.4000″E	KT884628	KT884654	–
CUPVOUCHER-FB-2014-2	AP: Andhra Pradesh (Mehbubnagar)	Dr Anil Kumar Mantha	02–12–2013	16°27′36.0000″N	077°33′36.0000″E	KT884621	KT884650	KT924352
CUPVOUCHER-FB-2014-3	BR: Bihar (Sheikhpura)	Ravi Shankar	20–10–2013	25°08′41.7000″N	085°52′09.8000″E	KT884627	KT884642	KT924354
CUPVOUCHER-FB-2014-4	KA: Karnataka (Mysore)	Ashish Kumar Pandey	13–11–2013	12°18′00.0000″N	076°42′00.0000″E	–	KT884649	KT924361
CUPVOUCHER-FB-2014-5	JH: Jharkhand (Palamu)	Ashish Kumar Pandey	15–11–2013	23°21′00.0000″N	085°19′48.0000″E	KT884625	KT884644	KT924359
CUPVOUCHER-FB-2014-6	RJ: Rajasthan (Jhunjhunu)	Prateek Sharma	09–10–2013	26°34′21.7200″N	073°50′20.4000″E	KT884637	KT884651	KT924365
CUPVOUCHER-FB-2014-7	RJ (NRC): Rajasthan (NRC)	Satej Bhushan	09–10–2013	28°00′36.0000″N	073°13′12.0000″E	KT884623	KT884648	KT924366
CUPVOUCHER-FB-2014-8	KL: Kerala (Pyannur)	Dr Felix Bast	10–06–2013	12°06′00.0000″N	075°12′00.0000″E	KT884619	KT884652	KT924362
CUPVOUCHER-FB-2014-9	DL: Delhi	Dr Felix Bast	25–11–2013	28°37′58.0800″N	077°13′10.5600″E	KT884629	KT884638	KT924355
CUPVOUCHER-FB-2014-10	HR: Haryana (Palri)	Renu Yadav	9–06–2013	28°46′48.0000″N	076°07′48.0000″E	KT884622	KT884645	KT924358
CUPVOUCHER-FB-2014-11	AS: Assam (Bokajan)	Anamika Das	19–09–2013	26°01′12.0000″N	093°46′48.0000″E	KT884632	KT884639	KT924353
CUPVOUCHER-FB-2014-12	PB: Punjab (Abohar)	Pooja Rani	31–05–2013	30°08′00.2400″N	074°12′00.3600″E	KT884633	KT884643	–
CUPVOUCHER-FB-2014-13	PB: Punjab (Bhatinda)	Sheetal Sharma	27–07–2013	30°10′12.0000″N	076°27′00.0000″E	KT884620	KT884641	KT924364
CUPVOUCHER-FB-2014-14	HP: Himachal Pradesh (Gandhi Nagar)	Anil Rana	12–08–2013	31°40′48.0000″N	076°31′12.0000″E	KT884634	KT884640	KT924357
CUPVOUCHER-FB-2014-15	MH: Maharashtra (Viman Nagar)	Digvijay Singh Yadav	28–02–2014	18°34′04.8000″N	073°54′52.2000″E	KT884636	KT884657	KT924363
CUPVOUCHER-FB-2014-16	JK: Jammu (Bantalab)	Shokit Amin Poswal	03–02–2014	32°48′51.0000″N	074°50′01.0000″E	KT884626	KT884656	KT924360
CUPVOUCHER-FB-2014-17	TN: Tamil Nadu (Murugunagar)	Digvijay Singh Yadav	18–02–2014	12°58′18.0000″N	080°12′51.0000″E	KT884631	KT884655	KT924368
CUPVOUCHER-FB-2014-18	GJ: Gujrat (Chandkheda)	Digvijay Singh Yadav	20–03–2014	23°25′60.0000″N	072°40′00.0000″E	KT884635	KT884646	KT924356
CUPVOUCHER-FB-2014-19	AP: Andhra Pradesh (Hyderabad)	Dr Felix Bast	07–03–2014	17°21′57.6000″N	078°28′33.6000″E	KT884630	KT884653	KT924351
CUPVOUCHER-FB-2014-20	TN: Tamil Nadu (Adyar)	Dr Felix Bast	20–02–2014	13°00′22.6800″N	080°15′26.6400″E	KT884624	KT884647	KT924367

– No data available.

likelihood model test in MEGA v7. Pairwise distances between sequences were calculated using p-distance model in MEGA v7 (http://www.megasoftware.net/). After analysis, a consensus tree was generated using the consensus tree builder with Geneious v6.1.5.

Morphological assessment of leaves and DNA sequencing data were congruent in the identification of the test samples as *F. benghalensis*. There were no significant intraspecific morphological differences observed in the samples, suggesting that the species is phenotypically least influenced by climatic variations.

In total, 58 sequences for three primers sets, i.e. ITS1, rps16 and trnL were generated. The length of these sequences was varied from 700 to 1300 bp. Ambiguous sequences of low quality were excluded. This study reports the barcoding record of *F. benghalensis* with rps16 intron. Phylogenetic reconstruction using Bayesian inference for rps16 dataset, with *F. formosana*, *F. erecta*, *F. vaccinoides*, *F. benguetensis* and *F. variegata* as outgroup, generated a single monophyletic clade within section *Conosycea* consisting of all isolates of *F. benghalensis* (Figure 2). Likewise, the phylogenetic reconstruction for ITS1 loci with *F. crassiramea* and *F. pubilimba* as out group, generated a single monophyletic clade within section *Conosycea* (Figure 3). Due to the fast-evolving nature of ITS1 and rps16, these primers are generally expected to be more variable than the conserved and slow evolving ones like trnL, but nesting of all sequences in a single clade suggests poor resolution of these markers for *Ficus* spp. The lower genetic variation observed in ITS1 and rps16 as well as the presence of a single widespread haplotype in India are suggestive of long-distance nuclear and chloroplast gene flow via pollen.

However, phylogenetic analysis performed for trnL sequences generated two haplotypes having zero within-group mean distance and 2×10^{-3} between-group mean distance, calculated using Tamura-3-parameter with 500 bootstrap replications (Figure 4). The population differentiation into two haplotypes depicted by trnL data suggests that gene flow in this case must have been restricted. This intraspecific variation can possibly be attributed to climatic variations[16]. These two haplotypes may also pertain to two different cohesion species arising from the genetic and ecological factors creating different reproductive communities[17]. However, reproductive lineage data would be required to validate them as two cohesion species.

The present study provides evidence of substantial phylogeographic distribution of *F. benghalensis* into two haplotypes. Although posterior probability values are not convincing, they leave us with the curiosity of further studies on the same. Earlier reports have also emphasized the plastid gene flow; however, conflicts arising because of existing understanding of *Ficus* and nuclear phylogeny could not be resolved. Despite issues of ancient hybridization, plastid-based phylogeny could address more conflicts in comparison to nuclear, suggesting plastid-mediated gene flow[18]. Our result is congruent with the

Figure 2. Phylogenetic position of *Ficus benghalensis* isolates among other related species in rps16 dataset using Bayesian inference phylogenetic reconstruction (LnL0 = –1308.789) with Hasegawa–Kishino–Yano model of molecular evolution with equal distribution. Numbers near nodes represent Bayesian posterior probabilities. Scale bar represents average nucleotide substitutions per site.

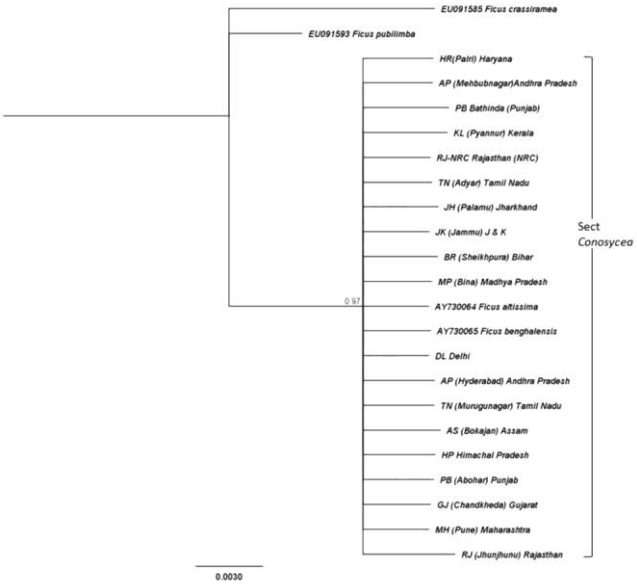

Figure 3. Phylogenetic position of *F. benghalensis* isolates among other related species in ITS1 dataset using Bayesian inference phylogenetic reconstruction (LnL0 = –525.529) with Jukes–Cantor model of molecular evolution with equal distribution. Numbers near nodes represent Bayesian posterior probabilities. Scale bar represents average nucleotide substitutions per site.

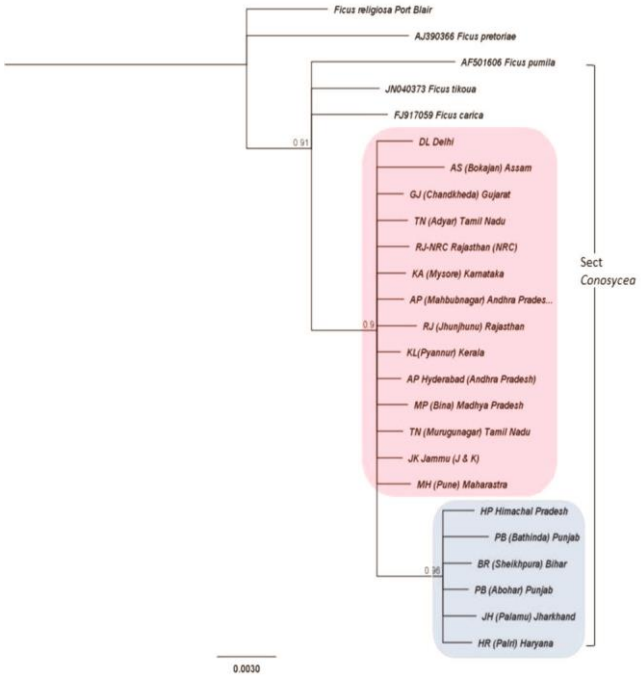

Figure 4. Phylogenetic position of *F. benghalensis* isolates among other related species in trnL dataset using Bayesian inference phylogenetic reconstruction (LnL0 = –1581.828) with Hasegawa–Kishino–Yano model of molecular evolution with equal distribution. Numbers near nodes represent Bayesian Posterior Probabilities. Scale bar represents average nucleotide substitutions per site.

previously described notions of molecular markers, further categorizing the geographical isolates into two putative haplotypes. Similar edaphic heterogeneity was also observed in *Protium* spp. (Burseraceae) across Amazon Basin, where varied degree of edaphic specialization and division of the population in eastern and western zones was reported[19]. There are several reports available for the clonal variations and adaptive differentiation of tree species in quantitative traits reflecting local environmental origins[20]. Structured genetic diversity was observed amongst two groups of foundation species in South Western Australia following by climatic gradient and newly formed and expanded central refugia. The succeeding contraction at the Last Glacial Maximum (LGM) was identified in *Eucalyptus wandoo* Blakely[21].

Pleistocene glacial events generated periodic contraction and expansion of species ranges, leaving genetic fingerprints on existing populations[22,23]. The low level of genetic diversity observed in *F. benghalensis* can be attributed to genetic bottlenecks during palaeo-historical events after LGM[24]. However, to validate this hypothesis, species distribution modelling (SDM) based on the principle of climate-restricted species distribution within their evolutionary and biogeographic history is suggested, which can serve as an important tool to provide better understanding about the evolutionary patterns of current populations of *F. benghalensis*[25]. Various niche-modelling algorithms such as artificial neural networks and regression models can be used to implement SDM, such that spatio-temporal distribution of the species can be predicted from environmental data provided through GIS. SDM-coupled phylogeography is also expected to forecast the predictive species distributions of the concerned species on future climate models and help in devising better strategies to preserve the genetic diversity[21].

This study provides insights into genetic heterogenity of *F. benghalensis* across India, and contributes to the sequence data of the species by barcoding with rps16. The study also validates the efficacy of trnL for molecular phylogeny for *Ficus* species. For a better understanding of the causes of the lower genetic variation observed, SDM-coupled phylogeography is suggested.

Conflict of interest: The authors declare that they have no conflict of interest.

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Magmatic epidote in the Grenvillian granitoids of North Purulia Shear Zone, Chhotanagpur Gneissic Complex, India and its significance

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Magmatic epidotes of granitoid pluton from North Purulia Shear Zone, eastern India, are identified by textural and chemical criteria. The accessory sphene, epidote, allanite and magnetite in the granitoid indicate high fO_2 during crystallization. Hornblendes were crystallized between 1.2 and 4.8 kbar, 753°C and 783°C as well as high fO_2 (>Ni–NiO buffer). Preservation of magmatic epidote in pluton emplaced at low pressure is due to rapid magma ascent (>3.1 km/year). Fast upward transportation of parental magma of the pluton took place through extensional voids along the regional shear zone.

Keywords. Granitoids, magmatic epidote, oxygen fugacity, shear zone emplacement.

THE petrological implications of magmatic epidote (mEp) have been petrographically and experimentally demonstrated by several workers[1–4]. The presence of mEp in tonalites and granodiorites suggests that the plutons crystallized under lithostatic pressure >6 kbar (refs 2, 3). Survival of mEp in calc-alkaline granitoids implies rapid upward movement of the magma[4]. Consequently, mEp can be used for estimating crystallization pressure, oxygen fugacity and rate of upward movement of melt. Studies on mEp in the Neoarchaean granitoids of Srinivaspura (Eastern Dharwar Craton) and the Paleoproterozoic Malanjkhand Granitoid (Central India) revealed upward magma migration rate of 27.65 and 0.45 km/year respectively[5,6]. In the present study, mEp is distinguished from secondary epidote based on textural and chemical criteria in the granitoids of Agarpur pluton lying in the North Purulia Shear Zone (NPSZ) of Chhotanagpur Gneissic Complex (CGC) of eastern India. The significance of mEp on emplacement mechanism of granitoids is also discussed.

The ENE–WSW-trending Central Indian Tectonic Zone and CGC mark the Grenvillian collsional zone between the North Indian Block and the South Indian Block[7–9] (Figure 1 *a*). The CGC is mainly composed of granitoid and migmatitic gneisses with older enclaves of para- and ortho-metamorphic rocks[10]. The structural trend of the rocks is E–W with steep northern dip. Younger

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