

# Rediscovery and Characterization of *Citrus Indica* Tanaka, A Wild Endangered and Progenitor Species in Dailong Forest, Manipur, and Recommendations for Its Conservation.

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## Short Report

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# Abstract

*Citrus indica* Tanaka is a wild endangered and one of the progenitor species of citrus endemic to North East (NE) India, which is reported in Nokrek Biosphere Reserve in the Garo hills of Meghalaya. A wild orange morphologically claimed to be *C. indica* was encountered in the Dailong forest of Tamenglong district, Manipur which is located in the Indo-Burma biodiversity hotspot region. Surveys were conducted to establish the exact identity of possible *C. indica* and study the natural habitat in Dailong forest area. The traditional morpho-taxonomic description for the identification of the plant is performed along with the molecular characterization using three chloroplast regions (*trnL-F*, *psbK-l*, *matK-5'trnK* spacer) and one nuclear (ITS) region. Phylogenetic analysis revealed that the *C. indica* found in Dailong Forest is monophyletic with the earlier reported *C. indica* sequence from Garo hills, Meghalaya. Both morphological and molecular data support the identity of *C. indica* that has been collected from Manipur. However, lack of knowledge and clearing of forest cover has led to an urgent need for the implementation of conservation strategies to safeguard this endangered species. This study supports the evidence that NE India is one of the origin centres for citrus and this finding can guide the conservationists to initiate priority for adopting possible methods of proper conservation of *C. indica* in the region.

## 1. Introduction

*Citrus indica* Tanaka belongs to the genus *Citrus* of the family Rutaceae and subfamily Aurantiodeae which was first discovered and characterized by Tanaka (1928). It is believed to be the most primitive and progenitor of all citrus species and is endemic to NE India (Singh 1981). Presently, it is reported to be endemic to the Tura range of Garo Hills of Meghalaya (Singh and Singh 2003) and the Garo tribes used the fruits of *C. indica* for medicinal and religious purposes (Malik et al. 2006). As the natural population of *C. indica* got drastically decreased due to large scale deforestation, Citrus Gene Sanctuary was established in the Nokrek Biosphere Reserve to protect the area (Singh 1981). Conservation of this species is required as it is endemic to only a specific region with a particular microclimate. Citrus genetic resources of NE India need appropriate conservation strategies as the wild and indigenous population is being eroded due to various anthropogenic activities like jhumming and introduction of exotic species, etc. *C. indica* is not only exposed to habitat destruction but also has extremely low genetic diversity, high habitat specificity, and low regeneration which pose a serious threat in its natural population (Kumar et al. 2010). Previously, besides Garo hills, *C. indica* was reported to be found wild in Naga hills of Nagaland, Manipur, and Kaziranga Reserve forest in Assam (Tanaka 1937; Singh 1981; Sharma et al. 2004). However, Singh and Singh (2003) reported the presence of this species only in Garo hills of Meghalaya which fall under the Nokhrek biosphere reserve. Thus, *C. indica* is one of the seven citrus in India which is enlisted as endangered as per IUCN norms (Singh and Singh 2003; Malik et al. 2006). Though, a recent study in Behali Reserve Forest of Assam recorded the occurrence of few *C. indica* species (Borah et al. 2018).

NE India has a rich biodiversity of citrus germplasm including 23 taxa and 68 varieties and it is gifted with highly favorable agro-climatic conditions for the growth of citrus (Sharma et al. 2004). 33 members

of citrus have been enlisted in the digital database on the citrus biodiversity of Manipur (Sanabam et al. 2012). Bhattacharya and Dutta (1956) reported 17 species, 52 cultivars, and 7 probable natural hybrids of citrus are found growing in natural wild conditions in the NE India region. Many citrus species and progenitors are believed to have originated in this region (Malik et al. 2012). In a study to trace the proposed origin of citrus and its ancient dispersal routes, it was found that the ancestral citrus species are migrated originally from NE India, northern Myanmar, and north-western Yunnan (Wu et al. 2018). Thus, the NE India region is considered to be one of the centres of origin of citrus. However, citrus germplasm of NE India has been minimally utilized in the improvement programs due to a lack of exploration and characterization (Sharma et al. 2004). An approach for systematic exploration, characterization, and conservation of *C. indica* is still lacking (Malik et al. 2006). Systematic characterization including morphological and molecular identification should be implemented to assess the conservation and management policy of such rare and wild species. Molecular identification in higher plants has now become far simpler with the advent of various molecular markers and plant barcoding genes. The nuclear and chloroplast region had been extensively used as genetic markers for molecular taxonomic studies and plant identification (Yang et al. 2017). Since morphological characterization sometimes creates confusion in the identification of citrus taxonomy, implementation of appropriate molecular approaches have now been encouraged like molecular markers (Kumar et al. 2013), analysis of non-coding chloroplast DNA regions (Araujo et al. 2003; Chase et al. 1999; Lu et al. 2011; Morton et al. 2003) and ITS region (Hynniewta et al. 2014; Kyndt et al. 2010; Pessina et al. 2011; Xu et al. 2006). There are few previous studies in which *C. indica* is involved in the molecular phylogeny analysis like using RFLP and RAPD (Federici et al. 1998), RAPD, SCAR and cpDNA markers (Nicolosi et al. 2000), chloroplast DNA (Bayer et al. 2009; Jena et al. 2009), nuclear DNA (Kumar et al. 2013) and ISSR polymorphism (Kumar et al. 2010). These previous findings paved the way for taxon sampling in the phylogeny analysis for the present study.

Dailong is located in Tamenglong district of Manipur, North East India (NE India) which is part of the Indo-Burma biodiversity hotspot region. Tamenglong is the least densely populated district of Manipur which has a forest cover of 88%. Dailong forest covers an area of 11.35 km<sup>2</sup>. In May 2017, the Government of Manipur declared Dailong village as the 'Biodiversity Heritage Site' under the Biological Diversity Act, 2002 Sect. 37(1) because of its rich biodiversity of flora and fauna. This village is home to the Rongmei Naga tribes of Tamenglong district. Even though there was an earlier report of the occurrence of *C. indica* in Manipur (Tanaka 1937; Singh 1981; Sharma et al. 2004), systematic efforts for characterization and conservation status has not been carried out in detail. The present study attempts to analyse the distribution, characterization and conservation of *C. indica* along with its associated local indigenous knowledge. Both the morphological and molecular analyses were employed to characterize the species as proper identification of species is fundamental for biodiversity conservation.

## 2. Methods

### 2.1. Survey and morphological characterization

During the surveys undertaken for citrus germplasm collection and citrus greening disease in Tamenglong district of Manipur, few patches of a type of wild citrus (suspected to be Indian wild orange or *Citrus indica*) were encountered in a moist tract of Dailong forest. Further exploration was continued from 9th to 13th April 2018 to record the presence of any other population inside the forest. Information was collected from people who dwelled near the forests and subsequently two other sites were detected inside the forest (Table 1). A possible threat associated with the natural habitat and conservation issues had been analyzed through interaction with the community of Dialong village. Indigenous and traditional information of the species was collected by interviews with the local tribes inhabiting the area. Meteorological data of the natural habitat of this species was recorded to know the region's microclimate. Photographs of the plants were taken in its natural habitat area (Fig. 1). The coordinates and elevations of the locations were recorded using a GPS unit (Garmin) (Table 1). The survey was undertaken in such a manner that involved minimal disturbance and harm to the target plant and also the overall forest environment in Dailong area. General field observations along with the natural population were briefly analyzed. All the individual plants were critically studied to determine their correct taxonomic identity and treated as a representative specimen. Young leaf tissues and fruits from all the samples were immediately stored in silica gel for subsequent DNA isolation in the laboratory. One representative voucher specimen of the collected samples is authenticated, and stored in the herbarium of the Institute of Bioresources and Sustainable development (IBSD), Imphal with the herbarium accession number 263. Morphological characters of the citrus were recorded using 'Descriptors for citrus' by the International Plant Genetic Resources Institute (IPGRI) Rome, Italy. 14 quantitative and 27 qualitative characters were characterized (Table 2a and b).

## 2.3. Molecular characterization

### 2.3.1. DNA extraction and PCR

The leaf samples were taken for total plant genomic DNA isolation. About 100 mg of each sample was ground in liquid nitrogen using a pre-cooled mortar and pestle. Total DNA extraction from this powdered tissue was further performed with the DNeasy Plant Mini Kit (Qiagen, Germany) by following the user's instructions. Nucleic acid was quantified by spectrophotometry (Nanodrop 2000, Thermo Scientific) and its quality checked by electrophoresis on 0.8% agarose gel.

PCR amplification of three chloroplast regions (*matK-5'trnK* spacer, *psbI-psbK*, and *trnL-F*) and one nuclear region ITS (ITS1, 5.8S, and ITS2) was performed by using the respective primers (Table 3). The PCR reaction was performed in a C1000 Touch™ Thermal cycler (Biorad) with 25µL reaction volume. The reaction mixture consists of 10X PCR buffer, 2–25 mM of MgCl<sub>2</sub>, 5–20 pmol of each primer, 0.125 mM-1.25M for each dNTPs, 0.5-1U Taq DNA polymerase (Sigma- Aldrich), and genomic DNA of 10–50 ng. PCR profile followed for different primer pairs was given in Table 3. The PCR products obtained were electrophoresed in 1.2% agarose gel for 1 hour and 30 minutes at a constant volt of 60V.

### 2.3.2. Sequencing and phylogenetic analysis

The bands obtained were excised and eluted using Qia quick gel extraction kit (Qiagen) following the manufacturer's instructions. The eluted products were sent for direct sequencing at Bioserve Technologies, Hyderabad. Using BLAST analysis of the NCBI database (Altschul et al. 1997), the identity of sequences was confirmed. The consensus sequence of the four genomic regions (three chloroplast and one nuclear) was obtained using Bioedit software 7.05 (Hall, 1999). Closely related sequences were retrieved from GenBank database and used for the construction of phylogeny (Supplementary Table 1). Multiple alignments of sequences in this study and sequences from the GenBank database were performed with the ClustalW program incorporated in MEGA X (Kumar et al., 2018). One representative sequence of each of the marker sequences (i.e., four sequences) used in this study was annotated and deposited in the National Centre for Biotechnological Information (NCBI) GenBank database.

Phylogenetic analysis of the ITS, *trnL-F*, *psbK-psbI* and *matK-5'trnK* spacer regions were generated using the Maximum Likelihood method incorporated in the software IQ-TREE version 1.6.12 (Nguyen et al. 2015) with a bootstrap approximation of 1000 replicates. The final depiction of the tree was produced using FigTree version 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree>). Outgroup taxa were selected based on the analyses of Chase et al. (1999), Samuel et al. (2001), and Morton et al. (2003). *Zanthoxylum monophyllum*, *Poncirus trifoliata*, *Atalantia monophylla*, and *Koelreuteria paniculata* were chosen as outgroups.

## 3. Results

### 3.1. Sampling and Morphological characterization:

The Indian wild orange or *C. indica* was found distributed in Dailong village of Tamenglong district, Manipur (Fig. 2). *C. indica* is locally known as 'Garuan-thai' ('Garuan' means cane, 'thai' means fruit) among the Rongmei tribes. The word 'cane' is given because the fruit stem is woody, slender, flexible, and sometimes climber. From interactions with senior members of the community, it was learnt that the particular citrus specimen had a distinct local name. The existence of a local name further indicated that the particular genotype/specimen has been associated with the community for a long time. During the survey, a total of thirty-two specimens of wild *C. indica* were identified from three spots in the forest area of Dialong village (Table 1). In one spot, 20 specimens were recorded from, where *C. indicis* is found maximally concentrated while the other two spots had a sparse and scattered occurrence. The type of plants varied from a thorny bush to a climber of 3 to 4 meters in height. Among the specimen, only five of them had a thick and bushy canopy and certainly bore fruit. Plants from the same spot were separated at least by 5 meters. The natural population associated with the wild orange in the forest includes *Xanthoxylum sp*, *Melocanna bambusoides*, *Melocanna baccifera*, *Castanopsis spp.*, *Medusferrea sp*, *Albizia spp.*, *Phoebe hainesiana*, *Albizia lebbeck*, *Tectona grandis*, *Quercus spp.*, orchids species like *Dendrobium sp*, *Vanda sp*, and several edible wild mushrooms.

The village of Dailong enjoyed high rainfall conditions of 3,135 mm with humidity of 76% and average temperature ranging from 4 to 31°C (Directorate of Environment & Climate Change, Govt of Manipur).

Preference for a unique shady microclimate comes to light as all the three locations come within the canopy of tall and dense forest plants. Morphological observation of the specimens does not reveal any distinctive character among them. However, variation in their habitat is quite significant in that some were small bushy shrubs, others straight tall to climbers type with spread canopy. Despite existing in wild conditions, the citrus plants were relatively free from any symptoms of disease.

The quantitative and qualitative morphological characters of fruit, leaf, and seed were recorded (Fig. 3) and given in Table 2a and b respectively. Fruits were obloid which weighed ranging from 21.60 to 30.71 gm. The fruit rind is whitish with a thickness of 0.17 to 0.25 cm. The outer dark orange fruit skin was smooth and shiny consisting of conspicuous oil glands with density 34.30 to 44.23 cm<sup>2</sup>. The number of fruit segments ranges from 9.00 to 10.00. Leaf division is simple with elliptic lamina length 8.35 to 12.00 cm, tapering at both ends. Seeds are ovoid, smooth, and creamish ranging from 7.00 to 10.00 per fruit. They are monoembryonic with green cotyledon and brown chalazal spot color.

## 3.2. Molecular Characterization

The four regions (three chloroplast and one nuclear) of citrus were utilized to confirm the taxonomic identity of the Indian wild orange 'Garuanthai' and also inferred their phylogenetic affinities to related taxa. Using the four pairs of primers for the three chloroplast sequences *psbK-psbI* intergenic sequence, *trnL-F* intergenic spacer region, *matK-5' trnK* spacer region, and one nuclear region ITS region, we obtained sequences from multiple samples from the three locations. The sequence alignment revealed sequence identity in all of the samples (n = 32). Based on the sequence analysis for each marker genes, all the specimens collected were identical. So, only one representative sequence was taken for each gene to annotate and submit in the NCBI GenBank with accession numbers KY963832, MN447519, MN443629, and MT782064 and further used for phylogenetic analysis. The phylogenetic analysis revealed that the sequence of 'Garuanthai' grouped with the *C. indica* in all the Maximum Likelihood trees consistently with all the four sequences (Fig. 4a, b, c, and d), thereby confirming the species to be *C. indica*. *Zanthoxylum monophyllum*, *Poncirus trifoliata*, *Atalantia monophylla* and *Koelreuteria paniculata* remained as outgroups in the respective phylogenetic trees in which they are incorporated.

## 4. Discussion

### 4.1. Sites and morphological characterization

A recent survey to Dailong Forest Tamenglong district reported the natural wild population of *C. indica*, locally known as 'Garuanthai' among the Rongmei tribes. In Fig. 2, the collection site of the *C. indica* in the present study is shown in the red triangle while the sites of earlier explorations are depicted in red circle. Among the earlier exploration, the presence of the species were recorded only in the Garo hills of Meghalaya (Singh and Singh 2003). Our present study reports the re-occurrence of this species in Manipur in Tamenglong District where it was said to be found in earlier studies. Such findings show that detailed research and studies should be carried out in various parts of NE India which remain largely unexplored because of its inaccessibility and difficult topographic nature. The meteorological data of

Dailong forest area also support the microclimate of *C. indica* with low temperature, high rainfall, and humidity which is similar to that of the specific microclimate of Garo hills, which may be the probable reason for occurrence of the plants in this forest.

Morphological characterization of citrus often creates confusion among the taxonomists as there are innumerable hybrids and mutants of citrus due to frequent hybridization, mutation, and polyploidy among the citrus species throughout the world (Susheel Kumar et al. 2013). In the present study, the morphological characters are characterized based on the Descriptors for citrus (IPGRI 1999) protocol which is a standardized tool for the characterization system. In earlier works, morphological classification has relied on a combination of few characters of leaf, seed, flower, and fruit (Swingle 1943; Tanaka 1954; Tanaka 1977; Bhattacharya and Dutta 1956; Hodgson 1965; Swingle and Reece 1967; Singh 1967; Singh and Nath 1969). In the present survey, all the *C. indica* species observed have identical morphology except for the height and nature of the plants viz., bushy or climber, tall or stunted. The tallest plants were found in Jhoulangpang area of the forest, where the density of the plant is more compared to other locations where the plants are stunted. Potential causes for the differences may be variations in the age of the plants as the forest area was used for jhum cultivation till 1978 except the Joulangpang area. Maybe the plants were newly regenerated from seeds or stem and started acclimatizing in the area. Most of the plants were found in shady and moist slopes under the canopy of the forest. Kumar et al. 2010 observed that the plants preferred cool and shady places under the canopy of other trees. The quantitative and qualitative characterization of leaf, seed, and fruit samples showed that the characters of *C. indica* described in the present study are more or less similar with the characters of the species found in Garo hills (Malik et al. 2006; Kumar et al. 2010) except for some minor variations. Such differences may be due to variations in meteorological data like temperature, precipitation, location, soil thickness and forest age (Yang et al. 2006).

## 4.2. Molecular characterization

In the four DNA regions analysed (*trnL-trnF*, *psbI-psbK*, *matK-5'trnK* spacer, and ITS), we detected the sequences obtained from the present study clustered with the *C. indica* that is found in Garo hills of Meghalaya. The emergence of the DNA barcoding technique has helped in calibrating current taxonomic resolution and provided a straightforward identification system when there exist troubleshooting in morphology-based taxonomy (April et al. 2011). The CBOL plant working group proposed to use *rbcL* and *matK* as the standard DNA barcode for plants (CBOL plant working group). However, this DNA barcode-based identification of biologically complex plant groups like Citrus remains challenging due to frequent natural hybridization. So the use of intergenic spacer of chloroplast regions had been preferred for the rapid identification of other secondary citrus species (Mahadani and Ghosh 2014). The ITS region and *trnL-trnF* regions were successfully used to study the detailed phylogenetic and biogeography of the *Zanthoxylum* L. and *Ivodea* which belong to the Rutaceae family (Appelhans and Wen 2020; Appelhans et al. 2018). The ITS region, *trnL-trnF* intergenic spacer and *matK-5'trnK* spacer regions were successfully used to resolve the taxonomic uncertainty of Aurantoid Rutaceae (Nguyen et al. 2019; Bayer et al. 2009). The chloroplast *psbK-psbI* intergenic spacer was proven not only to be the most reliable barcode (Suzuki

et al., 2014) but it was also useful for phylogenetic relationships analysis at the taxonomic level (Enan and Ahmed 2016). Moreover, chloroplast intergenic spacer *psbK-psbI* was included as a potential candidate locus for DNA barcode because of its evolutionary rates and high capability to distinguish independently evolving beings corresponding to taxonomic species (Lahaye et al. 2008).

### 4.3. Conservation

*Citrus indica* is unique citrus within the true citrus species. Known for its poor regeneration and endemic nature, this citrus genotype preferred a distinct microclimate for its survival and existence. Though the presence of *C. indica* has earlier been reported from different restricted pockets in NE India, its existence and conservation cannot be ascertained in many of these locations now. After a long period, the prevalence of *C. indica* was only recently reported from Behali Reserve Forest of Assam (Dipankar et al. 2018) and thus it is scantily reported even from NE India though the region is regarded as a natural home for Citrus. An assessment of ecological conditions, habitat, and interaction with people inhabiting the locality will enable us to give a clear view for the survival of such rare and endemic citrus genotype. Apart from this, morphological data and clear molecular analysis remain essential for correct identification and further conservation efforts.

Various wild and semi-wild citrus grow in the NE India forest regions which still need to be explored for their proper identification, collection, and conservation (Sharma et al. 2004). Dailong is one such area, which is endowed with a rich biodiversity of flora and fauna. The forest consists of montane wet temperate, tropical evergreen, semi-evergreen and riparian forest. *C. indica* is one of the seven species of Indian citrus enlisted in the endangered list, which requires urgent and special consideration for conservation because of its endemic nature and also the degree of threat perception is high (Malik et al. 2006). In situ conservation seems to be the most suitable option for *C. indica* as the previous study by Malik et al. 2006 reported that the plants growing outside its habitat did not show flowering fruiting. This can be related to the complex phenomena of the lack of adaptation to the man-made environment (Zeven and de Wet 1982). Kumar et al. 2010 also observed that after propagation of *C. indica* from seeds, its growth and survival outside its natural environment became difficult. A study in Canary Island, Spain recommended reintroduction, habitat management, and protection as the most important tools for conservation and management of endangered plants (Marrero-Gómez et al. 2003). However ex situ methods of conservation are also given importance to protect the endangered species (Laskar et al. 2009). National Bureau of Plant Genetic Resources (NBPGR), Delhi has preserved the pollens and seeds of *C. indica* in Cryogene Bank for long term preservation (Malik et al. 2006). Such an initiative for ex-situ germplasm conservation of citrus can help in the rehabilitation of endangered species (Kumar et al. 2010). Cryopreservation technique and other ex situ conservation actions have been developed for long-term conservation of critically endangered species *Rubus humulifolius* in Finland (Edesi et al. 2020). Sharma et al. 2004 also suggested the need for more nature reserves, gene sanctuaries, and parks with the inclusion of citrus species. In Burkina Faso, the conservation strategy and survival rate of *Zanthoxylum zanthoxyloides* seedlings were suggested to be managed by establishing nurseries, botanical gardens, and target places for safeguarding (Ouédraogo et al. 2019). Those sites were

entrusted to the local communities thereby facilitating financial support for the local communities to encourage them to actively participate in the conservation of the species. Likewise, the conservation strategy for *C. indica* can also be implemented in the present study area. Moreover, any conservation strategies related to natural resources needs public education and proper awareness to the people residing in the area for maintaining a healthy environment (Kaya and Raynal 2001). Thus a holistic approach including both the ex-situ and in situ conservation methods should be developed to contribute to Citrus improvement programs (Malik et al. 2006).

Singh and Singh (2003) also stated that the anthropogenic activities are causing a serious threat to the citrus biodiversity in the NE India region for which, it has been classified as a hotspot. *C. indica* is seldom cultivated and commercialized as the fruits are small, rarely edible (Bhattacharcharya and Dutta 1956; Tanaka 1937). The present exploration in Dailong forest area also indicated no domestication and no utility of the species known to the local tribes. It remained as ordinary wild citrus to them with no economic importance. So there was no specific conservation policy taken up in that area particularly for the citrus species. However, in the Nokrek Biosphere reserve buffer zone area, preliminary domestication and conservation measures have been taken up. *C. indica* locally known as Memang Narang in Garo language is well known to the local tribes of this region and is used for various medicinal and ritual purposes. So the local tribes had cultivated in the area (Singh 1981; Malik et al. 2006). Maybe we can assume that the species had been conserved in Garo hills but not in other parts of the NE India because of its conservation by domestication by Garo tribes. In the other parts of NE India, there are no specific uses and medicinal values known to them. Also, the Rongmei tribes of Dailong Forest have not used the part so far. They know its existence in the forest as wild form; however, there is no domestication history of the species in the place so the conservation motive is very low to the local people as domestication is one of the important ways to conserve the germplasm of species. During the survey, *C. indica* was observed to be healthy and free from any kind of disease. Such property of *C. indica* can provide rootstock of high quality for other commercial citrus varieties in the future (Malik et al. 2006). Conservation of *C. indica* was already initiated around ten decades ago in the Garo hills of Meghalaya by establishing the Citrus Gene Sanctuary which is a part of Nokrek Biosphere Reserve (Singh 1981). Likewise, the protection and rehabilitation of germplasm of the present study area should be proposed and Dailong forest should be recognized as an in situ 'Citrus Sanctuary'.

Detailed interviews with the local tribes and elders of Dailong area revealed that they have been practicing conservation activities for many generations in the form of protected sacred groves called 'Raengan'. *C. indica* was reported to be found in abundant according to the elders of Dailong village but now the plant has shrunk its population due to earlier destruction of forest for jhum cultivation. However, an initiative was taken way back in 1978 by Rongmei tribes of Dailong Village by preventing jhum cultivation and other exploitation from the forest. Further discussion was also continued with the members of Dailong Ecology & Environment Preservation Society (DEEPS; local NGO since 1978), we came to know that they played a critical role in spreading awareness among the Rongmei tribes for forest conservation. Despite all the efforts of the local tribes to protect the forest, recent development has hampered the forest ecosystem. There are road and railway construction, and extensive illegal timber

logging which led to the loss of large forest area and ultimately leading to the loss of rare species. Even though the local tribes are protecting the forest area, adequate conservation measures have not been implemented so far from the government or other related organizations. There is an urgent need for identifying the priority areas for protection. In southern China, priority protected areas (PPAs) planning methods have been proposed to conserve endangered and threatened plant species in the identified locations (Wang et al. 2015).

Efforts should be given in collection, characterization, and conservation of the wild and semi-wild species of citrus in NE India. The results of our present study try to give insight to develop a conservation management policy for such wild and endangered species. Future studies including these species will give deeper insights into their evolution and geographic origin. It is reported that *C. indica* is inferred to have originated from NE India region, further studies can be explored whether the ancestor species colonized which location first (likely from Nokrek, Meghalaya) followed by dispersal to other parts of other NE India. In the present study, results derived from morphology and sequence data indicated that 'Garuanthai' is identified as *C. indica* which is grown in wild habitat in Dailong forest, Manipur. The present work also supports the evidence of the origin of citrus in NE India thereby locating a new habitat of the ancestral citrus species. Both the in situ and ex situ conservation strategies should be approached to protect and conserve this species. These measures should also involve the local population by giving them proper information and awareness campaigns about the importance of habitat conservation and management to protect this precious habitat of *C. indica*.

## **Declarations**

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### **Compliance with ethical standards**

This work does not contain any studies with human participants or animals performed by any of the authors.

### **Informed Consent**

Informed consent was obtained from all individual participants included in the study.

### **Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### **CRedit Author contribution statement**

Elangbam Julia Devi: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft. Rajendra Kumar Labala: Data curation, Bioinformatics analysis, Rakesh Sanabam: Conceptualization, Writing - review & editing, Nandeibam Samarjit Singh: Experimental designing, Writing - review & editing, Rahul Modak: Writing - review & editing, Huidrom Sunitibala Devi: Supervision, Conceptualization, Methodology, Writing - review & editing, Project administration.

### **Data availability statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Tables

**Table 1** Details of the sampling location and size.

Co-ordinates	Spot no	Elevation	Sample size (n)	Status
N25°00'22.5" E093°30'56.3"	1	838 mASL	20	Wild
N25°00'24.1" E093°31'13.9"	2	951 mASL	10	Wild
N25°00'19.1" E093°31'39.7"	3	1115 mASL	12	Wild

**Table 2a** Quantitative characters of fruit, leaf, and seed of *Citrus indica*

<b>Characters</b>	<b>Value range</b>	<b>Characters</b>	<b>Value range</b>
<b>Fruit</b>		<b>Leaf</b>	
fruit weight	21.60- 30.71gm	leaf lamina length	8.35-12.00 cm
fruit diameter	0.45-0.53cm	leaf lamina width	3.46- 5.00cm
fruit length	3.25-3.80 cm	petiole	2.00- 3.00cm
fruit rind thickness	0.17- 0.25cm	<b>Seed</b>	
density of oil gland per cm <sup>2</sup>	34.30- 44.23	seed length	1.00-1.24 cm
diameter of fruit axis	0.38-0.41 cm	seed width	0.78-0.80cm
number of segments	9.00-10.00	seed per fruit	7-10
		Number of embryo per seed	1.00

**Table 2b Qualitative characters of fruit, leaf, and seed of *Citrus indica***

<b>Characters</b>	<b>Value range</b>	<b>Characters</b>	<b>Value range</b>
<b>Fruit</b>		<b>Leaf</b>	
fruit shape	Obloid	leaf division	Simple
shape of fruit base	Truncate	leaf lamina shape	Elliptic tapering at both ends
fruit rind colour	White	leaf lamina attachment	Brevipetiolate
shape of fruit apex	Truncate	leaf margin	Dentate
fruit skin colour	Dark orange	leaf apex	Acuminate
fruit axis	Solid	Vegetative life cycle	Evergreen
fruit surface texture	Smooth	<b>Seed</b>	
pulp colour	White	seed shape	Ovoid
pulp colour intensity	Light	seed surface texture	Smooth
nature of oil gland	Conspicuous	seed colour	Cream
cross section shape of axis	Irregular	Cotyledon colour	Green
style scar	Present	Chalazal spot colour	Brown
areola	Absent	Seed embryony	Monoembryonic
segment shape uniformity	Present		
Taste	Sour		

**Table 3 List of primer sequences used for phylogenetic analyses and PCR profile.**

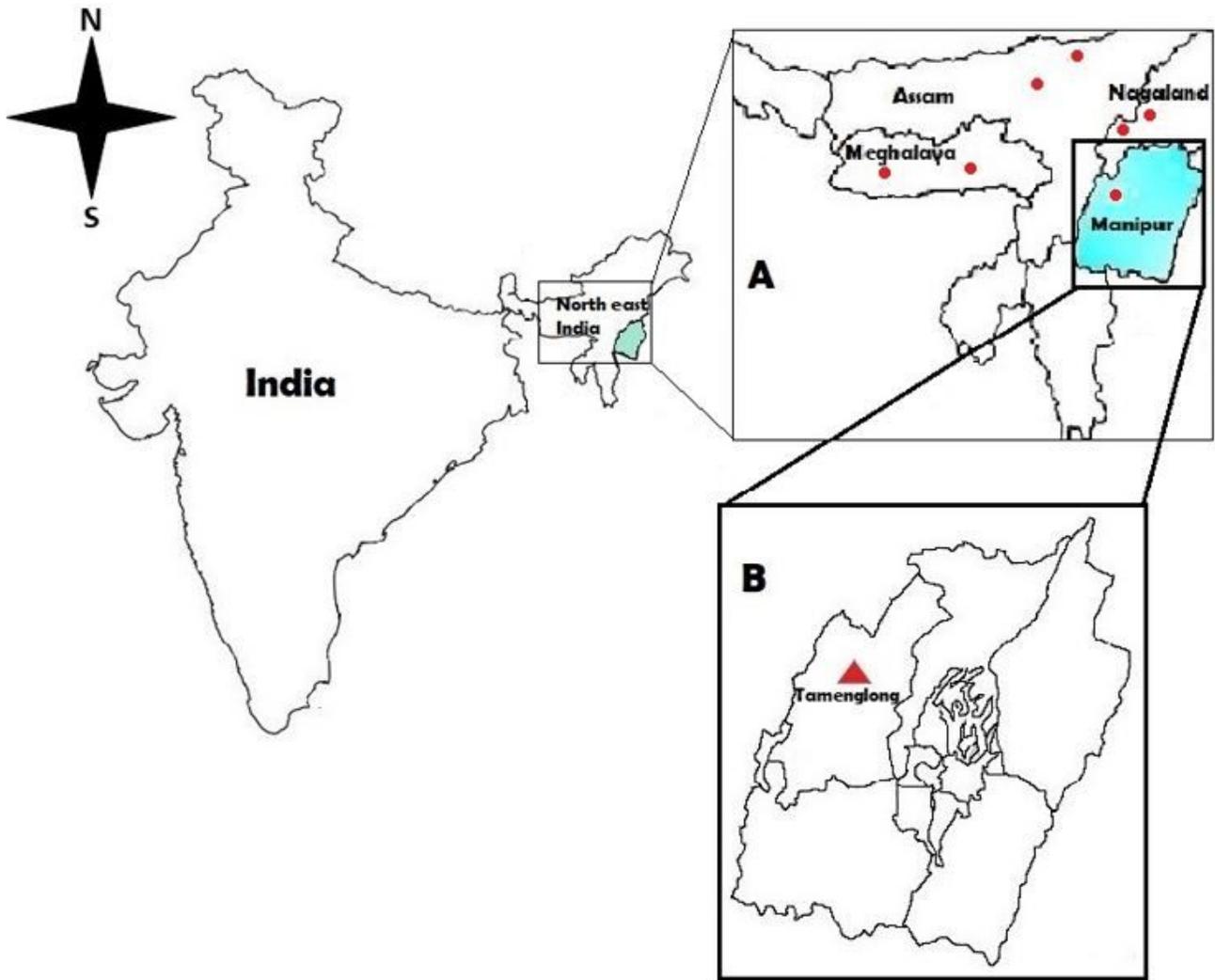
Target sequence	Primer name	5' -3' primer sequence	References	PCR conditions
<i>trnL-F</i> intergenic spacer region	c	CGA AAT CGG TAG ACG CTA CG	Taberlet et al., 1991	Initial denaturation at 94°C for 3 min followed by 30 cycles at 94°C for 60 sec, 48°C for 60 sec, 72°C for 120 sec and a final extension at 72°C for 7 min
	f	ATT TGA ACT GGT GAC ACG AG	Taberlet et al., 1991	
ITS region	ITSP4	TCCTCCGCTTATTGATATGC	White et al., 1990	Initial denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 90 sec and a final extension at 72°C for 5 min
	ITSP5	AAGTCGTAACAAGGTTTCCGTAG	Kollipara et al., 1997	
<i>psbI-psbK</i> intergenic region	psbk	TTAGCCTTTGTTTGGCAAG	Lahaye et al., 2008	Initial denaturation at 94° C for 5 min followed by 35 cycles at 94°C for 30 sec, 51°C for 40 sec, 72°C for 45 sec and a final extension at 72°C for 7 min
	psbI	AGAGTTTGAGAGTAAGCAT	Lahaye et al., 2008	
<i>matK-5' trnK</i> spacer	matK5'R	GCA TAA ATA TAY TCC YGA AAR ATA AGT GG	Shaw et al., 2005	30 cycles of denaturation (94°C for 1 min), primer annealing (48°C for 1 min), extension (72°C for 2 min) and final extension (72 ° C for 7 min)
	matK6	TGG GTT GCT AAC TCA ATG G	Johnson and Soltis, 1994	

## Figures



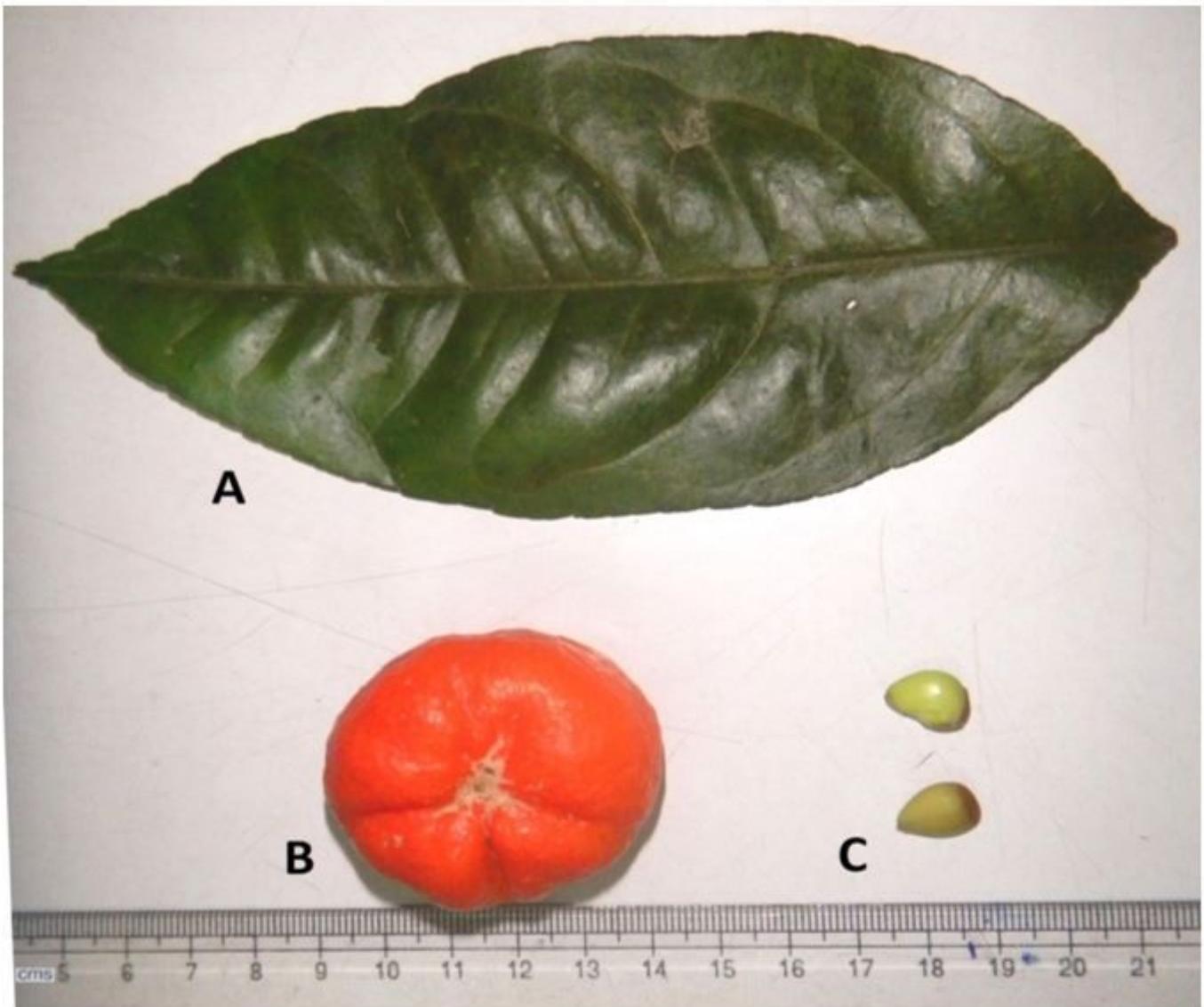
**Figure 1**

Citrus indica view in the surveyed area A) closed view of the plant B) whole plant view in its natural environment.



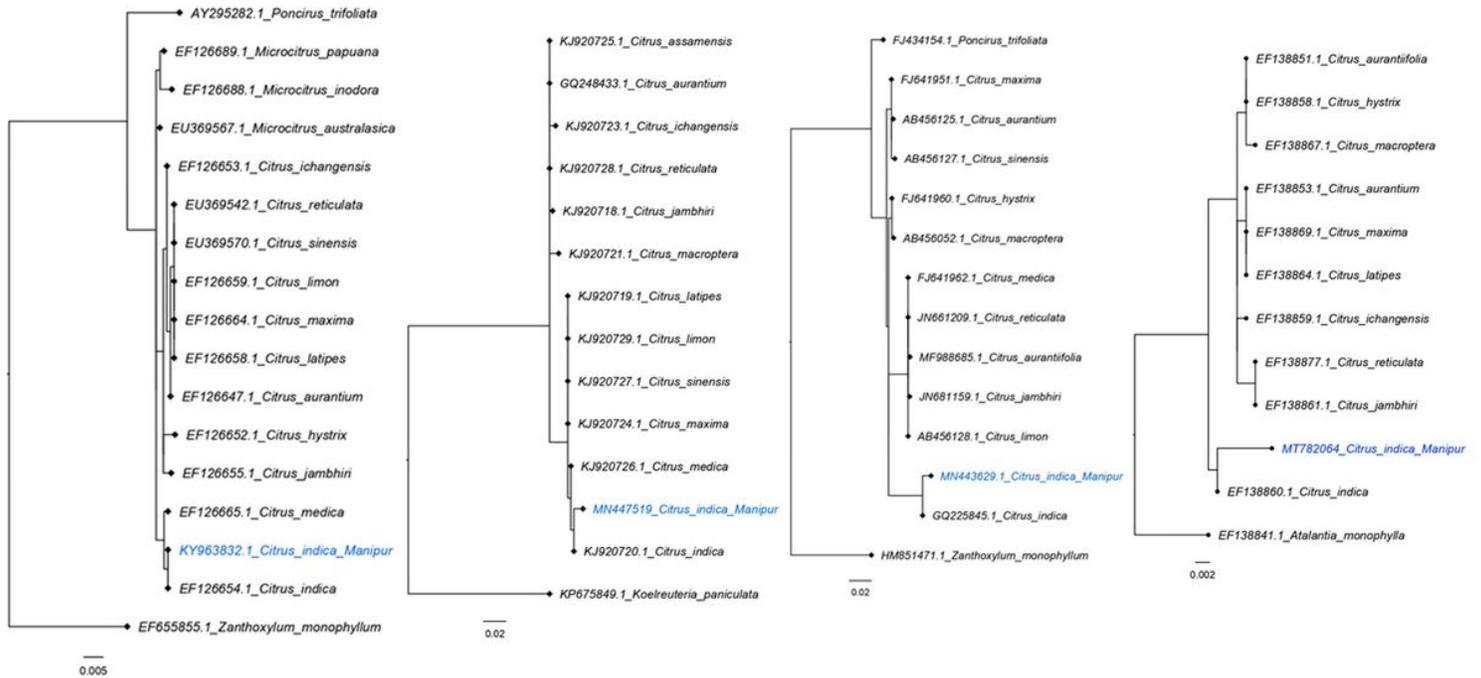
**Figure 2**

Locations of the *Citrus indica* in NE India. (A)- red circles indicate the sites from where *C. indica* was reported to occur earlier (Malik et al., 2006), (B)- red triangle shows the present study site. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



**Figure 3**

(A) Leaf, (B) fruit and (C) seeds of *Citrus indica* collected from Dailong forest, Tamenglong district, Manipur.



**Figure 4**

4a. Genetic relationship of *Citrus indica* (from the present study) in blue colour with other 14 citrus species and two outgroups *Zanthoxylum monophyllum* and *Poncirus trifoliata* based on partial sequences of trn L-F region. 4b. Genetic relationship of *Citrus indica* (from the present study)) in blue colour with other 12 citrus species and one outgroup *Koelreuteria paniculata* with other citrus species based on sequences of psbK-I region. 4c. Genetic relationship of *Citrus indica* (from the present study) in blue font with other 11 citrus species and two outgroups *Zanthoxylum monophyllum* and *Poncirus trifoliata* based on sequences of ITS region. 4d. Genetic relationship of *Citrus indica* (from the present study) in blue font with other 10 citrus species and one outgroup *Atalantia monophylla* based on sequences of matK-5'trnK spacer region.

## Supplementary Files

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