households cannot afford it and hence, they continue to remain dependent on dry wood collected from the sanctuary.

In conclusion, we strongly recommend the plantation of fast-growing firewood species, such as *Dalbergia sissoo*, *Acacia auriculiformis*, *Gmelina arborea* and *Ailanthus excelsa* in the villages adjoining the sanctuary and along the boundary of the tea plantations to meet the fuelwood needs of the people. Additionally, the use of alternative fuel sources, such as LPG, should be encouraged under government schemes, such as the Pradhan Mantri Ujjwala Yojana. Previous attempts in this direction have, however, failed because of the significant cost involved in refilling gas cylinders. Finally, appropriate alternative livelihood options need to be urgently considered for those involved in the commercial harvest of NTFP. An example of such options includes the training and recruitment of members of the local communities as forest guides by government agencies and non-governmental organizations, as the sanctuary attracts a significant number of wildlife tourists every year. These individuals can also regularly be employed to monitor the flora and fauna of the sanctuary, important indicators of the health of this unique, though increasingly threatened, lowland rainforest patch of northeastern India.

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Genetic homogeneity in brown planthopper, *Nilaparvata lugens* (Stål) as revealed from mitochondrial cytochrome oxidase I

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Brown planthopper, *Nilaparvata lugens* (Stål) is a seasonal migrant pest in North India. The present study analyses the genetic diversity of *N. lugens* by employing a partial fragment of the mitochondrial gene encoding cytochrome oxidase I (COI) using samples from 16 different localities of India. Total of 16 full-length COI gene sequences generated from this study

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with 16 COI gene sequences retrieved from GenBank were analysed for genetic differentiation and haplotypes of *N. lugens* populations in order to determine the genetic structure. Based on the partial COI gene, high genetic homogeneity was detected in *N. lugens* populations of India and they form a single genetic group. The Tajima's *D* test and Fu's *F* test also support our result, and indicate recent population expansion, while the phylogenetic tree suggests that geographically distinct populations of *N. lugens* do not exist in India.

Keywords: Brown planthopper, cytochrome oxidase I, genetic homogeneity, phylogenetic tree.

THE brown planthopper (BPH), *Nilaparvata lugens* (Stål) is one of the serious pests of rice causing an annual yield loss worth US$ 1.06 billion in Southeast Asia[1]. The major damage symptom of *N. lugens* in rice involves yellowing and drying of plants in patches, also known as 'hopper burn'[2], due to phloem sapping. *N. lugens* is known to transmit rice virus diseases such as rice ragged stunt (RRSV) and rice grassy stunt (RGSV) in India[3,4].

Due to extremely low winter temperatures in North India and the absence of rice crop, *N. lugens* is not expected to survive in the North Indian states, but it infests the new rice crop every year[5]. Further, there are no alternate hosts of *N. lugens* in North India on which it can survive to complete its life cycle and to act as the nucleus population of the pest for infestation in the next season of rice. Migration of *N. lugens* during November to West Bengal[6], and two population peaks of the pest and associated insect predators were observed in net catches placed 150 m above ground level during May and October respectively, in the state, indicating the migratory behaviour of the pest[7]. *N. lugens* does not overwinter in temperate Asian countries such as Korea, Japan and northern China. It annually migrates into Korea and Japan during the rainy season from northern Vietnam and southern China where rice is cultivated throughout the year. It has been reported that northern Philippines is also the source for *N. lugens* migration to Japan and Taiwan[8,9]. Therefore, it is possible that *N. lugens* must be migrating to North India from the southern or eastern parts of the country during May–July, immediately after the harvest of *rabi* rice crop in these regions, with the aid of southwest monsoon winds[10]. *N. lugens* has developed resistance to important groups of insecticides, including organophosphates and neonicotinoids, particularly imidacloprid[11] and has shown distinct virulence reactions to some rice varieties[12]. Therefore, the present study was undertaken to evaluate the molecular genetic structure and diversity of *N. lugens*, which is essential in designing effective management strategies for its suppression.

Mitochondrial cytochrome oxidase I (COI) is a widely used marker for understanding population structure, species identification and phylogenetic relationships of insects because of its rapid evolutionary rate, maternal inheritance and lack of introns[13–15]. The population genetic structure of various species such as finless porpoises[16], *Reticlitermes*[17], *Amrasca biguttula biguttula*[18], etc. has been revealed using mitochondrial DNA sequences[16,17]. In this study we examined genetic diversity among *N. lugens* populations that were collected from 16 different geographical regions of India, along with 16 sequences retrieved from NCBI, including samples from India, Pakistan and Bangladesh.

N. lugens populations were collected from 15 different states of India using sweep net during 2017–18 and 2018–19 rainy seasons (Table 1). The samples were transferred to glass vials containing 70% ethanol and the date and place of collection were noted. Further, samples were studied under a microscope for identification. The *N. lugens* populations were stored at –20°C in the laboratory for DNA extraction and further analysis.

DNA was extracted from individual *N. lugens* using DNAeasy Blood and Tissue Kit (Qiagen GmbH, Germany), following the manufacture's protocol. The universal barcode primer used in the present study (LCO-1490-5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′; HCO-2198-5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) has been described by Folmer *et al.*[19] specific to COI. The PCR composition consists of (per 25 µl) *Taq* DNA polymerase (Fermentas Inc., USA) were 2.5 µl of 10× PCR buffer with 2 µl of 25 mM MgCl$_2$, 0.5 µl of 10 mM dNTPs, 0.5 µl each of forward and reverse primer, IU of *Taq*, and 17 µl of UltraPure water (Invitrogen, Thermo Fisher Scientific, USA). Thermal cycling was performed with the following conditions: pre-denaturation for 5 min at 94°C followed by 35 cycles of 30 sec at 94°C (denaturation), 40 sec at 47°C (annealing) and 40 sec at 72°C (extension), with a final extension for 8 min at 72°C. PCR products were visualized on 1% TAE-agarose gel electrophoresis (Figure 1). Single bands were purified using a QIAquick PCR purification kit (Qiagen GmbH, Germany). Purified PCR products were sequenced directly using an automated sequencer (ABI prism® 3730 XL DNA analyzer; Applied Biosystems, USA) at Scigenomics Lab, Cochin, India. To verify the authenticity of the COI sequences obtained from this study, they were cross-checked with the established reference sequences of GenBank. The sequences were analysed carefully and submitted to NCBI (Table 1).

All the sequences were arranged in FASTA format for analysis using MEGA 6.0 software. Totally 32 sequences were used in diversity analysis (16 sequences of mitochondrial COI gene of *N. lugens* generated in this study and the remaining 16 sequences were retrieved from NCBI, including sequences of *N. lugens* from different localities of India, Pakistan and Bangladesh). The sequences were subjected to multiple alignments using Clustal W (ref. 20). The phylogenetic neighbor-joining tree based on Kimura-2-parameter[21] distance was

Table 1. Details of sample data used in the analysis of *Nilaparvata lugens* populations

Accession no.	Specific collection locality	Latitude	Longitude
MN148724	Delhi, India	28.7041	77.1025
MN148725	Khonsa, Arunachal Pradesh	26.9929	95.5014
MN148726	Umiam, Meghalaya	25.6768	91.9270
MN148727	Ranichauri, Uttarkhand	27.1750	85.9815
MN148713	Berthin, Himachal Pradesh	31.4188	76.6420
MN148714	Bapatla, Andhra Pradesh	15.9039	80.4671
MN148715	Ranchi, Jharkhand	23.3441	85.3096
MN148716	Ludhiana, Punjab	30.9010	75.8573
MN148717	Pantnagar, Uttarkhand	29.0222	79.4908
MN148718	Kishanganj, Bihar	26.0917	87.9384
MN148719	Coimbatore, Tamil Nadu	11.0168	76.9558
MN148720	Varanasi, Uttar Pradesh	25.3176	82.9739
MN148722	Cuttack, Odisha	20.4625	85.8830
MN148721	Mohanpur, West Bengal	21.8398	87.4232
MN148723	Mahabubnagar, Telangana	16.3841	78.1108
MN148728	Tirupathi, Andhra Pradesh	13.6288	79.4192
MG775040	Panna, Madhya Pradesh*		
MK032794	Solapur, Maharashtra*		
MG775041.1	Madhya Pradesh, India*		
MK032794.1	Pune, Maharashtra, India*		
MK922239.1	New Delhi, India*		
BOLD:ACA8955	Nellore, Andhra Pradesh, India*		
BOLD:ACA8955	Odisha, India*		
MK033512.1	Delhi, India*		
KX351398.1	Umiam, Meghalaya, India*		
KT879869.1	Assam, India*		
BOLD:ACA8955	Odisha, India*		
MK301229.1	Faisalabad, Pakistan		
BOLD:ACA8955	Chittagong, Bangladesh*		
BOLD:ACA8955	Chittagong, Bangladesh*		
MH052644.1	Dhaka, Bangladesh*		
MH052645.1	Dhaka, Bangladesh*		

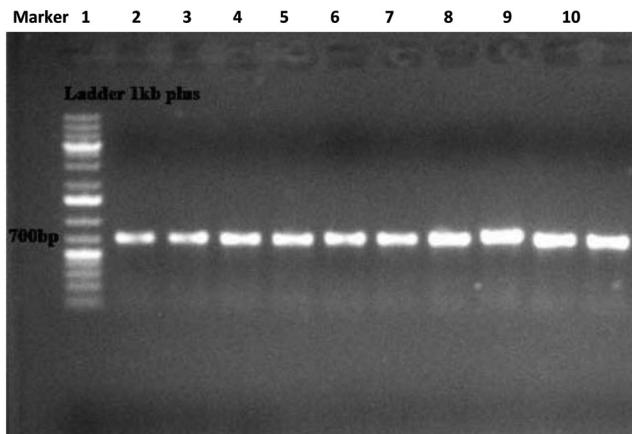*Sequences retrieved from the NCBI database.

Figure 1. *Nilaparvata lugens* COI gene (PCR amplicon) samples showing 700 bp product in 1% agarose gel with 1 kb DNA ladder.

constructed using MEGA 6 software[22] with 2000 bootstrap replication. DnaSP5.10.01 was used to estimate a series of population-level parameters, including pairwise nucleotide diversity (π) and haplotype diversity (h)[23]. Neutrality tests like Tajima's D[24] and Fu's FS[25] were also per-formed using DnaSP 5.10.01 (ref. 23) for evaluating the range of historical population expansion and testing whether the sequences conformed to the expectations of neutrality.

Adults of *N. lugens* were collected from 16 different localities of 15 states of India (Table 1). Totally 32 sequences of *N. lugens* were used for the analysis, excluding two sequences of *Nephotettix* genus used as outgroups (MH052646.1, HM160144.1). The mean total nucleotide composition in the sequences was found to be A 31%, T 36.2%, G 12.8% and C 20%. The average AT content accounted for 67.2% and GC for the rest 32.8% of the sequences. The bias towards AT content (67.2%) is a general feature of the COI region in arthropods. The maximum pairwise genetic distance was found to be 0.7%, and there existed only one segregating site. Further, test statistics were generated for the 32 COI sequences (Table 2). A total of four haplotypes were observed with nucleotide diversity as 0.00491 and haploid diversity as 0.7319, indicating lower nucleotide diversity and higher haplotype diversity.

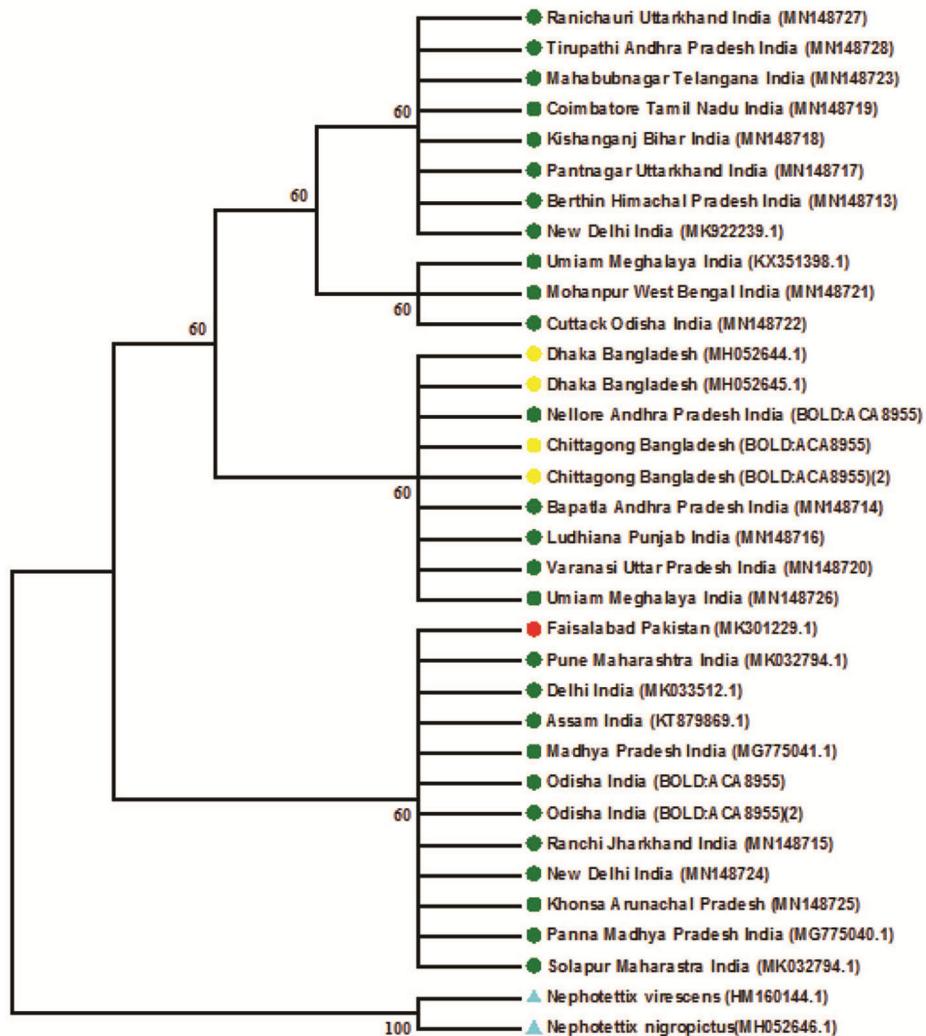The phylogenetic neighbor-joining tree was constructed based on 32 sequences using MEGA 6.0 software[22]. The

Figure 2. Phylogenetic neighbor-joining tree constructed from COI gene sequences of *N. lugens* and *Nephotettix* species used as outgroup.

Table 2. Genetic diversity of *N. lugens* populations based on COI gene sequences

Population parameters	Total population
Sample size	32
No. of haplotypes (N_h)	4
Haplotype diversity (h)	0.7319
Nucleotide diversity (π)	0.00491
No. of segregating sites (S)	1
Tajima's D	−0.04056
Fu's F	−0.286

phylogenetic tree had a total branch length of 0.33077592 base substitutions per site (Figure 2). The *N. lugens* populations from India, Bangladesh and Pakistan clustered in four different clades and are clearly depicted in the tree. There was no clustering of sequences from the same geographical region. However, samples from the same locality grouped together, except in case of New Delhi and

Umiam, Meghalaya samples. Further, samples from different localities of India including the southern, eastern and northern populations, were mixed in different clades without forming clades of geographical distinction (Figure 2).

N. lugens is a serious migratory pest of rice in tropical and temperate Asia. It is widely distributed across the region: in the west from Pakistan to northeastern Australia and in the south from the Malay Archipelago to New Guinea and the Solomon Islands to central Japan. It is a serious pest of rice in India, Bangladesh, Japan, China, Philippines, South Korea, Sri Lanka, Taiwan, Indonesia, Thailand and Vietnam[26]. In India it is also migratory in nature, infesting the new rice crop in North India every year and disappearing after November due to freezing temperatures in winter. However, information on genetic diversity of this pest is lacking in India.

The genetic variation in *N. lugens* populations based on 32 sequences of the COI gene in the present study

indicated high genetic homogeneity. The maximum genetic distance among the populations was found to be 0.7%, which is below the limit of 2% generally considered for species delineation[18,27]. The COI sequence analysis of cotton leafhopper, *Amrasca biguttula biguttula* collected from cotton-growing areas of India suggested the presence of single species in the country[18]. Similar reports of genetic variation of 0.3–1.2% was reported among populations of tea green leafhoppers, *Empoasca onukii* and *Jacobiasca formosana* from China, Japan and Taiwan based on 16SrDNA and COI, corroborating the hypothesis that geographically separated populations of the pest represent a single species[27]. On the other hand, high haplotype and low nucleotide diversity observed in *N. lugens* in India suggest rapid demographic expansion of the pest from a small effective population size; similar results were reported earlier in *Bicyclus anynana*[28]. The negative values of Tajima's D and Fu's F tests in the present study indicate recent population expansion that has also been observed in *Bicyclus anynana*[28] and in North Indian cotton leafhopper[18]. On the other hand, the phylogenetic tree did not depict any clustering of sequences based on geographic distinction such as North, North East and South Indian populations of *N. lugens*. However, samples from the same locality clustered together, except for a few sub-populations. The sub populations of *N. lugens* of India, Pakistan and Bangladesh were mixed in different clades without forming distinct clades. This result clearly indicates that *N. lugens* populations from different regions of India formed a single genetic group. *N. lugens* is known to migrate during two-seasons of the year: once in May when the southwest monsoon aids migration and another during October to November in eastern India due to harvest of rice crop[7]. This migration might be responsible for gene flow among the *N. lugens* populations that helped in maintaining their genetic homogeneity. It has been previously observed that sufficient gene flow among populations of the same species could slow down or prevent geographic differentiation and result in small population size over larger areas, as commonly observed in migratory insects or good dispensers such as Monarch butterflies *Danaus plexippus*[29], bumblebee *Bombus terrestris*[30], dragonflies *Anax junius*[31] and cotton leafhopper *A. biguttula biguttula*[18,32].

From the present study, it can be concluded that owing to the migratory nature of *N. lugens*, genetic homogeneity was maintained among the populations in India with less genetic variability. It is not possible to pinpoint the geographic source of *N. lugens* migration to northern India based on this level of variability. However, the future work with other molecular markers may help find the migration source of the pest. Further, studies are essential to explore the offseason survival and migratory pathway of *N. lugens* in India that will be helpful in designing regional pest management strategies for its suppression.

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