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Authors: Roopa, H. K., Kumar, N. K. Krishna, Asokan, R., Rebijith, K. B., Mahmood, Riaz, et. al.

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PHYLOGENETIC ANALYSIS OF TRIALEURODES SPP. (HEMIPTERA: ALEYRODIDAE) FROM INDIA BASED ON DIFFERENCES IN MITOCHONDRIAL AND NUCLEAR DNA

H. K. ROOPA1, N. K. KRISHNA KUMAR1,*, R. ASOKAN2, K. B. REBIJITH2, RIAZ MAHMOOD3 AND ABRAHAM VERGHESE1

1Division of Entomology and Nematology and 2Division of Biotechnology, Indian Institute of Horticultural Research, Hessaraghatta Lake P.O Karnataka, Bengaluru, 560 089, India
3Department of Biotechnology and Bioinformatics, Kuvempu University, Jnanasahyadri, Shankaraghatta, Shimoga-577 451, India.

*Corresponding author’s; E-mail: kumarhort@icar.org.in

ABSTRACT

About 55 species of Trialeurodes have been described with only a few viruliferous species like T. vaporariorum and T. abutilonea, which are able to transmit Criniviruses (Closteroviridae) and T. ricini, which is able to transmit Begomovirus spp. (Geminiviridae). Quick and accurate identification of whitefly vectors in the early life stages is important from the point of view of understanding the epidemiology of crinivirus associated plant diseases, and in their management and quarantine. Morphological identification is further strengthened by DNA barcoding. In this regard, we examined genetic differences in mitochondrial cytochrome oxidase-I (COI) gene and the internal transcribe spacers (ITS) sequences of ribosomal DNA among various populations of T. vaporariorum collected from different geographic locations in the state of Karnataka, Tamil Nadu, and Andhra Pradesh in India. Our studies are the first to analyze the genetic variation of T. vaporariorum populations in India, which showed no appreciable differences. This clearly indicated that there are no cryptic species or biotypes in T. vaporariorum in India in contrast to the studies of B. tabaci, where there is evidence for many biotypes. The phylogenetic analyses comprised of 4 Trialeurodes species showed 2 clades. Clade I is comprised of T. vaporariorum and T. abutilonea which are genetically close. Clade II consists of the remaining 2 species, viz., T. lauri and T. ricini. Also the current study provided evidence of the suggested emergence of biotypes T. ricini.

Key Words: Criniviruses, Trialeurodes vaporariorum, DNA barcoding, cryptic species, biotypes

RESUMEN

Se han descrito cerca de 55 especies de Trialeurodes y de estas hay unas cuantas especies que son reportadas como vectores de Crinivirus (Closteroviridae). Trialeurodes vaporariorum y Bemisia tabaci son dos vectores importantes del virus de enrollamiento amarillo de las hojas del tomate (TYLCV) y del virus de enanismo amarillo de la papa (PYDV). Una identificación rápida y precisa de los vectores de la mosca blanca en las etapas tempranas de vida es importante desde el punto de vista de la comprensión de la epidemiología de los crinivirus, su manejo y también la cuarentena. Utilizando las técnicas moleculares, la identificación morfológica ha sido reforzada aun más por el uso de la codificación de barras del ADN utilizando primers universales de códigos de barras. En este estudio se analizaron las diferencias genéticas en el COI y ITS en 14 poblaciones de T. vaporariorum recolectadas de diferentes sitios geográficos en Karnataka, India. Nuestros estudios sobre T. vaporariorum mostró por primera vez que no hay diferencias apreciables genéticas entre las diferentes poblaciones. Esto indica claramente que no existe especies crípticas o biotipos en T. vaporariorum al contrario de las observaciones en B. tabaci, donde se han reportado varios biotipos. Un filograma fue construido para cuatro especies de Trialeurodes utilizando información disponible del nucleótidos del COI en el NCBI-GenBank junto con nuestros datos.

Palabras Clave: Crinivirus, Trialeurodes vaporariorum, codificar bandas de ADN, especies crípticas, biotipos
Whiteflies (Hemiptera: Aleyrodidae) are important sap sucking insect pests of agricultural and horticultural crops, and can cause crop damage either by direct feeding or by transmission of plant pathogenic viruses. The three most damaging and ubiquitous species are the cotton whitefly or sweet potato whitefly (*Bemisia tabaci* Gennadius), sliver leaf whitefly (*Bemisia argentifolii* [Bellows and Perring]) and the green house whitefly (*Trialeurodes vaporariorum* Westwood). Their rapid population growth is due in part to arrhenotoky or arrhenotokous parthenogenetic reproduction of whiteflies in which unfertilized eggs develop into males and fertilized eggs to females. The piercing-sucking feeding behavior of whiteflies can cause considerable crop damage. Currently 55 species in the world are assigned to the genus *Trialeurodes* (Mound & Halsey 1978), which is one of the largest genera in the family Aleyrodidae. The greenhouse or glasshouse whitefly, *Trialeurodes vaporariorum*, was described by Westwood in 1856 as *Aleyrodes vaporariorum*. It is a polyphagous insect, and plants belonging to 82 families serve as its hosts (Mound & Halsey 1978). Feeding by nymphs and adults draws proteins and other nutrients of plant sap, and they excrete surplus sugars as ‘honeydew’. At high populations, the copious honeydew produced leads to the growth of sooty mold, which restricts photosynthesis, stunts plants and causes leaf necrosis (Byrne et al. 1990). *T. vaporariorum* can also act as a vector by transmitting viruses to plants, viz., *Beet pseudo yellow virus* (BPYV) (*Closterovirus*) (Duffus et al. 1965.; Tzanetakis et al. 2003, 2004a), *Potato yellow vein virus* (PYDV) (Alba 1950), *Tomato infectious chlorosis virus* (TICV), *Tomato chlorosis virus* (ToCV) Duffus et al. 1995; Wisler et al. 1998a, 1998b) and *Strawberry pallidosis associated virus* (SPaV) (Tzanetakis et al. 2003). Recent observation on *B. tabaci* states, it’s a complex of 24 morphologically indistinguishable species/biotypes and defined by analyzing consensus sequences and 3.5% of mt-COI sequences pairwise genetic divergence. (Paul J. De Barro 2011). Development of biotypes in *B. tabaci* may be due to various causes including insecticide selection pressure and development of resistance, host variation, and geography; all of which may influence vector potential for various viruses (Bird 1957; Mound 1963; Costa and Russell 1975; Bird & Maramorosch 1978). In contrast to the case of *B. tabaci*, fewer studies were carried out with *T. vaporariorum*, which is becoming a serious pest in glasshouse or polyhouse cultivation of agricultural crops. In addition through the characterization of esterase banding patterns, Idriss et al. (1997) reported the existence of 5 different biotypes in *T. ricini* populations.

This study investigates the evolutionary relationship of *Trialeurodes* spp. based on sequence variation of the mitochondrial cytochrome oxidase-I (CO-I) gene, and internal transcribed spacer (ITS) sequences of ribosomal DNA. This is one of the first studies to examine the genetic population structure of *T. vaporariorum* over a broad range.

**Materials and Methods**

**Collection of Whitefly Populations**

Adults and nymphs of the whitefly, *T. vaporariorum*, were collected using a hand-held aspirator from 2009 to 2011 on ornamental and vegetables at 22 different locations in South India (Table 1). The specimens were preserved in 70% ethanol for further analysis.

**Morphological Identification**

Specimens were slide mounted with Canada balsam using the procedure described by Brown (1997). The taxonomic characters were used for identification based on Martin (1987).

**DNA Extraction and Polymerase Chain Reaction (PCR)**

*Trialeurodes vaporariorum* were taken individually and a small part of each abdomen was used to extract the genomic DNA using the modified cetyl trimethyl ammonium bromide buffer (CTAB) method (Saghai-Marroof et al. 1984). The rest of the insect body was retained as the specimen voucher and placed in the Division of Biotechnology, IISR, Bengaluru. Briefly, a part of the abdomen was ground with 1.0 mL of CTAB 2%, 100 mM Tris-HCl (pH 8.0), 1.4 M sodium chloride, 20 mM EDTA, 0.1% of 2-mercaptoethanol (added just prior to use) and suspended in the same buffer. The suspension was incubated at 65 °C for 2 h and then an equal volume of chloroform: isomyalcohol (24:1) was added. The suspension was centrifuged at 10,000 rpm (Eppendorf 5415 C) for 10 min at 8 °C. The upper aqueous layer was transferred to a fresh micro centrifuge tube taking care not to disturb the middle protein interface. DNA was precipitated by adding an equal volume of ice-cold 95% ethyl alcohol. The precipitated DNA was spun at 10,000 rpm (Eppendorf 5415 C) and the resultant DNA pellet was washed with 70% ethanol and dissolved in 50 μL DNase-, RNase- and Protease-free molecular biology water (5 PRIME Hamburg, Gaithersburg, Maryland, US). Extracted DNA was further purified to be free of RNA contaminants by addition of 10 μL of RNase. The intact genomic DNA was visualized using a fluorometer (DyNa quant 200™, Hoefer, www.hoeferinc.com) following standard procedures. Depending upon the concentration, the DNA samples were diluted with sterile water.
| Species          | Voucher number | Host plant         | Site of collection | GenBank accessions |
|------------------|----------------|--------------------|--------------------|-------------------|
| T. vaporariorum  | ORPRP-01       | Gerbera jamesonii  | Tubgere            | JQ339309 JQ339323 JQ339337 |
| T. vaporariorum  | ORPRP-02       | Rosa spp           | Arallu mallige     | JQ339310 JQ339324 JQ339338 |
| T. vaporariorum  | ORPRP-03       | Gerbera jamesonii  | Byrasandra         | JQ339311 JQ339325 JQ339339 |
| T. vaporariorum  | ORPRP-04       | Gerbera jamesonii  | IIHR               | JQ339312 JQ339326 JQ339340 |
| T. vaporariorum  | ORPRP-05       | Gerbera jamesonii  | Moodal Palya       | JQ339313 JQ339327 JQ339341 |
| T. vaporariorum  | ORPRP-06       | Cucurbita moschata | Malur              | JQ339314 JQ339328 JQ339342 |
| T. vaporariorum  | ORPRP-07       | Gerbera jamesonii  | Malur              | JQ339315 JQ339329 JQ339343 |
| T. vaporariorum  | ORPRP-08       | Rosa spp           | Saragapalli        | JQ339316 JQ339330 JQ339344 |
| T. vaporariorum  | ORPRP-09       | Rosa spp           | Kodiyala           | JQ339317 JQ339331 JQ339345 |
| T. vaporariorum  | ORPRP-10       | Rosa spp           | Kurabatti          | JQ339318 JQ339332 JQ339346 |
| T. vaporariorum  | ORPRP-11       | Gerbera jamesonii  | Srinivaspura       | JQ339319 JQ339333 JQ339347 |
| T. vaporariorum  | ORPRP-12       | Solanum lycopersicum | Solur         | JQ339320 JQ339334 JQ339348 |
| T. vaporariorum  | ORPRP-13       | Capsicum annuum    | Solur              | JQ339321 JQ339335 JQ339349 |
| T. vaporariorum  | ORPRP-14       | Rosa spp           | Solur              | JQ339322 JQ339336 JQ339350 |
| T. vaporariorum  | ORPRP-15       | Hibiscus spp       | Yercaud            | JX841200 JX841208 JX841216 |
| T. vaporariorum  | ORPRP-16       | Ipomea spp         | Yercaud            | JX841201 JX841209 JX841217 |
| T. vaporariorum  | ORPRP-17       | Gerbera jamesonii  | Dharmapuri         | JX841202 JX841210 JX841218 |
| T. vaporariorum  | ORPRP-18       | Rosa spp           | Dharmapuri         | JX841203 JX841211 JX841219 |
| T. vaporariorum  | ORPRP-19       | Solanum lycopersicum | Guntur         | JX841204 JX841212 JX841220 |
| T. vaporariorum  | ORPRP-20       | Gerbera jamesonii  | Guntur             | JX841205 JX841213 JX841221 |
| T. vaporariorum  | ORPRP-21       | Nicotiana tabacum  | Bengaluru          | JX841206 JX841214 JX841222 |
| T. vaporariorum  | ORPRP-22       | Phaseolus vulgaris | Bengaluru          | JX841207 JX841215 JX841223 |
to get a working solution of 20-25 ng/μL. A portion of the total DNA was preserved in glycerol (10%) at -80 °C for future reference.

The polymerase chain reaction (PCR) was carried out in a thermal cycler (AB-Applied Biosystems, Veriti 96 wells) with the following cycles; 94 °C for 4 min as initial denaturation followed by 35 cycles of 30 s at 94 °C, for 45 s at 47 °C, 54 °C, and 52 °C for COX-I, mtCO-I and ITS-1 respectively, for 45 s at 72 °C and for 20 min at 72 °C as the final extension. The primers as mentioned in Table 2 were specific to mitochondrial DNA; and nuclear DNA fragments were used in the present study. PCR was performed in 25-μL total reaction volume containing 20 picomoles of each primer, 10 mM Tris HCl (pH -8.3), 50 mM KCl, 2.5 mM MgCl2, 0.25 mM of each dNTP and 0.5 U of Taq DNA polymerase (Fermentas GmBH, St. Leon-Rot, Germany). The amplified products were resolved in 1.5% agarose gel, stained with ethidium bromide (10μg/mL) and visualized in a gel documentation system (UVP).

Molecular Cloning and Sequencing

The PCR amplified fragments were eluted using Nucleospin® Extract II according to the manufacturer’s protocol (Macherey-Nagel, Duren, Germany) and ligated into the general purpose-cloning vector, InsT/Aclone (Fermentas GmBH, St. Leon-Rot, Germany) according to the manufacturer’s protocol. The transformed cells were spread on LB agar plates containing 20 picomoles of each primer, 10 mM Tris HCl (pH -8.3), 50 mM KCl, 2.5 mM MgCl2, 0.25 mM of each dNTP and 0.5 U of Tag DNA polymerase (Fermentas GmBH, St. Leon-Rot, Germany). The amplified products were resolved in 1.5% agarose gel, stained with ethidium bromide (10μg/mL) and visualized in a gel documentation system (UVP).

Phylogenetic Analysis

All the sequences generated in the present study, corresponding to COX-I, mtCO-I and ITS-1 was aligned using Clustal W. The alignment was further analyzed using MEGA.4.0 (Kumar et al. 1993). Maximum- Parsimony (MP) and Neighbour-Joining (NJ) trees were constructed using the Kimura-2-parameter (K2P) distance model (Kimura 1980; Saitou & Nei 1987). All the corresponding sequences for different mitochondrial and ribosomal markers of *T. vaporariorum* were deposited in the NCBI-GenBank.

RESULTS AND DISCUSSION

Molecular diversity studies in various insects of importance in agriculture pave the way in identifying new species (Ball & Armstrong 2006), biotypes (Perring 2001), cryptic species (Hebert et al. 2004) and haplotypes (Toda & Murai 2007), which are difficult to identify through morphological

**Table 3. Maximum composite likelihood estimate of the pattern of nucleotide substitution from different populations of *Trialeurodes vaporariorum*.**

|   | A   | T   | C   | G   |
|---|-----|-----|-----|-----|
| A | —   | 6.38| 5.35| **12.28** |
| T | 6.45| —   | **11.83** | 5.51 |
| C | 6.45| **14.12** | — | 5.51 |
| G | **14.4** | 6.38| 5.35| — |

Each entry shows the probability of substitution from one base (row) to another base (column) instantaneously. Only entries within a row should be compared. Rates of different transitional substitutions are shown in bold, and those of transversional substitutions are shown in italics.
characters. Insect pests with piercing-sucking mouthparts are of major concern both agricultural and horticultural crops because many of them possess immense vectoring capacity and they are developing insecticide resistance (Denholm et al., 1996, 1998). One of the invasive species, *B. tabaci* is reported as ‘species complex’ with the existence of 24 biotypes (Paul J. De Barro et al 2011) and also is an important vector for many begomo viruses. Attempts to define these biotypes were made by using esterase banding patterns and a range of biological characters (Bedford et al. 1994). Subsequently many biotypes have been defined primarily by differences in both or either mitochondrial COI or ribosomal ITS1 DNA sequences with little or no meaningful biological data to support their uniqueness (Boykin et al. 2007). In India, another whitefly species, *T. vaporariorum*, also has been gaining importance to rival *B. tabaci* in developing insecticide resistance, especially in polyhouses on ornamentals, vegetables and rarely in open fields. Considering their pest potential, we judged it to be necessary to analyze the molecular diversity in *Trialeurodes* species viz. *T. vaporariorum*, *T. ricini*, *T. lauri* and *T. abutilonea*. Recently, Idriss et al. (2009) proved the existence of 5 different biotypes in *T. ricini* using esterase banding patterns, and proved that these biotypes have varying efficiencies in transmitting *Tomato yellow leaf curl virus* (TYLCV).

COX-I, mtCOI and ITS-1 sequencing yielded 658bp, 825bp and 525bp nucleotide sequences, respectively, and a total of 22 specimens for *T. vaporariorum* collected from various hosts from different geographical regions in Karnataka, Tamil Nadu and Andhra Pradesh (Table 1). All of these sequences have been deposited in the NCBI-Genbank database under the accession numbers JQ339309 to JQ339350 (Table 1).

COX-I and ITS-1 sequences were aligned using Clustal X (Version 1.83, 2003; Thompson et

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Fig. 1. Maximum Parsimony tree using COI and ITS sequences with bootstrap support (1000 replicates) showing phylogram for *Trialeurodes vaporariorum* collected from different hosts and different geographical locations in South India. *Bemisia tabaci* was used as an out group in the analysis. (MEGA. 4.0)
al. 1997) with default settings. The Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution for *T. vaporariorum* sequences was performed using MEGA 4.0 (Tamura et al. 2007). The reliability of the clustering pattern in the trees was determined by the bootstrap test, with 1000 replications. Each entry showed the probability of substitution from one base (row) to another base (column) instantaneously. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics (Table 3). The *T. vaporariorum* nucleotide frequencies are 0.272 (A), 0.269 (T), 0.226 (C) and 0.232 (G). The base composition of the COX-I gene fragment was biased toward Adenine (A) and Thymine (T), which together constituted 54.1% of the total. The overall transition (ti)/transversion (tv) bias of *T. vaporariorum* nucleotide sequence is $R = \frac{[A^*G^*k_1 + T^*C^*k_2]}{[(A+G)^*(T+C)]]}$. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated from the datasets (complete deletion option). All calculations were conducted in MEGA 4.0 (Tamura et al. 2007). Summary statistics for the different substitution changes are shown in Table 3. Maximum Parsimony (MP) and maximum likelihood (ML) analysis were performed with PAUP*4.0b10 (Swofford 1998), using the heuristic search procedure with 1000 random additions of sequences and 10 trees held at each pseudo-replicate, and the tree bisection reconnection (TBR) branch swapping method; all characters were treated as unordered and equally weighted for the MP analysis.

In India, *T. vaporariorum* is gaining importance as a major direct pest on crops in polyhouses. Therefore, specimens were collected from different geographical locations in Karnataka, Tamil Nadu and Andhra Pradesh and the corresponding sequences for CO-I region and ITS-1 revealed that there are very little sequence variations in the nucleotides. The phylogram suggested that *T. vaporariorum* is a single species (Fig. 1), which contrast with corresponding observations of the *B. tabaci* species complex (Boykin et al. 2007) and *Thrips tabaci* (Brunner et al. 2004). The present study clearly indicated that there are neither biotype nor host associated genetic differences in *T. vaporariorum*. For the phylogenetic analysis various *Trialeurodes* spp. sequences of the mt-CO1 gene were acquired from NCBI- GenBank (Table 4) and were used along with our data generated in the present study. Four different *Trialeurodes* species viz. *T. vaporariorum*, *T. ricini*, *T. lauri*, and *T. abutilonea* were analyzed and the mt-CO1 sequences revealed that, there are 2 major clades; i.e., clade I consists of *T. vaporariorum* and *T. abutilonea* and clade II consists of *T. ricini* and *T. lauri* (Fig. 2). Another interesting observation resulted from our molecular data was the strong support for the evidence of biotypes emer-

Table 4. Details of mtCO-I Sequences for *Trialeurodes* spp. Acquired from NCBI-GenBank for Phylogenetic Analysis.

| Species                | Host plant           | Country     | mtCO1     |
|------------------------|----------------------|-------------|-----------|
| *T. vaporariorum*      | Unknown              | China       | JF512474  |
| *T. vaporariorum*      | Unknown              | China       | JF693934  |
| *T. ricini*            | *Ricinus communis*   | Egypt       | AM179430  |
| *T. ricini*            | Unknown leaves       | Gambia      | AM179428  |
| *T. ricini*            | *Ricinus communis*   | Spain       | AM179426  |
| *T. ricini*            | *Ricinus communis*   | Spain       | AM179424  |
| *T. ricini*            | *Ricinus communis*   | Spain       | AM179422  |
| *T. ricini*            | *Ricinus communis*   | Spain       | AM179447  |
| *T. ricini*            | Unknown leaves       | Egypt       | AM179431  |
| *T. ricini*            | Unknown leaves       | Nigeria     | AM179429  |
| *T. ricini*            | *Ricinus communis*   | Spain       | AM179427  |
| *T. ricini*            | *Ricinus communis*   | Spain       | AM179425  |
| *T. ricini*            | *Ricinus communis*   | Spain       | AM179432  |
| *T. ricini*            | Unknown leaves       | Egypt       | AM179430  |
| *T. ricini*            | Unknown leaves       | Gambia      | AM179428  |
| *T. lauri*             | *Laurus nubilis*     | Croatia     | AM179440  |
| *T. lauri*             | *Arbutus andrachne*  | Israel      | AM179438  |
| *T. lauri*             | *Arbutus unedo*      | Turkey      | AM179436  |
| *T. lauri*             | *Arbutus unedo*      | Turkey      | AM179434  |
| *T. lauri*             | *Arbutus unedo*      | Turkey      | AM179432  |
| *T. lauri*             | *Laurus nubilis*     | Croatia     | AM179439  |
| *T. lauri*             | *Arbutus andrachne*  | Israel      | AM179437  |
| *T. lauri*             | *Arbutus unedo*      | Turkey      | AM179435  |
| *T. lauri*             | *Arbutus unedo*      | Turkey      | AM179433  |
Fig. 2. Maximum Parsimony tree using COI sequences with bootstrap support (1000 replicates) showing two major clades for Trialeurodes spp., viz., T. vaporariorum, T. abutilonea, T. lauri and T. ricini. Clade I consists of T. ricini (●) and T. lauri (○), and Clade II consists of T. vaporariorum (▲) and T. abutilonea (♦). Bemisia tabaci (■) was used as an out group in the analysis. Gp-I and Gp-II are the two new groups of T. ricini cryptic species/biotypes, according to Hebert’s barcoding gap of 10X interspecific-to-intraspecific distances.
gence as in earlier case of T. ricini by Idriss et al, 2009. The phylogenetic analysis showed that two populations from Egypt, collected on Ricinus communis formed the clade I and the rest of the populations collected on various geographical locations viz. Spain, Gambia and Nigeria formed the Clade II. According to Hebert et al., (2004), sequence thresholds of 10X, the mean intraspecific mtCOI variation for group under study as an approximate method of screening for potentially different (cryptic) species or biotypes. Using MEGA 5.0, intraspecific and interspecific distances were calculated for both group-I and group-II observed in the MP tree for T. ricini. The intraspecific distance ranges for group-I and group-II of T. ricini 0.00-0.35. The interspecific distances for group-I and group-II of T. ricini is 6.25%. Thus our studies showed that T. ricini is not a single polyphagous species but on contrary, by the standards of genetic and ecological differentiation in other species groups and also based on Hebert's barcoding gap of 10X interspecific-to-intraspecific distances, the recognition of a distinct biotypes may be considered. On the other hand T. lauri also formed two clades with the variations in the nucleotide sequences, however unable to satisfy the sequence thresholds of 10X rule.

Our study indisputably proved that T. vaporariorum is a single species, and unlike B. tabaci species complex. Also the phylogram showed that T. vaporariorum is more closely related to T. abutilonea than to T. ricini and T. lauri, which formed the second clade in the phylogram (Fig. 2). This is a much needed starting point with reference to the molecular identification of the greenhouse whitefly, T. vaporariorum, and to further attempts to determine if biotypes or cryptic species occur in this globally distributed species.

ENDNOTE

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