DNA Barcoding and Phylogenetic Relationships of Spodoptera litura and S. exigua (Lepidoptera: Noctuidae)

Authors: Shashank, P. R., Thomas, Asha, and Ramamurthy, V. V.

Source: Florida Entomologist, 98(1) : 223-228

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.098.0138

DNA barcoding and phylogenetic relationships of *Spodoptera litura* and *S. exigua* (Lepidoptera: Noctuidae)

P. R. Shashank, Asha Thomas and V. V. Ramamurthy

Abstract

Spodoptera spp. (Lepidoptera: Noctuidae) are highly polyphagous pests that inflict serious damage to a wide spectrum of crops. The ability of *Spodoptera* spp. to thrive on diverse host plants is an adaptive advantage for their survival in the ecosystem, which is achieved by its high mobility, fecundity and capacity to develop resistance to wide spectrum of chemical insecticides. In this study, we present molecular diversity and phylogenetic relationship of *S. litura* (Fabricius) and *S. exigua* (Hübner) inferred from mitochondrial cytochrome oxidase-I (COI). Alignment of the sequences of COI from various life stages of the 2 species of *Spodoptera* shows that the molecular identification is independent of life stages and polymorphism of the target species. Maximum likelihood analyses of *S. litura*, *S. exigua* and *S. mauritia* (Boisduval) reveal that there exist significant variations among these. *Spodoptera exigua* showed intraspecific variations with respect to different geographic locations. Present study proves the utility of COI for identification of *S. litura* and *S. exigua* irrespective of their life stages, and also draws inferences on the phylogenetic relationships between the 3 pest species.

Key Words: *Spodoptera* sp.; lifecycle stages; COI; phylogeny; mtCOI; tobacco cutworm

Resumen

El género *Spodoptera* incluye especies plaga altamente polífagas que infligen graves daños a una amplia gama de cultivos. La capacidad de las especies de *Spodoptera* para prosperar en diversas plantas hospederas es una ventaja adaptativa para su mejor sobrevivencia en el ecosistema, el cual es facilitado por su gran movilidad, fecundidad y capacidad para desarrollar resistencia a una amplia gama de insecticidas químicos. En este estudio, se presentan datos de diversidad molecular y la relación filogenética de *S. litura* y *S. exigua* deducida del citocromo oxidasa mitocondrial -I (mtCOI). La alineación de las secuencias del mtCOI de varios estadios de vida de las 2 especies de *Spodoptera* muestra que la identificación molecular es independiente del estadio de vida y el polimorfismo de la especie objetivo. El analisis de la probabilidad máxima de *S. litura* y *S. exigua*, junto con *S. mauritia* revelan que existen variaciones significativas entre estas especies. En particular *S. exigua* mostró variaciones intraespecíficas con respecto a las diferentes ubicaciones geográficas. El presente estudio demuestra la utilidad del mtCOI para la identificación de *S. litura* y *S. exigua*, independientemente de su estadio de vida y también extrae conclusiones sobre las relaciones filogenéticas entre las 3 especies de plagas.

Palabras Clave: identificación; estadios del ciclo de vida; mtCOI; filogenia; gusano cortador del tabaco

The genus *Spodoptera* (Lepidoptera: Noctuidae) occurs throughout the warmer regions of the world (Mitchell 1979). The tobacco cutworm, *Spodoptera litura* (Fabricius), is a polyphagous pest of diverse vegetable and field crops; it is known to damage more than 120 species worldwide (Thomas et al. 1969; Knipling 1980). The species is native to Asia and is distributed throughout tropical and temperate regions of Asia, Australia, Africa, the Middle East, southern Europe, and the Pacific Islands. The high insecticide resistance combined to high adult dispersal and migration capacity allows *S. litura* to utilize various types of host plants including tobacco, castor, groundnut, maize, cotton and rice to a number of grain legumes and vegetable crops (Sparks 1979; Johnson 1988). In India *S. exigua* (Hübner) is a serious pest of jute and tobacco. It is commonly known as beet armyworm and one of the important crop pests in the tropics. Using diagnostic morphological keys requires microscopic examination of adult male genital structures, a tedious procedure when screening large numbers, and one that requires substantial sample preparation and undamaged specimens (Pogue 2002). Unambiguous keys are frequently not available for females or immature stages, and substantial overlap in host range and attraction to pheromone blends limit the use of behavioral criteria (Meagher et al. 2008). Therefore, finding an alternative method to supplement morphometric analyses is of practical interest for the identification of *Spodoptera* complex.

DNA barcoding has been proposed as a molecular method for assigning individual specimens to known species (Hebert et al. 2003). The barcode involves DNA sequence analysis of a portion (typically between 600-900 bp) of the mitochondrial gene cytochrome *c* oxidase subunit I (COI).Most of the molecular work was concentrated on *S. frugiperda* (Levy et al. 2002; Meagher & Gallo-Meagher 2003; Nagoshi & Meagher 2003; Prowell et al. 2004; Martinelli et al. 2006), which is a Western Hemisphere species. Recently, population genetic structure of *S. litura* from 6 Korean and 5 Chinese localities using COI and internal transcribed spacer 2 (ITS2) were studied and results revealed absence of genetic variance between Korean and Chinese populations

Network Project on Insect Biosystematics, Division of Entomology, Indian Agricultural Research Institute, New Delhi 110 012, India
Corresponding authors; E-mail: spathour@gmail.com; ashabio@gmail.com, vvrento@gmail.com
Supplementary material for this article in Florida Entomologist 98(1) (March 2015) is online at http://purl.fcla.edu/fcla/entomologist/browse

(Wan et al. 2011). Nagoshi et al. (2011) analyzed DNA sequences from a portion of the COI region from 5 *Spodoptera* spp. (*S. frugiperda*, *S. eridania* (Stoll)*, S. exigua, S. dolichos* (Fabricius) and *S. pulchella* (Herrich-Schäffer)) native to Florida along with 2 potentially invasive species, *S. littoralis* (Boisduval) and *S. litura* to show feasibility of using DNA barcodes for identifying these species. The objective of this study was to assess the applicability of DNA barcoding to *Spodoptera* species from India. The results were assessed for the likelihood of barcode gaps sufficient to discriminate the native from the foreign populations and thereby justify the expansion of the barcode databases for these and other related species. The potential role of DNA barcoding in monitoring the species at all lifecycle stages has also been exemplified.

Materials and Methods

STOCK CULTURE

For the present study, the samples *S. exigua* and *S. litura* were collected from cotton field of Indian Agricultural Research Institute, New Delhi, India during Sep 2012. The stock cultures were maintained in transparent jars with fresh cotton leafs and bolls with enough aeration. Adults were examined for accurate species identity through dissecting the genitalia and using these, pure cultures were maintained from the freshly emerged adults for only 1 generation. Samples of eggs, first to fourth larval instar, pupa, adult male and female were drawn from the F1 of culture for DNA isolation.

PCR AMPLIFICATION AND SEQUENCING

The DNAeasy Blood and Tissue Kit (Quiagen GmbH, Germany) method was used to extract DNA from 1-3 legs of each adult, and from eggs (10 eggs/egg mass), larvae and pupae. The DNA the method provided by Fukova et al. (2008) was followed. The voucher specimens of these and of those used for mtCO1 analysis are deposited with the National Pusa Collection (NPC), Division of Entomology, Indian Agricultural Research Institute, New Delhi, India. The genomic DNA was visualized using 0.8% agarose gel and quantified by fluorometer using standard procedures. Depending upon the concentration, the DNA samples were diluted with molecular gradient water to get a working solution of 10-30 ng/µL. A portion of the total DNA was preserved in glycerol (10%) in −80 °C for future reference purposes. The mtCO1 region was amplified using LCO 1480 and HCO 1298 (Folmer et al. 1994). The optimized PCR conditions (per 25 µL) using Taq DNA polymerase (Fermentas Inc., USA) were 2.5 µL of 10 X 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, 17 µL of UltraPure water (Invitrogen). Thermocycler conditions were as follows: initial denaturation for 5 min at 94 °C followed by 35 cycles of denaturing for 30 sec at 94 °C, annealing for 40 s at 54 °C and extension time of 40 s at 72 °C, with a final extension for 5 min at 72 °C. PCR products were visualized on agarose gel after electrophoresis. Single bands were purified using a QIAquick PCR purification kit (Quiagen GmbH, Germany). Purified PCR products were sequenced directly in both directions using an automated sequencer (ABI prism® *3730 XL DNA Analyzer; Applied* Biosystems, USA) at Scigenomics Lab, Cochin, India. All sequences were aligned using BioEdit 4.0 program, using ClustalW 1.8 (Thompson et al*.* 1994). The sequences were used in BLAST search to confirm the sequence identity. The alignment was further analyzed employing MUSCLE in MEGA 5.0.

SEQUENCE DIVERSITY AND PHYLOGENETIC ANALYSIS

Overall 63 sequences of *S. litura* (34)*, S. exigua* (24) and *S. mauritia* (5)*,* were used in diversity analysis. The number of haplotypes (*h*) and nucleotide (π) diversities for the populations of each *Spodoptera* sp. were estimated using the software DnaSP 5.10.01 (Librado & Rozas 2009). Sequence divergences among *S. litura* and *S. exigua* individuals were calculated using the Kimura 2-Parameter distance model (Kimura 1980) and graphically displayed in Maximum Likelihood (ML) tree by the program MEGA 5.05 (Tamura et al. 2011). Tree robustness was evaluated by bootstrapping (Felsenstein 1985) with 2,000 replicates and *S. mauritia,* obtained from NCBI GenBank was used as the outgroup.

Results

Mitochondrial cytochrome oxidase 1 sequencing yielded a 650 bp fragment from the egg, larva, pupa and adults of *S. litura* and *S. exigua*. A comparison of the triplicate sequence showed no evidence of mismatch which showed no sequencing errors. A total fragment of 650 bp of the COI was analyzed from all life stages. Evidence of nuclear copies was not found, which was supported by the absence of a stop codon within the sequence, and base composition was similar with no indels. The 8 sequences generated in the study were deposited in the NCBI GenBank (Table 1). Pairwise alignment of *S .litura* and *S. exigua* showed variation in 45 nucleotides out of 645 bp amounting to 7% (Fig. 1).

Blast search for the sequences showed the highest hits for the respective species. Multiple sequences of COI were aligned using Clustal W (BioEdit 4.0,). These results on COI sequences in addition to corroborating the ones already available in the NCBI GenBank, also provided confirmation that stage specific identification of *S. litura* and *S. exigua* is possible with the data generated in this study.

SEQUENCE DIVERSITY

The 63 sequences of *S. litura* (34)*, S. exigua* (24) and *S. mauritia* (5)used in diversity analysis led to the following: from 34 COI gene fragments of *S. litura* 7 haplotypes were identified, with a haplotype diversity (Hd) of 0.647 ± 0.054 and nucleotide diversity (π) of 0.00196 (Table 2). The haplotype frequency and nucleotide diversity within *S. litura* from different locations was very small. In *S. exigua*, among 24 COI sequences 10 haplotypes were recognized with 0.873 ± 0.044 haplotype diversity, 0.01286 nucleotide diversity and 28 total numbers of mutations indicating complex genetic variability. *Spodoptera mauritia* included 5 COI sequences, which gave 2 haplotypes with Hd value 0.400 ± 0.237 and nucleotide diversity of 0.00392.

PHYLOGENETIC ANALYSIS

The Maximum Likelihood tree (ML tree based on Kimura 3 parameter distance at 1,000 iterations) was constructed based on 63 sequences including 34 from *S. litura*, 24 from *S. exigua* and 5 from *S. mauritia* obtained from GenBank and BOLD, and from 4 sequences each of *S. litura* and of *S. exigua* from the present study using MEGA 5.0. Based on the ML tree, 3 major clades were recognized which differentiate the 3 *Spodoptera* spp. (Fig. 2). The *S. litura* sequences produced a cladogram that did not cluster with sequences from different countries, showing no clear differentiation among the populations. Further, the cladogram revealed that in *S. litura* clade there were 2 subclades, the first in which our sequences were grouped with populations from Taiwan, Japan, China, Australia; and in the second subclade, populations from India, Taiwan, Bangladesh, Thailand, Pakistan and United Kingdom were grouped. The second clade was *S. mauritia,* which included populations from Australia and Japan; and because we did not sequence *S. mauritia,* a population from India was not included. In the *S. exigua* clade, there were 2 subclades, both supported by a 100% bootstrap

Table 1. *Spodoptera* spp. populations analyzed, their countries of origin, and GenBank and BOLD accession codes.

Species	GenBank Accession	BOLD Accession	Country
S. mauritia	HQ950503	ANICK414-10	Australia, Northern Territory
	KF389305	ANICK415-10	Australia, Western Australia
	AB733409	GBMIN12113-13	Japan, Okinawa, Taramajima, Nakasuji
	AB733407	GBMIN12114-13	Japan, Okinawa, Taramajima, Nakasuji
	AB733408	GBMIN12121-13	Japan, Okinawa, Taramajima, Nakasuji
S. litura	HQ950413	ANICK311-10	Australia, Northern Territory
	HQ950414	ANICK312-10	Australia, Queensland
	HM756090	GBGL10128-12	Taiwan
	HM756091	GBGL10129-12	Taiwan
	HM756092	GBGL10130-12	Taiwan
	HM756093	GBGL10131-12	Taiwan
	AB733672	GBMIN12112-13	Japan, Okinawa, Yaeyama, Isls
	AB733671	GBMIN12120-13	Japan, Okinawa, Yaeyama, Isls
	JX156331	GBMIN22009-13	China, Guangdong
	JN087373	GBMIN22884-13	Japan, Sapporo,
	JQ064569	GBMIN30168-13	India
	JQ064567	GBMIN30169-13	India
	JQ064565	—	India
	JQ064570	GBMIN30172-13	India
	JQ064568	GBMIN30173-13	India
	JQ064566	GBMIN30174-13	India
	JQ064564	GBMIN30175-13	India
	FN908025	GBMIN38571-13	India
	FN908021	GBMIN38573-13	Pakistan
	FN907969	GBMIN38599-13	Bangladesh
	FN907967	GBMIN38600-13	Bangladesh
	FN907965	GBMIN38601-13	Thailand
	FN908022	GBMIN38622-13	India
	FN908020	GBMIN38623-13	Bangladesh
	FN907994	GBMIN38636-13	United Kingdom York,
	FN907968	GBMIN38649-13	Bangladesh
	FN907966	GBMIN38650-13	Bangladesh
	—	LEPIN003-12	India, Punjab, Bathinda
	—	LEPIN014-13	India, Punjab, Sangrur
	—	LEPIN015-13	India, Punjab, Sangrur
	—	KF939043	India, Delhi
	—	KF939044	India, Delhi
	—	KF939045	India, Delhi
	—	KF939046	India, Delhi
S.exigua	HQ950504	ANICK416-10	Australia, Western Australia
	HQ950505	ANICK417-10	Australia, Western Australia
	HQ950506	ANICK418-10	Australia, South Australia
	GU707393	FBLMV381-09	Germany, Bavaria, Niederbayern
	HM756077	GBGL10115-12	USA, Florida
	HM756078	GBGL10116-12	USA, Florida
	HM756079	GBGL10117-12	USA, Florida
	HM756080	GBGL10118-12	USA, Florida
	AB733674	GBMIN12111-13	Japan, Kagoshima, Minamisatsuma, Kinpou-cho
	AB733675	GBMIN12118-13	Japan, Kagoshima, Minamisatsuma, Kinpou-cho
	AB733673	GBMIN12119-13	Japan, Kagoshima, Minamisatsuma, Kinpou-cho
	JQ064572	GBMIN30171-13	India
	FN907975	GBMIN38596-13	United Kingdom, York
	FN907973	GBMIN38597-13	United Kingdom, York
	FN908024	GBMIN38621-13	Spain
	FN908004	GBMIN38631-13	Thailand
	FN907974	GBMIN38646-13	United Kingdom, York
	JF415658	GWORO707-09	Germany, Bavaria, Oberbayern
	HM914242	GWORZ535-10	Germany, Bavaria, Oberbayern
	—	LEPIN012-12	India, Punjab,Ludhiana
	—	KF939047	India, Delhi
	—	KF939048	India, Delhi
	—	KF939049	India, Delhi
	—	KF939050	India, Delhi

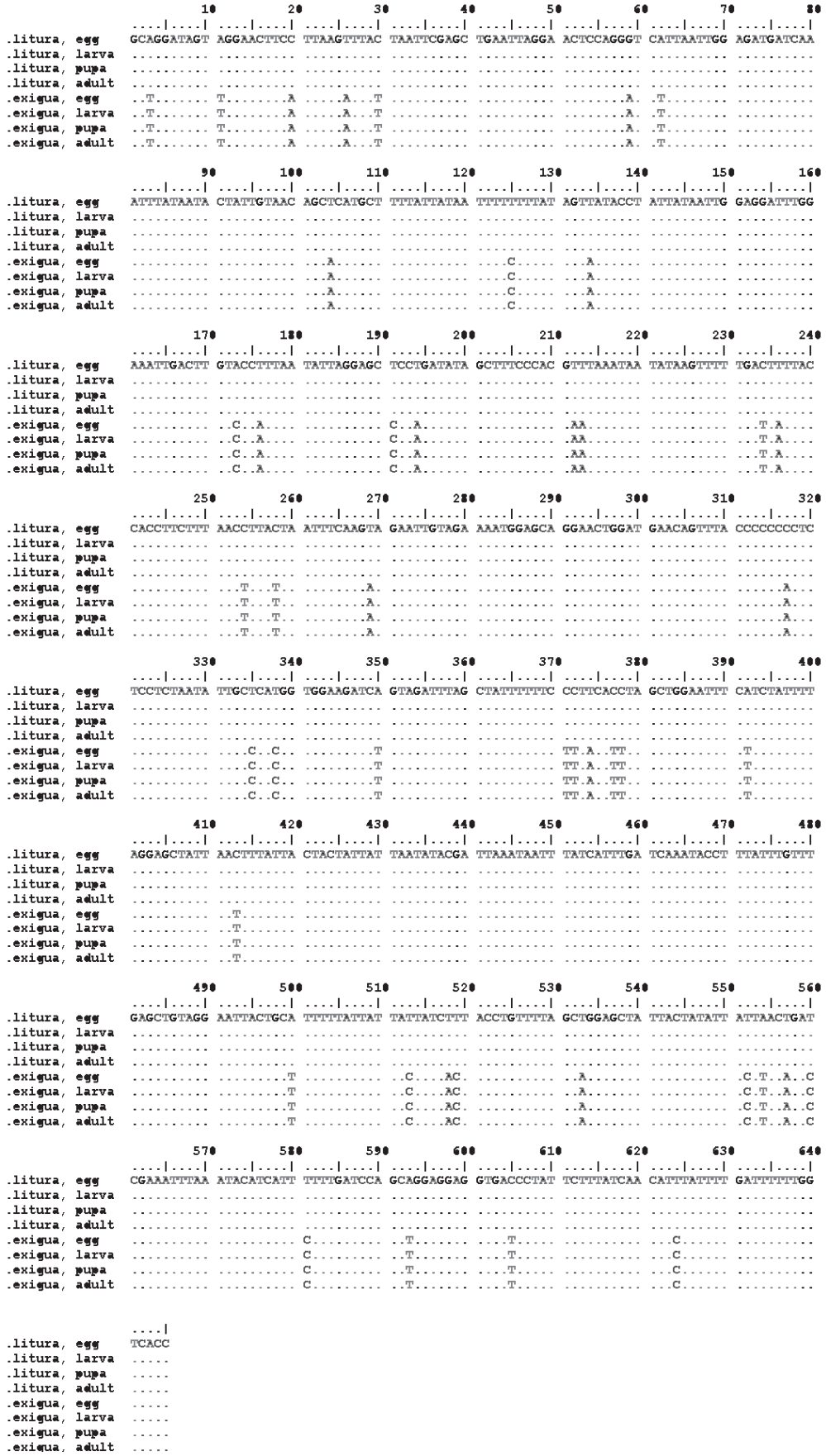

Fig. 1. MtCOI sequence comparison for *Spodoptera litura* and *S. exigua.* A color version of this graphic can be seen online in supplementary material for this article in Florida Entomologist 98(1) (March 2015) at http://purl.fcla.edu/fcla/entomologist/browse.

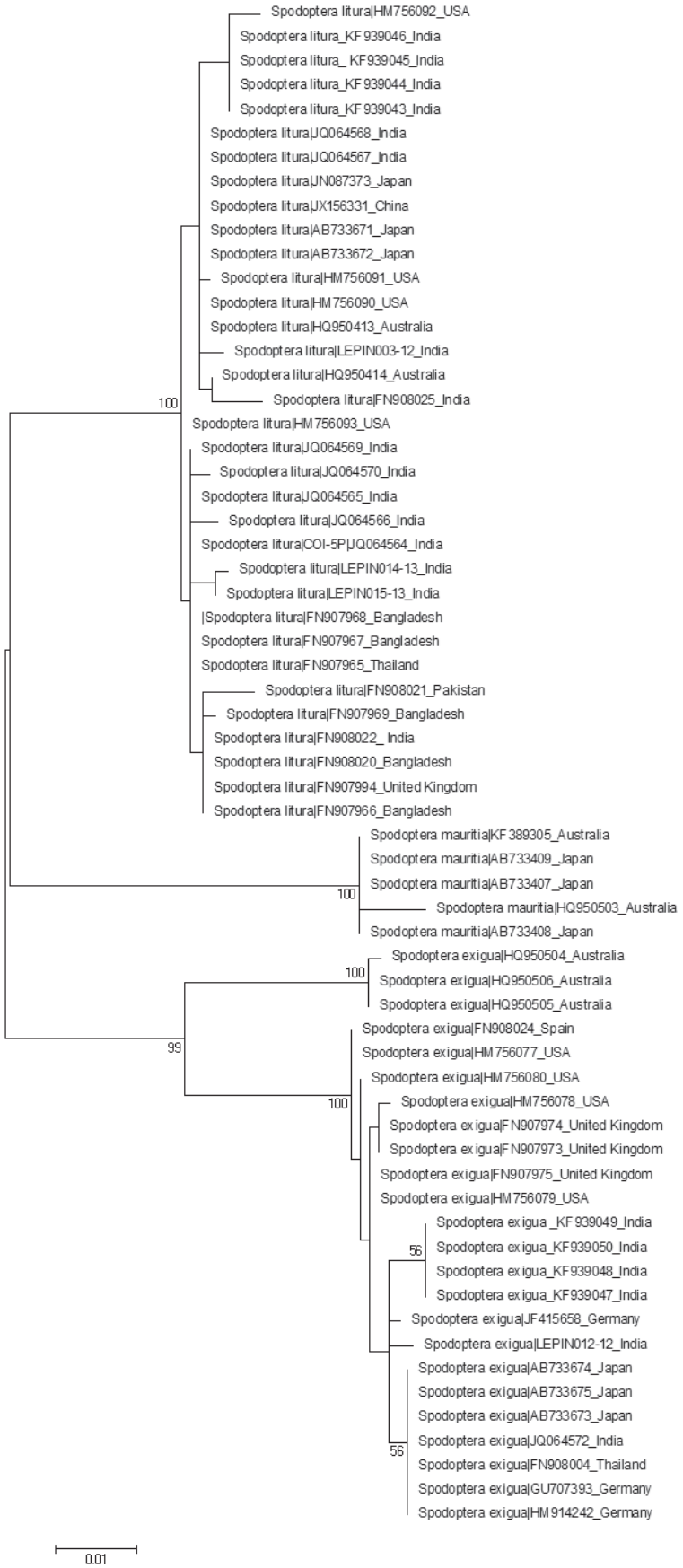

Fig. 2. Maximum likelihood tree with bootstrap support (2,000 replicates) showing clustering of *Spodoptera* spp. for mtCOI sequences. (Clade 1: *S. litura*; Clade 2: *S. mauritia*; Clade 3: *S. exigua*).

Table 2. COI gene polymorphism in *Spodoptera litura, S. exigua* and *S. mauritia.* Diversity analysis was based on a total of 63 DNA sequences of these three species.

Index*	*S. litura*	*S. exigua*	*S. mauritia*
n	34	24	5
Hn	7	10	2
Hd ± SD	0.647 ± 0.054	0.873 ± 0.044	0.400 ± 0.237
π	0.00196	0.01286	0.00392
k	1.041	6.754	2.000
S Number of segregating sites	9	27	5
Total number of mutations	9	28	5
Theta S	0.00419	0.01421	0.00474
Theta π	0.00197	0.01309	0.00394

*Indices: n, number of sequences; # of haplotypes (Hn); Hd, haplotype diversity; π, pi nucleotide diversity, k = average number of nucleotide differences (genetic distances) among haplotypes, and theta values based on the number of polymorphic sites (S) and the mean number of pairwise differences.

value. Subclade1 of *S. exigua* included individuals only from Australia. Subclade 2 included individuals collected from geographically distant locations, viz., India, Spain, USA, United Kingdom, Germany, Japan and Thailand.

Discussion

DNA barcoding makes possible the use of specimens from developmental stages where morphological keys for such species are not available or of poor quality. In addition, continued advances in molecular genetic technology will improve the efficiency and economics of barcode analysis, making the screening of even a large number of samples increasingly practical. These benefits combined with the observed applicability of barcode for species assignment in *Spodoptera* justify efforts to expand the barcoding database to become broader and more representative of relevant domestic and exotic species. The present study has confirmed that the COI barcode sequences from the immature stages are generally consistent with current adult morphological species concepts and are useful tools for species identification.

The ML tree of 3 *Spodoptera* species revealed that *S. litura* and *S. mauritia* are closer compared to *S. litura* and *S. exigua*. Further, the Australian population of *S. exigua* is entirely different from the populations used in the present study. Hence, a molecular study on the populations of these species from various hosts and geographical regions is urgently needed to elucidate the genetic relationships between them. DNA based identification in this group, especially of the immature stages, has the potential to provide a practical approach to pest control and interception activities that require timely and accurate identifications. Our study represents a needed starting point with reference to cutworms, and their molecular characterization towards a stable taxonomy for *Spodoptera* spp., and for solving intra and interspecific complexities.

Acknowledgments

Authors gratefully acknowledge the financial support received from the Indian Council of Agricultural Research (ICAR), New Delhi through the XIth Plan Network Project on Insect Biosystematics (NPIB). Our sincere thanks are due to Dr. Suresh Nebapure, Scientist, Division of Entomology, Indian Agricultural Research, New Delhi, India for providing the cultures of the *Spodoptera* spp.

References Cited

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791.

Folmer OM, Black M, Hoeh BW, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit 1 from diverse metazoan vertebrates. Molecular Marine Biology and Biotechnology 3: 294-299.

Fuková I, Neven LG, Bárcenas NM, Gund NA, Dalíková M, Marec F. 2009. Rapid assessment of the sex of codling moth *Cydia pomonella* (Linnaeus) (Lepidoptera: Totricidae) eggs to larvae. Journal of Applied Entomology 133: 249-261.

Hebert PDN, Cywinska A, Ball SL, Dewaard JR. 2003. Biological identifications through DNA barcodes. Philosophical Transactions of the Royal Society London B: Biological Science 270: 313-321.

Johnson SJ. 1988. Migration and life history strategy of fall armyworm, *Spodoptera frugiperda*, in the Western Hemisphere. Insect Science and Its Application 8: 543-549.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16: 111-120.

Knipling EF. 1980. Regional management of the fall armyworm: a realistic approach? Florida Entomologist 63: 468-480.

Levy HC, Garcia-Muruniak A, Muruniak JE. 2002. Strain identification of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) insects and cell line: PCR-RFLP of cytochrome oxidase C subunit I gene. Florida Entomologist 85: 186-190.

Lirado P, Rozas J. 2009. DnaSP V5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451-1452.

Martinelli S, Barata RM, Zuch MI, Silva-Filho MC, Omoto C. 2006. Molecular variability of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations associated to maize and cotton crops in Brazil. Journal of Economic Entomology 99: 519-526.

Meagher RL, Brambila J, Hung E. 2008. Monitoring for exotic *Spodoptera* Species (Lepidoptera: Noctuidae) in Florida. Florida Entomologist 91: 517-522.

Meagher RL, Gallo-Meagher M. 2003. Identifying host strains of fall armyworm (Lepidoptera: Noctuidae) in Florida using mitochondrial markers. Florida Entomologist 86: 450-455.

Mitchell ER. 1979. Migration by *S. exigua* and *S. frugiperda*, North American style (Lepidoptera: Noctuidae). Journal of the Australian Entomological Society 18: 363-372.

Nagoshi RN, Brambila J, Meagher RL. 2011. Use of DNA barcodes to identify invasive armyworm *Spodoptera* species in Florida. Journal of Insect Science 11:154.

Nagoshi RN, Meagher RL. 2003. FR tandem-repeat sequence in fall armyworm (Lepidoptera: Noctuidae) host strains. Annals of the Entomological Society of America 96: 329-335.

Pogue MG. 2002. A world revision of the genus *Spodoptera* Guenée (Lepidoptera: Noctuidae). Memoirs of the American Entomological Society 43: 1-202.

Prowell DP, McMichael M, Silvain JF. 2004. Multilocus genetic analysis of host use, introgression, and speciation in host strains of fall armyworm (Lepidoptera: Noctuidae). Annals of the Entomological Society of America 97: 1034-1044.

Sparks AN. 1979. A review of the biology of the fall armyworm. Florida Entomologist 62: 82-87.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28: 2731-2739.

Thomas MJ, Jacob A, Nair MRGK. 1969. Host-biology relations of *Spodoptera litura* (F.) (Lepidoptera: Noctuidae). Indian Journal of Agricultural Science 39: 400-402.

Thompson JD, Higgins DG, Gibson TI. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673-4680.

Wan X, Li J, Kim MJ, Park HC, Kim SS, Kim I. 2011. DNA Sequence variation of the tobacco cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae), determined by mitochondrial A+ T-rich region and nuclear ITS2 sequences. Biochemical Genetics 49: 760-787.