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INFESTATION, GENETIC VARIATION ANALYSIS AND BIOLOGY STUDY OF BIOCONTROL AGENT *ISTURGIA DISPUTARIA* (GUENEES) ON *ACACIA NILOTICA* IN PAKISTAN

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ABSTRACT

In Pakistan, Trees of *Acacia nilotica* are extremely valuable sources of fuel, small timber and have important pharmaceutical and medicinal value. But on the other hands, in Australia, *Acacia nilotica* subsp. *indica* is the most serious weed of national significance because of its potential of spread, invasiveness, economic and environmental impacts. Different strategies including leaf feeding insects as biological control agents are being used against such wild plant species. Surveys for leaf feeding insects on *Acacia nilotica* during 2018-2019 revealed the enhanced infestation (15%-38%) with the presence of a potential biological control agent, *Isturgia disputaria* in various districts of Punjab, Pakistan. For proper molecular identification, DNA was extracted from collected samples and polymerase chain reaction (PCR) was performed to amplify a 710bp fragment of the mitochondrial COI gene. The amplified PCR products were sequenced and phylogenetic examination and genetic evolutionary divergence (GD) showed that studied species of *I. disputaria* exhibited 99-100% homology (NCBI Acc. No MK301226) with other submitted sequences of *I. disputaria (*KX861182.1, KF147289.1) on NCBI GenBank database. Further, feeding and development potential of this species in no-choice tests on foliage of *A. nilotica spp.* indicated a good larval feeding and developmental capability for prickly acacia, *A. nilotica indica* and *A. tomentosa* indicating future threat for acacia forest in Pakistan. The larvae of *I. disputaria* completed their development life cycle 80-100% more successfully on *A. nilotica* subsp. *indica and A. nilotica tomentosa* than the larval development tested on other related species of Acacia existed in Pakistan. This is the first report of infestation, identification and biological features of potential biological control agent *I. disputaria* in Pakistan. This insect can also be reared to control wild species but, its increasing infestation on *A. nilotica* can be problematic for billion tree Project initiated by Government of Pakistan

Keywords: *Acacia nilotica, Isturgia disputaria*, biological control, DNA barcoding, invasive species, PCR
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INTRODUCTION

Acacia nilotica subsp. *indica*, (Benth.) also called prickly acacia *(Vachellia nilotica*), is a vigorous multipurpose plant that occurs naturally and cultivated throughout the country (Balu *et al*., 2014; Kaur *et al*., 2005). Genetic studies have revealed that the invasive prickly acacia population found in Australia is native to India and Pakistan (Wardill *et al*., 2005; Brenan, 1983; Hannan-Jones, 1999). *A. nilotica* is broadly utilized in field of agroforestry, such as firewood, fuel, food, fodder, tannin, gum, and furniture, pharmaceutical and has medicinal value (Puri *et al*., 1994; Pandey *et al.,* 1999; Pandey and Sharma, 2003). Prickly acacia, *Acacia nilotica* subsp. *indica* is a most serious weed of National significance in Australia because of its potential of spread, invasiveness, economic and environmental impacts (Thorp and Lynch, 2000). Mechanical and herbicide treatments are not economical in spite of their availability in Australia (Jeffrey, 1995; Spies and March, 2004). Classical biological control program is considered a low-cost and permanent alternative solution for the long-term sustainable control of this weed. In early 1980s, biological control of *A. nilotica* was initiated in Australia when surveys to find out effective bio control agent were conducted on *A. nilotica ssp. indica* in Pakistan (Mohyuddin 1981, 1986). Then, similar surveys were also conducted on *A. nilotica ssp. Subalata,* and *A. nilotica ssp. leiocarpa* in Kenya (Marohasy, 1992, 1995) and *A. nilotica ssp. kraussiana* in South Africa (Stals, 1997). Some insects belonging to Coleopteran and Lepidoptera orders such as *Bruchidius sahlbergi* Schilsky (Coleoptera: Chrysomelidae), and *Cuphodes profluens* (Meyrick) (Lepidoptera: Gracillariidae) are considered as potential bio control agents against *A. nilotica*, (Mohyuddin 1986). The Geometrid moth, *Isturgia*

disputaria is also used as abiological control agent on unfocused plant species (Balu *et al*., 2014) and is acknowledged as extremely host-specific on *Acacia* (Catania, 2010).

The morphological identification is difficult because it requires sample preparation for observation under microscopic using male genital structures of adults (Pogue, 2002). Further, for females or immature stages of many insects' pests, unambiguous keys are not frequently available for correct identification (Meagher *et al*. 2008). Therefore, molecular technique have been developed for the purpose of barcoding and bio-identification of organisms and employed to investigate genetic discrepancy in numerous species of arthropods from individual specimens (Hebert *et al*., 2003) using cytochrome oxidase I (COI) based primer pairs (Brower, 1994; Nagoshi & Meagher, 2003; Hebert *et al.,* 2003; Perera *et al*., 2015; Ashfaq *et al*., 2017; Manzoor *et al*.. 2018; Ahmad *et al*., 2018, 2020c). Molecular detection of *I. disputaria* is reported only from Kenya (Miller *et al*., 2014). The morphological recognition of *I. disputaria* from Malta was done by Dr. Hausmann (Catania, 2010). The identification and differentiation of potential bio control agent against weeds worldwide is of utmost importance. Molecular identification and characterization of several important insect pests have been conducted from Pakistan (Ahmad *et al.,* 2018ab, 2019, 2020ab). In previous years, no occurrence of *I. disputaria* on *A. nilotica* subsp. *indica* was reported in Pakistan (Mohyuddin, 1986). Recently, billion tree project has been initiated by government of Pakistan therefore here, for the first time, after 1980s, we conducted a survey to find out insect pest of important tree as well as potential bio control agent against wild tree species of prickly acacia in Punjab, Pakistan.

MATERIALS AND METHODS

Field surveys and percentage Infestation: Field surveys were conducted to observe the damaging level of *I. disputaria* on *A. nilotica* subsp. *Indica* in different districts of Punjab including Faisalabad, Multan, Lodhran, Bahawalpur and Rahim Yar Khan (Fig. 1). For this purpose, during April-August, twenty-forty trees were surveyed randomly at possible hot spots from each region and collection of *I. disputaria* population was made for laboratory rearing to study different parameters. Distribution range of the taxon was worked out in each visited district (Fig. 1). Twenty-Fourty *A. nilotica* trees from each district were visited, and observed for *I. disputaria* infestation. The percentage infestation (Table 1) was calculated for each region by using following formula.

Percentage infestation= No. of infested trees / total no. of trees×100

Figure 1. Map showing surveyed zones of Punjab, Pakistan where *I. disputaria* population were observed (surveyed zones).

Insect Collection and Rearing on Host plant: Collections of *I. disputaria* larvae from *A. nilotica* subsp. *indica* trees were made in Punjab during 2018-2019 and brought for rearing in the Dr. Jam Laboratory (DJL), Department of Entomology, University of Agriculture Faisalabad. After collection, 50-60 larvae were kept in clean jars of dimensions (30cm x 15cm) and reared at 25 - 30 C and 12h light/12h. Larvae were fed with fresh cut branches of *A. nilotica*, having water and covered with white muslin cloth. Rearing of another set of larvae was done on group of live and young potted plants. Pupae were collected after two weeks, and then kept on moisturized cotton placed in individual vials for adult emergence.

Table 1. *A. nilotica* subsp. *indica* trees from each district of Southern Punjab and their percentage infestation (no. of infested trees/total no. of trees×100) due to *Isturgia disputaria* during 2018 and 2019.

Location (Districts)	Coordinates	No. of inf. Trees/total number of trees	%age infestation	No. of inf. Trees/total number of trees	%age infestation
		2018		2019	
RY Khan	28.4212 N, 70.2989 E	4/20	20	9/40	23
Lodhran	29.5363 N, 71.6317 E	6/20	30	13/40	33
Bahawalpur	28.5062 N, 71.5724 E	7/20	35	15/40	38
Multan	30.0168 N, 71.4774 E	5/20	25	12/40	30
Khanewal	30.3039 N, 71.9299 E	4/20	20	6/40	15
Faisalabad	31.4187 N, 73.0791 E	3/20	15	7/40	18
Total		29/120= 24.1%	140/6= 23.3%	62/240=25.8%	157/6= 26.2%

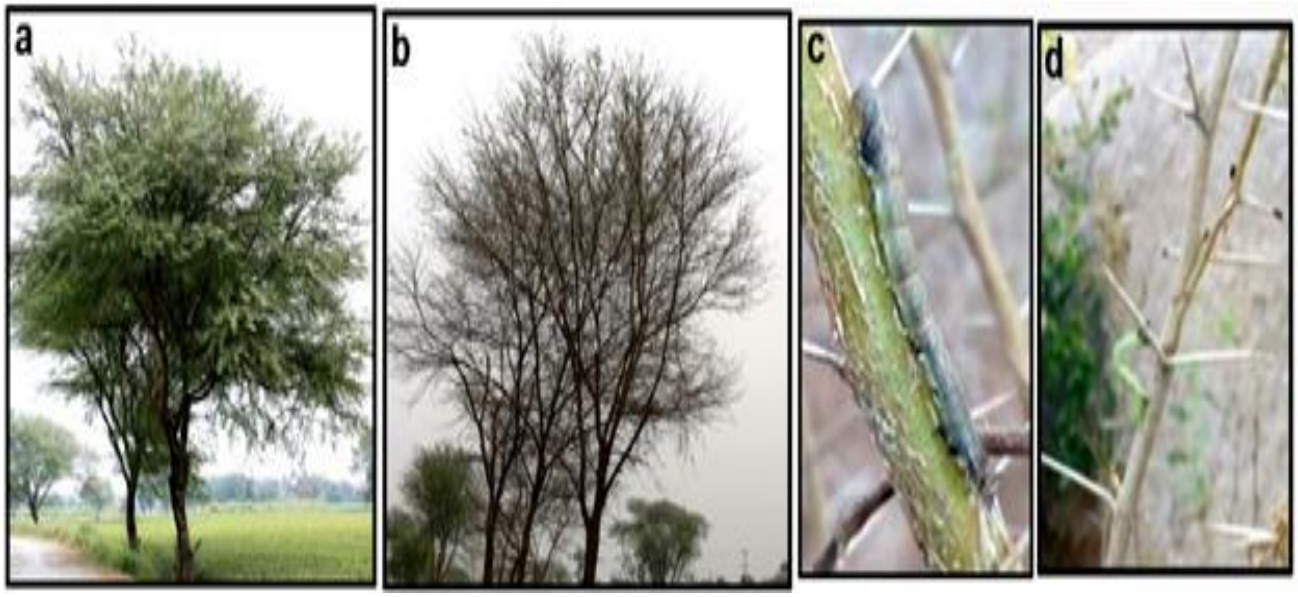

Figure 2. Picture exhibiting feeding on prickly Acacia (*A. nilotica* subsp. *Indica)* trees due to *I. disputaria*: (a) health trees,(b)infested trees, (c-d) *I. disputaria* presence on tree

Host-Specific Test: *I. disputaria* colony was established at Dr. Jam Laboratory, department of Entomology, University of Agriculture Faisalabad. Diluted honey solution was fed to the adults that emerged out of the reared colony. In insect proof netted rearing cages (60cm x 60cm x 100cm), newly emerged adults (n=10) were introduced in the jars containing cut foliage of *A. nilotica* of live plants. After four days, oviposition and fecundity were observed on cloth used for casing the culture jars and on wall of insect cages. Freshly hatched larvae from eggs were introduced on *A. nilotica* spp. insect proof caged plants. No-choice host testing of I. disputaria were conducted on four different species of Acacia (*A. nilotica indica, A. nilotica tomentsa, A. tortilis and A. catechu*). For this purpose, unfed neonates were kept on the *A. nilotica* plant grown in poly-pots (20 x 30 cm). Each time 25 unfed hungry larvae were fed on freshly cut foliage kept in glass vials holding water to retain the freshness of the leaves. Within two-three days, old foliage was replaced with fresh one. The observation on larval

survival and development, % pupation and adult emergence was examined. Further, in each experiment, newly formed pupae from the developed larvae were transferred individually to separate vials and the pupal and adult duration was also recorded. Each experiment was replicated five times keeping at 25 - 30 C and 12h light/12h dark photoperiod

Morphological Observation and Molecular Study: Careful visual examinations and protocol were observed to study different morphological features of *I. disputaria.* The features studied included size, bands pattern and colour variations of this moth at different developmental stages. The typical protocol used for wing span measurement was ruler-based as described by Van Hook *et al*., (2012). For molecular identification, extraction of DNA was carried out from *I. disputaria* insect samples (Ahmad *et al*., 2019). The legs of adults were initially crushed with the help of mortar and pestle in CTAB mixture following extraction protocol as documented by Doyle and Doyle (1990).

PCR amplification and Gel electrophoresis: Mitochondrial cytochrome oxidase I (mtCOI) based primers (LCO-1490/HCO-2198) were used for polymerase chain reaction (PCR) in PCR machine (PeqSTAR, Germany) following conditions as described by (Folmer *et al.*, 1994; Ahmad *et al*., 2020c). Simply, an initial denaturation temperature at 95 C for 5 minutes, then following 40 cycles at 94 C for 40 seconds (denaturation), 47 C for 40 seconds (annealing), and 72 C for 45 minutes (extension) and final extension at 72 C for 15 minutes were performed. The amplified PCR products were then tested for the confirmation of genomic DNA presence using gel electrophoresis on 1.5.0% agarose gel. The required size of DNA fragment from samples was estimated by comparing with DNA Ladders (Fig 3). The amplified band corresponding to the target PCR product was documented using SYNGENE Gel system under UV light.

Sequencing and Phylogenetic Analysis: The amplified PCR products were purified and sequenced directly in both directions from Macrogen (Korea). The sequences were analyzed through Lasergene v. 7.1 software package (DNASTAR, USA) and CLUSTAL W method of Bio-Edit software. The studied obtained sequences were compared with sequences available in GenBank using NCBI BLAST service. MEGA 6 software was performed for phylogenetic analysis and Homology studies employing a methodology names as "Maximum Likelihood method" (Tamura *et al*., 2013). The Evolutionary analyses were also conducted in MEGA6 (Tamura *et al*., 2013). The degree of deviation among sites was exhibited via gamma distribution (shape parameter = 5) and all those positions that comprising gaps as well as missing data were removed from the dataset (complete deletion option).

RESULTS

Field surveys and pest infestation: Field surveys from each surveyed district (Table 1) provided information about the number of infected trees due to infestation by *I. disputaria*. Maximum infestation (35%) during July-August was observed in Bahawalpur while 15% was found in Faisalabad during 2018 as compared to 38% infestation in Bahawalpur and 15% in Khanewal during 2019. . *I. disputaria* infestations observed in other districts during 2018 were 20, 25, and 30% respectively (Table 1). But during 2019, an increasing trend except Khanewal (15%) of trees infested was observed in nearly all districts, Rahim Yar Khan (23%), Lodhran (33%), Bahawalpur (38%), Multan (30%). *I. disputaria* nfestation was enhanced from 23.1% (2018) to 26.2% (2019) in all surveyed regions (Table 1)

Life History: The four Acacia trees, two hosts (*A. nilotica indica, A. nilotica tomentosa*) and two non-host (*A. tortilis and A. catechu*) were used for *I. disputaria* fitness and host specificity test. In an adult life cycle duration of 10-12 days (11.00 ± 1.00 $p<0.05$), a female moth of *I. disputaria* laid 60-70 eggs (65.40 ± 4.60) on *A. nilotica indica* and *A. tomentosa*. Fertility of the eggs was more than 90%, and the hatching of eggs took place within 3-4 days (3.20 ± 0.80 $p<0.05$). Freshly hatched neonates fed on delicate foliage, and five larval instars were observed during a period of 14-17 days (15.70 ± 1.30 $p<0.05$). The maximum larval weight 0.272g and 0.231g was observed on *A. nilotica indica* and *A. nilotica tomentosa* respectively as compared to *A. tortilis* and *A. catechu* 0.09 -0.03g (Fig. 4). The larvae feeding on the *A. nilotica indica and tomentosa* leaves resulted in complete defoliation of growing plants in the insectary. As the larvae developed to maturity, they started pupation. Pupae of a male and a female (n = 25) weighed to be 0.043-0.051g and 0.06-0.072g respectively. Maximum pupation % was also observed on *A. nilotica indica* and *tomentosa* (80-97%). The time interval for pupation lasted for 5-7 days (6.25 ± 0.75 $p<0.05$). The adult emergence from pupae was more than 85% target trees as compared to non-target (non-host). The results of no-choice host specificity test showed that the larvae of *I. disputaria* completed their development life cycle 80-100% more successfully on *A. nilotica* subsp. *indica and A. nilotica tomentosa* than the larval development tested on other related species of Acacia existed in Pakistan (Fig.4-5).

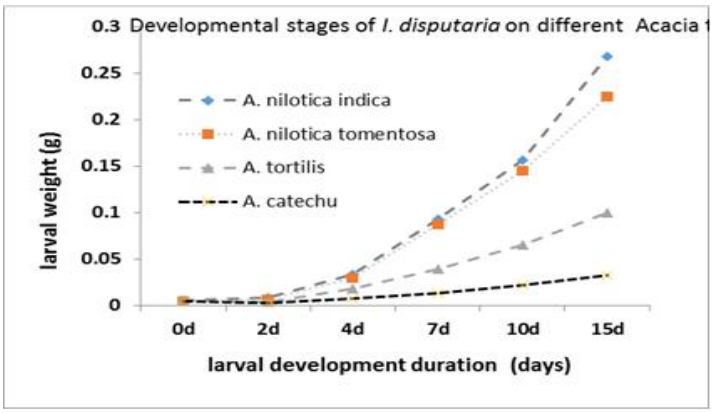

Figure 4. Survival of *I. disputaria* larvae on different target and non-target Acacia plants in no-choice host specificity tests.

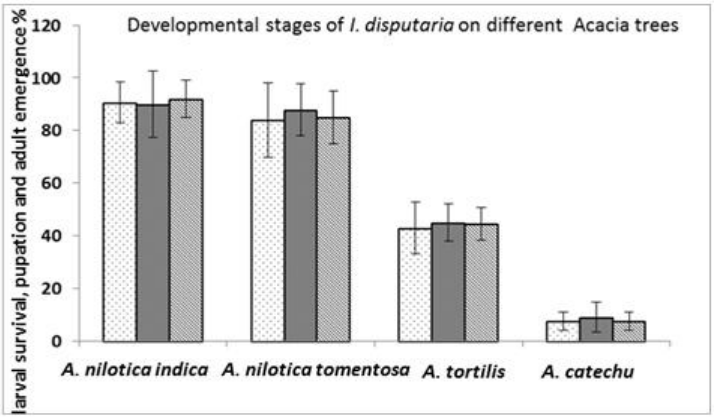

Figure 5. Performance fitness of of *I. disputaria* on different target and non-target Acacia plants in no-choice host specificity tests. White bars- (% of larval survival), dark grey bars-(Pupation %) and grey bars (Adult emergence %), (SE n=25)

Isturgia disputaria

Morphology: The wing span of newly emerged moth was about 22 mm which increased up to 29 mm at maturity. The wings were cloudy whitish in colour, and were comprised of three transverse brownish bands on the forewings. Color variation (greenish to brownish) was also observed for larvae hatched from greenish to brownish eggs laid by mature female of *I. disputaria*. Furthermore, the size of dark brown pupae was found about 7-8 mm (Fig 3).

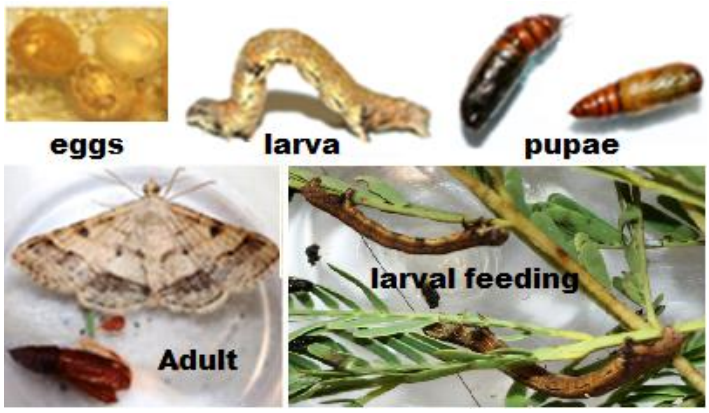

Figure 3. Different Life stages (eggs, larvae, pupae and adult) of *I. disputaria;* Insects were collected from the culture and used to study their life cycle.

PCR and Gel Electrophoresis: The primers based on COI used in PCR reaction amplified DNA fragment from all samples of *I. disputaria*. PCR reactions performed in PCR gradient machine (PeqSTAR, Germany) with a final reaction volume up to 50 µL amplified 710 bp DNA fragment. In all cases, the total DNA extracted from 4 insect samples by the CTAB method provided good results. The electrophoresis of PCR products showed well-defined (710 bp) amplified gel bands on 1.5 % agarose gel whereas negative control did not show amplification (Fig 6).

Sequencing and Phylogenetic Analysis: The DNA fragments of full amplified PCR product (710bp) were sequenced and aligned using CLUSTAL W Method and CLUSTAL OMEGA multiple alignment tool (Fig. 6). The studied *I. disputaria* DNA sequence (NCBI Acc. No MK301226) formed same clade with other reported same species (KX861465.1, KF147289.1, KX861182.1) on the phylogenetic tree constructed by ML methodology (Fig. 6) whereas different clusters were observed by different species of genus Isturgia (KX071495.1, KX072151.1, KX045431.1, KX71930.1, KX71890.1) as well as other insects of noctuid (*P. gossypiella, H. armigera*). Table 2 also showed very little evolutionary divergence (0.000-0.007) with the sequences of *I. disputaria* species (KX861465.1, KF147289.1, and KX861182.1) while when we calculated sequences divergence of studied sequences of *I. disputaria* with others isolates of closely related species (Access. nos: KX071495.1, KX072151.1, KX045431.1, KX71930.1, KX71890.1) exhibited increasing pattern of genetic divergence (0. 149) was observed.

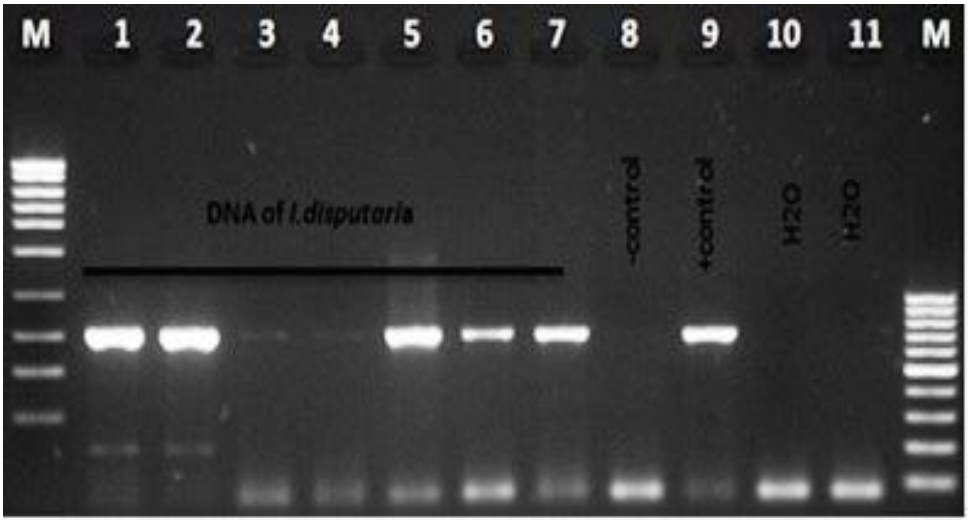

Figure 6. PCR based amplified DNA fragments (710bp) of mitochondrial Cytochrome Oxidase 1 (COI) gene using (LCO-1490/HCO-2198) primer pairs from *I. disputaria* samples in Gel electrophoresis. Wells M (Left) correspond to I kb Midranger DNA Ladders (Norgen) and M (right) 100bp DNA ladder (Thermo).

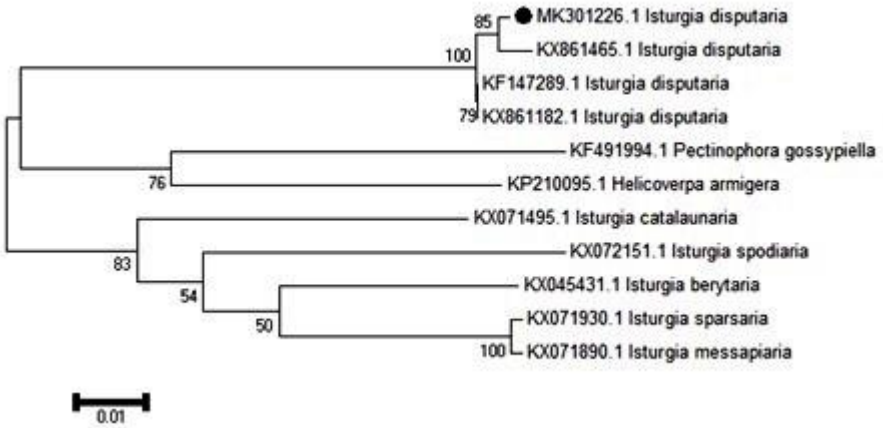

Figure 7. Molecular Phylogenetic analysis by Maximum Likelihood method constructed using NCBI Ac. No. from different countries (Pakistan: NCBI Acc. No MK301226). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura *et al.*, 2013).

Table 2. Estimates of Evolutionary Divergence between Sequences: The number of base substitutions per site from between sequences is shown. Analyses were conducted using the Tamura-Nei model. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 630 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

	1	2	3	4	5	6	7	8	9	10	11	12
1-MK301226.1												
2-KF147289.1	0.005											
3-KX861182.1	0.005	0.000										
4-KX861465.1	0.007	0.008	0.008									
5-KX071890.1	0.122	0.120	0.120	0.126								
6-KX071930.1	0.124	0.122	0.122	0.128	0.003							
7-KX045431.1	0.124	0.118	0.118	0.124	0.073	0.073						
8-KX863232.1	0.131	0.128	0.128	0.133	0.131	0.133	0.128					
9-KP210095.1	0.129	0.124	0.124	0.131	0.124	0.126	0.135	0.095				
10-KX071495.1	0.137	0.131	0.131	0.141	0.097	0.095	0.099	0.112	0.129			
11-KX072151.1	0.139	0.139	0.139	0.145	0.092	0.092	0.094	0.143	0.141	0.107		
12-KF491994.1	0.149	0.145	0.145	0.153	0.116	0.118	0.139	0.124	0.110	0.129	0.139	

DISCUSSION

Prickly acacia, *Acacia nilotica* subsp. *indica* is an invasive widespread weed of national significance in Australia (Thorp and Lynch, 2000). Several species of insects feed on *A. nilotica* in Australia, but none of them has any major impact (Palmer *et al*., 2005) because of non-availability of a potential suitable bio control agent. In the early 1980s, 71 phytophagus insect from Pakistan (Mohyuddin, 1981, 1986), 86 species from Kenya (Marohasy, 1992, 1995) and more than 400 insects from South Africa (Stals, 1997; Witt *et al.,* 2005, 2006) were reported for the control of *A. nilotica* in Australia. Thus far, six species of insects as bio control agent against *A. nilotica* have been released in Australia, but only three of them, *Bruchidius sahlbergi* and *Cuphodes profluens* from Pakistan (Wilson, 1985; Palmer, 1996) and *Chiasmia assimilis* from Kenya and South Africa (Lockett and Palmer, 2004) have been established successfully in *A. nilotica* growing regions (Mackey, 1997). In Europe, over 900 species of Geometridae have been documented, out of which, 65 species are recognized from Malta (Sammut 2000) including the species, *Chiasmia aestimaria* (Hubner, 1809), a common species *Isturgia pulinda* found breeding on *Acacia karroo* at Marfa Ridge (Catania *et al.,* 2008). *I. spodiaria* was also recorded from Rabat (Sammut *et al.,* 2008). The Geometrid moth, *I. disputaria* is also used as a biological control agent on unfocused plant species (Balu *et al.,* 2014) and is acknowledged as extremely host-specific on *Acacia* (Catania, 2010). *I. disputaria* has been considered as a potential bio-control agent of weeds including prickly acacia (*A. nilotica* spp. *indica*) (Palmer, 2004; Dhileepan *et al.,* 2013; Balu *et al.,* 2014). Likewise the seasonal incidence (34-52%) of *I. disputaria* and two other leaf feeding geometrid insects "*Ascotis infixaria* Walker" and "*Hyposidra successaria* Walker" on prickly acacia were also recorded in South India (Dhileepan *et al.,* 2006). The presence of *I. disputaria* has been reported on the Canary Islands and Malta as well as in the Afro-tropical region, and is a common pest on *Acacia caryophylla* and *Acacia karroo*.

Recent biological study of *I. disputaria* also revealed the similar host specificity pattern on *A. nilotica* subsp. *indica* (Marohasy,1992; Palmer, 2003;Dhileepan *et al.*, 2013; Balu *et al*., 2014) while morphological results including wingspan, bands appearance, and colour variations in adult and larval instars complement the results already stated according to Catania (2010) from Malta. Molecular identification of this invasive insect has only been previously conducted in Kenya (Miller *et al.,* 2014) that is in accordance with this molecular study. During previous surveys (1980-1985), *I. disputaria* was not found in Pakistan (Mohyuddin 1981, 1986) therefore it was not introduced in Australia for effective bio control program against *A. nilotica*. Now, recent survey revealed widely occurrence of this bio control agent in various regions of Punjab, Pakistan. Previously in 1980s, two insects *Bruchidius sahlbergi* Schilsky (Coleoptera: Chrysomelidae) and *Cuphodes profluens* (Meyrick) (Lepidoptera: Gracillariidae) (Mohyuddin, 1981, 1986) that causes severe damage to juveniles and adult shoots of *A. nilotica* (Mohyuddin 1986) were brought from Pakistan and released in Australia in 1982 (Mohyuddin, 1986) and became established in 80% of the Australian sites within four years (Wilson, 1985; Marohasy, 1995). Recently in Pakistan, several types of important insect pests including insect vectors and their associated

pathogens have been identified (Manzoor *et al*., 2018, 2020; Ahmad *et al*., 2020abc; Malik *et al*., 2019; Yaseen *et al*., 2020).

Because of similar climatic condition of Pakistan and Australia, good feeding and developmental potential of *I. disputaria* observed in host specificity tests indicated that, it can be effectively used to control *A. nilotica* in Australia than previously introduced insects. Moreover, the identified *I. disputaria* can also be used in the countries where *A. nilotica* is a threat and weed control program has been initiated. But its presence and infestation is quite alarming in Pakistan triggering complete defoliation of acacia seedlings and trees. There is also wide occurrence of Acacia trees in various regions of Punjab and Sindh but they are already at the brink of extinction (Abbas *et al*., 2013). This is the first report of widely occurrence and infestation of *I. disputaria* on *A. nilotica* as a pest in Pakistan. But, in Pakistan, this bio control agent may become serious pest and threat for *A. nilotica* trees. This is the first report of widely occurrence, biology and molecular identification of *I. disputaria* on *A. nilotica* in Pakistan.

Conclusion: First time, molecular identification and biology of African Moth (*I. disputaria)* was conducted using samples collected from the infested *A. nilotica* in Punjab, Pakistan. Mitochondrial Cytochrome C Oxidase subunit 1 (COI) confirmed that the sequenced segment of studied insect corresponded to *I. disputaria* species reported from other countries. This insect can also be reared to control wild species but, its increasing infestation on *A. nilotica* can be problematic for green Pakistan Project initiated by Government of Pakistan. *I. disputaria* is spreading very quickly and proper pest management measures should be taken by the forest department as soon as possible to prevent its further spread in other forest region of Pakistan.

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