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A molecular characterization of the invasive fig weevil *Aclees taiwanensis* in Italy

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Abstract

An economically important pest of *Ficus carica* L. is causing severe infestations in many fig nurseries and orchards in Italy. Belonging to the genus *Aclees* spp. (Coleoptera Curculionidae), this Asiatic species was accidentally introduced in Europe about 15 years ago, in a Tuscan nursery. Originally identified as *Aclees cribratus* Gyllenhal, it has been then reported as *Aclees* sp.cf. *foveatus* Voss and, more recently, identified as *Aclees taiwanensis* Kono. A serious damage to fig plants is caused mainly by the larvae, which drill tunnels into the wood and by adults that feed on buds, leaves and young fruits. The present survey applies molecular genetics techniques to reconstruct the genetic profile of the species. To this purpose, the partial sequence of the 18S rRNA gene and the hypervariable region ITS2 of the ribosomal cistron were used as molecular markers for specimens of *A. taiwanensis* collected in Italy and *Aclees hirayamai* Kono from Philippines. The analysis of the partial sequences of the 18S rRNA allowed the distinction of two haplotypes for each insect, except for a sample from Philippines, for which one haplotype does exist. The use of the ITS2 hypervariable region highlighted the existence of only one haplotype in the Italian accessions. Only in the sample collected in Lucca (2LU) two haplotypes were highlighted in ITS2. These results are discussed with the occurrence of *A. taiwanensis* in Italy.

Key words: Coleoptera, Curculionidae, molecular marker, haplotype, 18S rRNA, Internal Transcribed Spacer 2.

Introduction

Italian orchards and nurseries of *Ficus carica* L. have been recently suffering severe infestations and reduced crop production, due to the accidental introduction of an exotic pest belonging to the genus *Aclees* (Coleoptera Curculionidae) (EPPO 2009; Iovinella *et al*., 2020; Farina *et al*., 2021). Despite this Asian invasive beetle was originally identified as *Aclees cribratus* Gyllenhal (Ciampolini *et al*., 2005) it has been then reported as *Aclees* sp. cf. *foveatus* Voss (Benelli *et al*., 2014) and, more recently, identified as *Aclees taiwanensis* Kono (Meregalli *et al*., 2020a; 2020b). Damage to fig plants is caused by the adults feeding on buds, leaves and early-stage fruits, but overall by the xylophagous larvae, which feed in the woody parts of the tree, drilling large tunnels, so causing the complete destruction of xylem and phloem (Ciampolini *et al*., 2007). Larval feeding activity is undetectable at the beginning of the attack, so that fig trees do not show any sign of distress. Then, concurrently with the appearance of the first symptoms, the wood damage appears already irreversible resulting shortly after in the tree death. To date, no chemical nor biological control strategies have been able to reduce *A. taiwanensis* harmfulness and spreading. In addition, the species is currently not considered as a quarantine pest, and no preventive procedures in compliance with EU Regulation 2019/2072 are adopted against its passive diffusion due to plant trading for ornamental and cultivation purposes.

The use of molecular markers permits sample identification regardless of the stage of the insect biological cycle and its gender. Various types of marker techniques have been developed, e.g. the analysis of restriction fragment length polymorphisms (RFLPs), the rapid amplified polymorphic DNAs (RAPDs), the amplified fragment length polymorphisms (AFLPs), the simple sequence repeats (SSRs), and the single nucleotide polymorphisms (SNPs) (Lehmann *et al*., 1997; Kuhner *et al*., 2000; Black *et al*., 2001; Nagaraju *et al*., 2001; Brumfield *et al*., 2003; Morin *et al*., 2004)

Ribosomal DNA (rDNA) is the most widely used nuclear sequence in evolutionary analyses. Thanks to its high rate of evolution, the internal transcribed spacer (ITS) regions flanking the 18S, 5.8S and 28S regions of the rRNA gene cluster, ITS1 and ITS2, have been used in phylogenetic inference for closely related taxa (Miller *et al*., 1996) and phylogeographical and other population genetic studies (Navajas *et al*., 1998; Ji *et al*., 2003; Volkov *et al*., 2003; Long *et al*., 2004; Fritz, 2006; Mahendran *et al*., 2006; Yara, 2006; Kumar *et al*., 2018; Li *et al*., 2018; Viviani *et al*., 2019).

The conserved region rDNA 18S has been extensively used for evaluating relationships among taxa (Nyaku *et al*., 2013; Ávila-Rodríguez *et al*., 2013).

This paper aims to investigate the phylogeny of *A. taiwanensis* between the occurrence of one or more events of introduction of the weevil in Italy by comparing ITS2 sequences and conserved region rDNA 18S in specimens collected in various infested Italian locations.

Materials and methods

DNA extraction without destruction of the insect

DNA was isolated from five unsexed *A. taiwanensis* adult specimens belonging to the entomological collection of the Department of Agriculture, Food and Environment of the University of Pisa, Italy, and from two unsexed *Aclees hirayamai* Kono adult specimens from a Philippine collection (table 1).

Table 1. List of *Aclees* specimens analysed in this study. For each specimen is reported the year of collection.

Specimen	Species	Location	Year
1.RM	*A. taiwanensis*	Rome (Italy)	2012
2.LU	*A. taiwanensis*	Lucca (Italy)	2017
3.LI	*A. taiwanensis*	Isola d'Elba (Italy)	2009
4.MS	*A. taiwanensis*	Carrara (Italy)	2018
6.TV	*A. taiwanensis*	Treviso (Italy)	2018
7.SUB	*A. hirayamai*	Subic Zambales Western Luzon (Philippines)	2015
11.SIB	*A. hirayamai*	Sibagat Agusan del Sur Mindanao (Philippines)	2015

Genomic DNA was extracted from one whole single leg per specimen using the Quick-DNA Miniprep Plus Kit (Zymo Research, USA) following the manufacturer's instructions with one modification, the single leg after proteinase k treatment was recovered, washed in water, dried and reattached to the insect body. The concentration of each DNA sample was measured using a WPA biowave DNA spectrophotometer (Biochrom Ltd., Cambridge, UK), and the integrity was evaluated by agarose gel electrophoresis. The extracted DNA was stored at −20 °C.

PCR primers design and amplification

Amplification was carried out by conventional PCR in 20 µl reactions containing 1x 10X DreamTaq Buffer (Thermo Fisher Scientific, USA), 0.5 µM of each primer, 1U of DreamTaq (Thermo Fisher Scientific, USA), and 20 ng of template DNA. PCR was run in a PCR system 2700 (Applied Biosystems, USA).

The amplification of the 18S rRNA gene was performed with universal primers (Applied Biosystem / Ambion, USA).

Amplified DNAs were inserted into a pGEM-T Easy Vector System (Promega, USA), positive colonies were screened and inserted DNAs were sequenced by automated sequencing (MWG Biotech, Ebersberg, Germany) as in Viviani *et al*. (2019).

Primers specific to the variable region of 18S rDNA sequence (called LGACb and LGACa), were then designed from nucleotide sequences (GenBank accession numbers MK478005 and MK478006) using Primer3 software (https://primer3.ut.ee).

Sequences corresponding to the Internal Transcribed Spacer 2 (ITS2) of the ribosomal DNA were amplified using primers previously designed for amplification of ITS2 from DNA of *Torymus sinensis* Kamijo (Viviani *et al*., 2019). Amplified DNAs were cloned and sequenced as above.

All the primers used in this study are reported in table 2. The different PCR settings are described in table 3; all the amplifications had an initial denaturation step at 95 °C (5 minutes), and a final step at 72 °C (10 minutes).

All reactions were checked for amplification by gel electrophoresis. Amplified sequences of the haplotypes for the partial 18S rRNA gene were deposited in GenBank with the accession numbers MK478003 - MK478009. The ITS2 sequences of *Aclees* collected in Italy (1.RM, 2.LU, 3.LI, 4.MS and 6.TV) and in the Philippines (11.SIB), were deposited in GenBank with the accession numbers MK460274-MK460309.

Phylogenetic analyses

ITS2 sequences were multi-aligned using the CLUSTALW program (https://www.genome.jp/tools-bin/clustalw). Bayesian Inference was estimated using MrBayes 3.2. Two runs with 4 chains were run for 1 million generations, sampling every 200 generations. The chains were left free to sample all the models of the GTR family using

Table 2. List of specific primer sequences used in PCR.

Primer ID code	Primer sequence	Gene sequence Accession number
LGACa	F: 5'-GCTCTTTCTTGATTCGGTGGG-3' R: 5'-GAAGCCGCCTGTCCCTC-3'	MK478006
LGACb	F: 5'-CCAGGAGTGTGGTGCATGGC-3' R: 5'-GACAGTAAAAACCGCGCACG-3'	MK478005
ToITS2	F: 5'-TGTGAACTGCAGGACACATG-3' R: 5'-ATGCTTAAATTYAGCGGGTA-3'	Viviani et al. (2019)

Table 3. PCR setting conditions.

Primers	Denaturation step	Annealing step	Extension step	N° of cycles
Universal 18S rDNA	30 seconds 95 °C	30 seconds 57 °C	30 seconds 72 °C	30
LGACa	30 seconds 95 °C	30 seconds 57 °C	30 seconds 72 °C	30
LGACb	30 seconds 95 °C	30 seconds 57 °C	30 seconds 72 °C	30
ToITS2	30 seconds 95 °C	40 seconds 50 °C	40 seconds 72 °C	40

reversible jump Monte Carlo Markov Chain (MCMC) (Huelsenbeck *et al*., 2004). Heterogeneity of substitution rates among different sites was modelled with a 4 category discretized Γ distribution, with a proportion of invariable sites. The standard model (4 × 4) of DNA substitution in which there are only four states (A, C, G, T/U) was implemented. The first 25% of generations were discarded (burn-in) and convergence was evaluated with the average standard deviation of split frequencies. Goodness of mixing was assessed looking at the acceptance rate of swaps between adjacent chains, following (Ronquist *et al*., 2012).

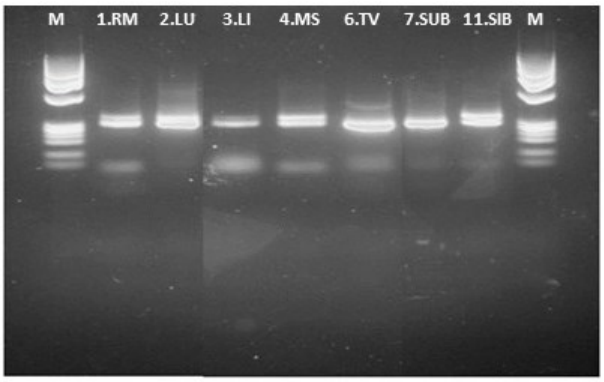

Figure 1. Electrophoretic analysis of PCR products of *Aclees* isolates with the universal primer 18S rRNA gene: 1.RM, 2.LU, 3.LI, 4.MS, 6.TV from Italy; 7.SUB, 11.SIB from Philippines. (M) Marker ΦX174 DNA-HaeIII digest.

Results

Polymorphism of 18S ribosomal RNA gene

DNAs of *Aclees* specimens collected in Italy and Philippines were amplified by PCR using the universal primer 18S rRNA gene. The presence of two different bands was observed for the samples 1.RM, 2.LU, 3.LI, 4.MS, and 11.SIB (figure 1) whereas one single band was found for samples 6.TV and 7.SUB (figure 1).

The PCR products of the DNA extracted from 2.LU were cloned and sequenced according Viviani *et al*. (2019). The alignment of the two sequences revealed two haplotypes, called A and B, for the partial 18S rDNA gene, as shown in figure 2.

Then, to rapidly analyse the insects without cloning the 18S rRNA gene, haplo-specific primers were designed in order to amplify separately the two haplotypes.

The DNA of insects from geographically distinct areas, 2.LU, 4.MS, 6.TV, 7.SUB, and 11.SIB, amplified with haplotype-specific primers, evidenced after gel electrophoresis the presence of the two haplotypes for the samples 2.LU, 4.MS, 11.SIB, only one haplotype for the sample 7.SUB and no amplification for the 6.TV sample (data not shown). Failure in the amplification of 6.TV sample is probably related to the presence of different haplotype/s. After sequencing amplification products, alignment of the sequences (supplemental material figure S1) confirmed that concerning the specific region of the 18S rDNA gene all insects showed two haplotypes except insect 7.SUB which presented only the B haplotype (supplemental material figure S1).

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2.LUA           TTAGGAATTGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACA
2.LUB           TTAGGAATTGACGGAAGGGCACCACCAGGAGTG--------------------------
                **************************** ***

2.LUA            CGGGAAACCTCACCAGGCCCGGACACCGGAAGGATTGACAGATTGAGAGCTCTTTCTTGA
2.LUB            ------------------------------------------------------------


2.LUA            TTCGGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTC
2.LUB            ------------TGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTC
                            ************************************************


2.LUA            CGATAACGAACGAGACTCTAGCCTGCTAACTAGGCGTATTTTGACATCCTAAAGGCCCGC
2.LUB            CGATAACGAACGAGACTCTAGCCTGCTAACTAGGCGTATTTTGACATCCTAAAGGCCCGC
                 ************************************************************


2.LUA            CGGCTTGGGCTTCGCCTTTAGTCGTGCGCGGTTTTTACTGTCGGCGTACAAACAATTCTT
2.LUB            CGGCTTGGGCTTCGCCTTTAGTCGTGCGCGGTTTTTACTGTCGGCGTATAAACAATTCTT
                  ************************************************ ***********


2.LUA            CTTAGAGGGACAGGCGGCTTCTAGCCGCACGAGATTGAGCAATAACAGGTCTGTGATGCC
2.LUB            CTTAGAGGGACAGGCGGCTTCTAGCCGCACGAGATTGAGCAATAACAGGTCTGTGATGCC
                 ************************************************************


2.LUA            CTTAGATGTCC
2.LUB            CTTAGATGTCC
                 ***********
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Figure 2. Alignment of partial 18S rRNA gene sequences: haplotype A (2.LUA) and haplotype B (2.LUB) of *A. taiwanensis* isolated in Lucca (2.LU), (acc. Nos. MK478006 and MK478005 respectively).

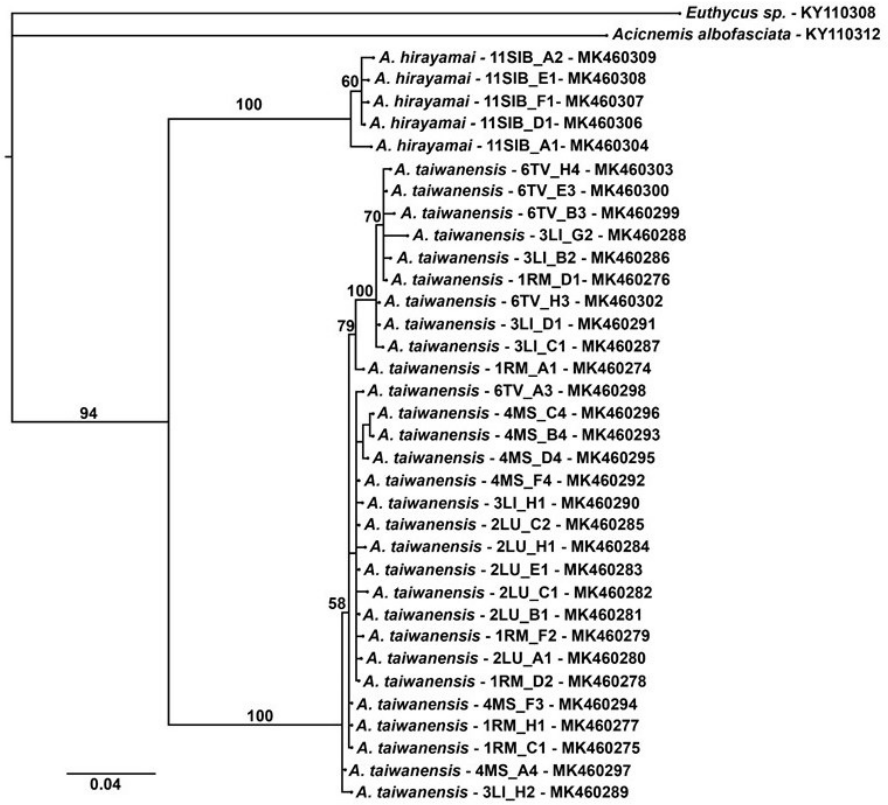

Figure 3. Molecular phylogenetic relationship among Internal Transcribed Spacer 2 (ITS2) sequences of *A. taiwanensis* and *A. hirayamai*. The organism, sample, the clone and accession number in gene bank are reported (e.g., *A. taiwanensis* - 6TV_H4_MK460303 is organisms '*Aclees taiwanensis'*, the sample '6TV', clone 'H4' and accession number in gene bank 'MK460303'. The sequences of *Acicnemis albofasciata* and *Euthycus* sp. were used as outgroups.

Polymorphism of ribosomal ITS2 sequence

The primer ToITS2 (table 2), which amplifies the internal transcribed spacer 2 of *T. sinensis* (Viviani *et al*., 2019) was successfully used to produce amplicons (471 to 474 nt long) of ITS2 in *Aclees* specimens. These DNA amplification products were cloned and sequenced. Sequence alignments (see FAS file in supplemental material) showed that all specimens, including *A. hirayamai*, are almost uniform with very few variations in the first 114 sites of the sequence, in particular only four A-G transitions, at sites 22, 41, 62 and 103, were observed. After site 114, *A. hirayamai* differs from Italian *A. taiwanensis*, because of several deletions and insertions. The central part of the sequenced ITS portion is the most variable, even among Italian specimens; in the last 55 sites, the sequences of the Italian specimens are again highly uniform, showing only an A/G transition in position 685 in 3 specimens. In the Italian specimens, 451 out of 474 sites are conserved. One specimen (4MS_F3) has an additional triplet, thus all the others have a 471 bp long sequence. The highest variation detected among the Italian samples corresponds to 15 sites out of 471, i.e., 3.18%.

Bayesian analysis of the ITS2 sequences showed that the two species of *Aclees* clustered in a separate clade with respect to the species of Molytinae used as outgroups, with a very high support (94 % post probability) (figure 3). Within the clade, they fell into two distinct groups, maximally supported. Considering the clade of the Italian specimens of *A. taiwanensis*, a few subgroups, differently supported, were determined by the small variation in the sequences, but they differ for very few sites. These subgroups were not correlated with the geographical origin of the specimens.

Discussion and conclusions

To get insights into phylogeny and biogeography of insect populations as well as their evolution, DNA markers have been widely exploited (Luque *et al*., 2002; Chatterjee and Mohandas, 2003; Mohandas *et al*., 2004; Prasad *et al*., 2004). Most importantly, they are used to assess genetic diversity, identify haplotypes and predict migration and colonization (Salvato *et al*., 2002; Llewellyn *et al*., 2003; Margonari *et al*., 2004; Bosio *et al*., 2005; Behura, 2006; Guo *et al*., 2017). Moreover, DNA markers have got great importance in studies concerning insect-plant interaction, insect-pathogen interaction and insect ecology studies as well (Caterino *et al*., 2000).

Indeed, the purpose of this work was to evaluate the genetic differences among Italian accessions of *A. taiwanensis* by using DNA molecular markers.

For such investigations, we used nuclear ribosomal DNA 18S gene, that occurs in many copies in every species and it is known to provide insights into the evolutionary history of different organisms (Nyaku *et al*., 2013; Costa *et al*., 2016; Zhang *et al*., 2017). In addition, the hypervariable ITS2 region of the ribosomal cistron

was used. ITS2 sequences evolve rapidly and are often used for intraspecific analyses of diversity of several species, including animals and plants (Gomulski *et al*., 2005; Venkatesan *et al.,* 2016; Viviani *et al*., 2019).

The presence of only two haplotypes using the conserved region of the 18S rRNA gene was highlighted in all the analysed Italian accessions. On the other hand, the use of the ITS2 hypervariable region highlighted the existence of a few genetic variations among the Italian accessions.

The variations shown by the ITS2 sequences reflect the analysis of the mitochondrial sequences (based on different specimens, not available for this study). Meregalli *et al*. (2020) also observed different haplotypes in the Italian specimens, differing by a few bp, and clustered in three clades, with a good statistical support (respectively, 79, 58 and 99% post probability). Specimens from Taiwan clustered together with the Italian specimens, but only in two of the three clades. The variation in the nuclear and mitochondrial genome of the Italian specimens indicates that they possess a good genetic variability, even with haplotypes that have not yet detected in the native country, and therefore the populations are well structured genetically.

It is plausible that, given the hypervariability of the ITS2 sequences, which evolves more rapidly than other DNA sequences (Mort *et al*., 2007; Zagoskin *et al*., 2014), and also considering that even intraindividual variations have been reported among copies of ITS2 sequences (Leo and Barker, 2002; Song *et al*., 2012), the few genetic variations observed among Italian *A. taiwanensis* specimens could be not related to different events of introduction of this insect in Italy.

As expected, our results showed that the *Aclees* specimens collected in different areas of Italy largely differ from the samples of *A. hirayamai* from Philippine, confirming that Italian accessions belong to another species, *A. taiwanensis*, as identified by Meregalli *et al*. (2020a; 2020b) according to morphological and molecular identification.

In conclusion, our results suggest that the specimens of *A. taiwanensis* collected in distinct geographically areas of Italy, belong probably to the same introduced population. Anyhow further investigations using specimens of *A. taiwanensis* coming from various Asiatic locations will take important insights into the introduction route of this pest in Italy.

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