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THE *ANOPLOPHORA CHINENSIS* (FÖRSTER) (COLEOPTERA CERAMBYCIDAE LAMIINAE) OUTBREAKS IN ITALY: A POSSIBLE GEOGRAPHICAL ORIGIN

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Strangi A., Binazzi F., Sabbatini Peverieri G., Roversi P.F. – The *Anoplophora chinensis* (Förster) (Coleoptera Cerambycidae Lamiinae) outbreaks in Italy: A possible geographical origin.

Anoplophora chinensis (Förster) (Coleoptera Cerambycidae) is a long-horned beetle native to Eastern Asia. It is a polyphagous pest responsible for severe damages to a wide range of woody plants of various sizes and ages and thus it is included in the quarantine lists of several countries of the world. Even though the current strengthening of inspection procedures has limited the spread of this species, three outbreaks have been recently reported in Italy. In this work, we identified five haplotypes in the barcode fragment of Cytochrome Oxidase I gene that characterize specimens collected in infested Italian sites. Furthermore, in order to locate the origin of the outbreaks, we reconstructed the haplotypic network between Italian populations and those reported for various geographical regions of East Asia. We found that haplotypes from Regions Lazio and Lombardy were closer to haplotypes harboured by Japanese populations of *A. chinensis* while those from Tuscany, were more closely related to the Chinese ones.

KEY WORDS: *Anoplophora chinensis*, Citrus Long-horned Beetle, quarantine pest, phylogeography.

INTRODUCTION

Anoplophora chinensis (Förster) (Coleoptera Cerambycidae) is a long-horned beetle native to Eastern Asia whose common name “Citrus Long-horned Beetle” (CLB) is due to severe damages caused in citrus orchards in Japan (ADACHI, 1988; ADACHI, 1990). However, *A. chinensis* is a polyphagous species able to attack a wide range of broadleaved taxa. Hosts include forest, urban, fruit and ornamental taxa and can be extremely variable in size and age, (e.g potted plants such as “bonsais” and shrubs, but also old trees) (LINGAFELTER and HOEBEKE, 2002; HAACK *et al.*, 2010; SABBATINI PEVERIERI and ROVERSI, 2010; VAN DER GAAG *et al.*, 2010). *A. chinensis* mostly colonizes healthy trees, and hosts can be exploited as a developing substrate for many generations. This species is particularly harmful since its woodboring larvae are able to disrupt the vascular tissue of the host causing physiological and structural weakness that eventually leads to plant death (HAACK, 2003). For these reasons, it was included in the quarantine lists of several countries of the world and it is currently subjected to severe phytosanitary regulations aimed at preventing its introduction and spread. Therefore, several eradication measures were undertaken including the complete elimination of infested trees and their root system (EU, 2012).

The routes of specimens intercepted at the entry ports or those recorded during *A. chinensis* infestations, have been tracked worldwide: results showed that they mostly start from East Asian countries such as China but also Japan and South Korea (HAACK *et al.*, 2010). In the light of the above evidences, several decisions and directives have been enacted in the UE against this pest. Particular emphasis has

been placed on controlling traded material from China due to the high number of interceptions made in various shipments from this country (EU, 2010; EU, 2012; CABI, 2016).

Even though the current strengthening of inspection procedures has limited the spread of this species, three outbreaks have been reported for Italy. The oldest one was detected in Parabiago (Lombardy, Northwest of Italy) in 2000 even if the first introduction had probably occurred 10 or 15 years before (COLOMBO and LIMONTA, 2001; HÉRARD *et al.*, 2005). Currently, the infested area covers 48.000 hectares and the most recently infested sites are Montichiari and Gussago (Eastwards from the main infested area) reported in 2007 and 2008 respectively (ERSAF, 2016). Another outbreak of *A. chinensis* was detected in 2008 in Rome (Region Lazio) but dendrochronological analysis of fully wood-enclosed adult exit holes showed that the actual year of introduction might date back to 2002 (VAN DER GAAG *et al.*, 2010; SABBATINI PEVERIERI *et al.*, 2012). Similarly, in Galciana (Tuscany, Central Italy), the infestation of *A. chinensis* was detected in 2014, but dendrochronological analysis evidenced that the first year of introduction could date back to 2009 (Strangi, personal communication).

Because of the severe implications on local and global plant trading and the high costs of eradication measures, detecting the main introduction routes of *A. chinensis* is a key factor in the prevention of further biological invasions. Therefore, identifying the origin of present and past outbreaks could improve custom regulations and inspection procedures aimed at detecting this species. In this work, we identified different regions in East Asia as potential geographical origins of the CLB outbreaks reported in Italy.

To this end, in order to compare the Italian specimens and locate a possible geographical origin of this pest, we used the COI barcode region as target of the phylogenetic reconstruction as previously done by OHBAYASHI *et al.* (2009)

MATERIAL AND METHODS

Samples of *A. chinensis* were collected in three Italian outbreak sites (Fig. I, 1). The samples from Rome (Lazio) and from Pogliano Milanese in Lombardy (the second location is close to the first CLB infestation site) were collected in 2011 (n = 15 and n = 5 specimens respectively), while the samples from Galciana (Region Tuscany) were collected in 2013 (n = 3 specimens). The samples from Rome and Pogliano Milanese were morphologically identified as *A. chinensis malasiaca*, while those from Galciana were identified as *A. chinensis chinensis*. All samples (adults and larvae) were stored in 95% ethanol and placed at +4°C until DNA extraction.

Tissue samples and body parts (third leg in adults or VIII-X abdominal segments of larvae) were dissected from each specimen under sterile conditions, frozen in liquid nitrogen and pulverized with Precellys 24 (Precellys). Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN, city) as per the manufacturer's instructions. Amplification and sequencing of barcode locus was performed as in STRANGI *et al.* (2012). Both strands of the

PCR products were sequenced at the Centro di Servizi per le Biotecnologie di Interesse Agrario Chimico ed Industriale (CIBIACI), Università degli Studi di Firenze, Italy.

We analysed 42 different sequences belonging to CLB from Japan and China retrieved from Genbank (most of them from OHBAYASHI *et al.* (2009) (Table 1) and compared them with the 23 sequences evidenced in the Italian outbreaks. The resulting sequences were aligned using ClustalOmega v1.2.1 (SIEVERS *et al.*, 2011). A DNA fragment of 453 bp long was considered for haplotypic network reconstruction. Gene genealogy was computed with PopARTv. 1.7.0 using TCS method (CLEMENT *et al.*, 2002).

RESULTS AND DISCUSSION

Since the Italian specimens from Pogliano Milanese and Rome belong to *malasiaca* form while those from Tuscany have been identified as *chinensis* form, it can be hypothesised that the Italian outbreaks have a different geographical origin. However, this assumption appears too simplistic in the light of the model proposed by OHBAYASHI *et al.* (2009) for CLB evolutionary history. In fact, this theory suggests that *A. chinensis malasiaca* originated in the Chinese mainland from *A. chinensis chinensis* and subsequently migrated into the Japanese archipelago through the Korean peninsula. This hypothesis could also explain the presence of a number of intermediate pheno-

Fig. I – 1. Italian outbreaks of *Anoplophora chinensis*. 2. Haplotype network inferred with TCS algorithm based on 5' fragment of COI gene. Haplotypes were grouped based on geographical data. In figure full dots represent the sampled haplotypes while white dots represents the unsampled ones. Perpendicular bars represent the number of differences between two linked haplotypes. 3. Geographical distribution of haplotypes in the native area of *A. chinensis*.

Table 1 – Sequence used in this work with sampling dates and geographical coordinates or bibliographical reference and their GeneBank Accession numbers.

Haplotype name	Sampling site	Sampling date	Number of specimens	Sampling coordinates	GeneBank Accession number
IT1	Italy, Tuscany, Galciana	2013	1	43.89 N 11.04 E	KX099759
IT2	Italy, Tuscany, Galciana	2013	1	43.89 N 11.04 E	KX099760
IT3	Italy, Tuscany, Galciana	2013	1	43.89 N 11.04 E	MF289533
JP4	Italy, Lazio, Rome	2011	15	41,88 N 12.50 E	KX099761
	OHBAYASHI <i>et al.</i> (2009)				AB439166 AB439164 AB439165 AB439163
JP5	Italy, Lombardy, Pogliano Milanese	2011	5	45.54 N 8.99 E	KX099762
	OHBAYASHI <i>et al.</i> (2009)				AB439168 AB439160 AB439169 AB439162
JP06	OHBAYASHI <i>et al.</i> (2009)				AB439191
JP07	OHBAYASHI <i>et al.</i> (2009)				AB439184
JP08	OHBAYASHI <i>et al.</i> (2009)				AB439174 AB439173 AB439181 AB439182
JP09	OHBAYASHI <i>et al.</i> (2009)				AB439177
JP11	OHBAYASHI <i>et al.</i> (2009)				AB439180 AB439179
JP13	OHBAYASHI <i>et al.</i> (2009)				AB439178
JP14	OHBAYASHI <i>et al.</i> (2009)				AB439175
JP17	OHBAYASHI <i>et al.</i> (2009)				AB439172
JP20	OHBAYASHI <i>et al.</i> (2009)				AB439171
JP21	OHBAYASHI <i>et al.</i> (2009)				AB439170
JP22	OHBAYASHI <i>et al.</i> (2009)				AB439169
JP28	OHBAYASHI <i>et al.</i> (2009)				AB439167
JP36	OHBAYASHI <i>et al.</i> (2009)				AB439161
JP39	OHBAYASHI <i>et al.</i> (2009)				AB439159
CH40	OHBAYASHI <i>et al.</i> (2009)				AB439158
CH41	OHBAYASHI <i>et al.</i> (2009)				AB439157
CH42	OHBAYASHI <i>et al.</i> (2009)				AB439156
CH43	Not published. An,Y., Yang,X. and Huang,X				DQ026041
CH44	Not published. An,Y., Yang,X. and Huang,X				DQ026043
CH45	Not published. An,Y., Yang,X. and Huang,X				DQ026042
CH48	NCBI Genome Project				NC_029230

types located in some geographical areas (LINGAFELTER and HOEBEKE, 2002). Likewise, either the coexistence in the same geographical region of phenotypic variation in elytral maculation and pronotal hairs or the existence in southern China of transitional forms of both morphological characters, (LINGAFELTER and HOEBEKE, 2002) might be explained on the basis of the same model. On the other hand, this hypothesis suggests that morphological characters alone could be insufficient to detect a different origin of CLB outbreaks and that both phenotypic characters and molecular data are necessary.

The sequence analysis of 23 Italian samples led to the identification of 5 unique haplotypes: IT1, IT2 and IT3 occurred in Galciana (Region Tuscany) (GenBank

Accessions: KX099759, KX099760, MF289533), JP4 in Rome (Region Lazio) (GenBank Accession: KX099761) and JP5 in Pogliano Milanese (Region Lombardy) (GenBank Accession: KX099762).

The alignment of 65 sequences belonging to a region located in the 5' end of Cytochrome Oxidase Complex I mitochondrial gene (COX1) of *A. chinensis* [mean nucleotide frequencies were 31.60% (A), 36.94% (T), 16.37% (C), and 15.08% (G)] resulted in a matrix of 43 segregating sites from which 20 were parsimony-informative. Using Tamura-Nei distance method, the average divergence over all sequences was found 0.010 ± 0.003 , while the average divergences within Chinese and Japanese sequences were 0.012 ± 0.004 and 0.06 ± 0.002 respectively. However,

the low number of specimens found in two of the three Italian outbreaks, does not allow an accurate estimation of haplotypic frequencies in all Italian sites.

In the TCS network, Japanese CLB haplotypes could be separated from Chinese ones with a clear demarcation line; this rule is respected in the entire network except for the CH44 haplotype (Fig. I, 2). In Fig. I, 3 we correlated known sampling coordinates with TCS haplotypic network groups in a geographical map of the region. It is worth noting that the haplotypic network approximately traces the geographical boundaries of the Asian continent and Japanese islands; in particular the lower part of the network is located along the borders of the Eastern China Sea, the central Korean peninsula and Kyushu island (Japan), while the upper part in Shikoku, Honshu and Hokkaido islands (Japan).

While IT1, IT2 and IT3 haplotypes were found only in the Italian CLB outbreaks, JP4 was reported in the Japanese archipelago in Hokkaido, Tokyo and Tokushima prefectures. JP5 haplotype, instead, was found in a more restricted area of Japan, in the Ehime, Aichi, Tokyo, Nagano and Mie prefectures. As regards the haplotypes occurring in Tuscany but not found in the native area, IT1 and IT2 were located in a region characterized by Chinese CLB haplotypes while IT3 was closer to CH44. This haplotype can be considered an exception in the network because, even though the geographical coordinates of the site were not provided, its sample was collected in China. Moreover, the number of substitutions separating Group A (Japan) haplotypes from both CH44 and IT03 (5 nucleotides) is the same that separates group B (Japan/Korea) from Group C (China). Therefore, we can hypothesize that different CLB populations characterized by a certain subset of haplotypes may live in the Chinese mainland where IT03 could come from. Alternatively this scenario might be the result of inadequate sampling in the northern Chinese coast (located in front of the main island of the Japanese archipelago).

Considering the results of the haplotypic network, it can be hypothesized that CLB has been introduced at least twice into Italy: the first time from Japan causing the outbreaks in Rome and Pogliano Milanese, and the second time from China causing the Galciana outbreak. The hypothesis of a Japanese origin of Rome and Pogliano Milanese outbreaks raises the question of whether a relationship exists between the Italian outbreaks (*i.e.* secondary spreading or recolonization).

Considering that the haplotypes found in Pogliano Milanese and Rome differed for only 1 synonymous substitution (A396T) and that no obvious geographical correlation was found between Japanese specimens closest to the Italian ones, further data are required to clarify this issue. However, the topic is still debated because no findings have been reported in Rome since 2013 (SFR LAZIO, 2013) and in Tuscany since 2014 when the outbreak was identified and the first prevention measures were applied. All specimens collected in these locations and stored for molecular investigations were analysed in the present work. Results suggest that a possible solution could be an extensive CLB barcoding program in the Pogliano Milanese infested area, searching for the presence of rare haplotypes that could be closer to those found in the Rome outbreak rather than to those from the other Japanese isolates.

Moreover, the occurrence of outbreaks in some of the neighbouring countries of Italy should be deeply investigated to determine whether they are related to the Italian ones. Particular attention should be placed on cases

occurred in France and in Croatia that were first reported in 2003 and 2007. In fact, it is worth noting that French and Croatian outbreaks occurred in a limited geographical area involved in extra-UE trades (bonsai and potted plants) and were quickly eradicated in 2004 and 2011 respectively (EPPO, 2017). Therefore, we suggest that the main CLB entry pathway in the UE might be associated with extra-UE trading activities while the European phytosanitary regulations might have limited its spread within the UE states. However, further data and information are required to confirm this hypothesis.

In particular, we think that a deeper phylogeographic reconstruction of Chinese CLB populations could be useful to understand the origin of different European outbreaks.

ACKNOWLEDGMENTS

This research was supported by a grant from the Italian Ministry of Agriculture, Food and Forestry national project "ASPROPI - Tools and Strategies for Plant Protection" (DM n.28635, 29682/12/19). We are grateful to the Regional Phytosanitary Services of Tuscany, Lombardy and Lazio for collection of specimens and assistance in the field.

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180 - Blank Page