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TITOLO TESI

MANAGING INVASIVE POPULATIONS OF *ANOPLOPHORA CHINENSIS* AND *A. GLABRIPENNIS* IN LOMBARDY REGION

Presentata da: **MATTEO MASPERO**

Coordinatore Dottorato
Ch.mo Prof.
Giovanni Dinelli

Relatore
Ch.mo Prof.
Mario Colombo

Correlatore
Dr Franck Hérard

Esame finale anno 2015

To *Franco Ezio Pallavicini*

President of Fondazione Minoprio

from 1999 to 2013

INTRODUCTION

The transport of alien arthropods associated with rapidly expanding global trade has led to an ever increasing list of quarantine pests establishing beyond their native range. A significant number of taxa have become serious forest pests, and some are directly threatening the viability of native tree species across their introduced ranges (Haack RA., 2006; Langor DW. *et al.*, 2009; Roques A. *et al.*, 2009; Humble L., 2010).

In recent years, the Asian longhorned beetle (ALB), *Anoplophora glabripennis* (Motschulsky) (Coleoptera, Cerambycidae) was unintentionally introduced in North America, and ALB and the citrus longhorned beetle (CLB), *Anoplophora chinensis* (Forster) were unintentionally introduced in Europe. Potentially, both pests are among the most destructive wood borers of many deciduous trees (Hajek AE, *et al.* 2004; Langor DW. *et al.*, 2009; Hérard F., *et al.* 2006). Both species are originating from Eastern Asia, mainly from China, where they cause serious damage to many broadleaf trees, specifically in the genera *Acer*, *Populus*, *Ulmus* and *Salix* (Sun *et al.*, 1990). The form *malasiaca* of *A. chinensis* is widely distributed in Japan and Korea. It is a major pest of Citrus in Japan (Lingafelter S. W. and E. R. Hoebeke, 2002; Adachi, 1994). CLB is usually introduced within living trees such as bonsais and maple rootstock, while ALB is introduced in wood packaging material.

Both pests are extremely polyphagous and currently considered as harmful quarantine organisms under the European Union (EU) legislation. In the initial EU Plant Health Council Directive 2000/29/EC the policy did not emphasize the quarantine status of these organisms, nor the measures to take against a defined list of their host plants. The council directive was later modified, and ALB and CLB have been added in the specific Annex I, Part A, Section 1 of Council Directive 2000/29/EC. According to Article 3(1) and (4) of Council Directive 2000/29/EC their introduction and spread within all Member States is banned regardless of the route and associated plant or commodity. EU member states must notify the European Commission about the presence of the pests in their territories when an outbreak is detected, and they have to take all the necessary measures to eradicate the populations and/or inhibit their spread to other plants in the surrounding environments. Every host plants, including plants for planting, plant products and other commodities listed in Part B of Annex V of Council Directive 2000/29/EC which are imported from a third country must be meticulously inspected by responsible official bodies to ensure they are not contaminated by the harmful organisms listed in Part A of Annex I of Council Directive 2000/29/EC. In 2008, as a consequence of a number of CLB interceptions in third countries, emergency measures came into effect to further prevent the introduction and spread of the pest in the EU. These emergency measures have been updated by the Commission Implementing Decision 2012/138/EU which focused on key issues where concrete and main risks have been identified within the EU. It was complementary to the general legal obligations against CLB as a regulated organism. The decision outlined specific requirements for internal movements and imports from third countries of a number of high-risk plants for planting with a stem thickness bigger than 1cm, and introduced more specific requirements for imports from China. All Member States are now required to carry out annual surveys for the presence of *A. chinensis*, the results of which are discussed in the appropriate regulatory committee with Member States representatives. If an outbreak is detected, an infested zone is demarcated and surrounded by a buffer zone. Infested plants and host plants within a radius of 100 m need to be felled and intensive surveys and monitoring within the demarcated area are carried out [Ref. Ares (2013) 3302050].

Several outbreaks of the pests were found in Europe, with breeding populations of *A. chinensis* in Croatia, France, Italy and Netherlands (Colombo M., Limonta L. 2001; Hérard F., *et al.* 2006; Maspero M., *et al.* 2005; van der Gaag D.J., *et al.* 2008; van der Gaag D, *et al.* 2010) and breeding populations of *A. glabripennis* in Austria, France, Germany, Switzerland, Italy, Netherlands and UK; some have been eradicated (Hérad F. *et al.* 2006; Benker U. and Boegel C., 2006; Benker U. and Boegel C., 2008; Cocquempot C. and Hérard F. 2009; Hoyer-Tomiczek U. *et al.*, 2006; Krehan H. 2003; Korean H. 2008; Maspero M. *et al.*, 2007; Schroeder T, *et al.*, 2006; Tomiczek C. and Hoyer-Tomiczek U. 2007; Dixon J. 2013). In Europe and North America the pests are controlled through physical methods: infested trees are felled and chipped or burnt, movements of trees and firewood are prohibited, and not necessarily infested susceptible trees are sometimes destroyed preventively. In the U.S.A., the systemic insecticide imidacloprid was often used by injection into the tree trunks, or into the soil at the base of trunks. The pesticide is effective against ALB adults feeding on small twigs, against ovipositing females and young larvae (Haack R.A. *et al.* 2010; EPPO, PM 9/15 (1), 2013; New Pest Response Guidelines, 2008; Hu J, *et al.*, 2009), but it is not effective at targeting older larvae or pupae in the sapwood. However, the use of imidacloprid was not approved in UK, Italy and the Netherlands, and was not considered during any eradication efforts against *Anoplophora* spp. in the EU (van der Gaag DJ, *et al.* 2008). Consequently, the identification of biological control methods that could target ALB and CLB would therefore be useful additional management tools (Brabbs *et al.* 2014).

This research report is the last one tied to the research projects financed by the Lombardy Region as a following to the outbreaks of CLB and ALB discovered in northern Italy in 2000 and 2007, respectively (Colombo & Limonta, 2001; Maspero M. *et al.* 2007). The pests were considered as very serious threats to the urban and natural forests, and the nursery tree production as well. The Plant Protection Service of the Lombardy Region implemented eradication measures to contain the pests and get a neat reduction of their geographical distribution areas. As quarantine pests, CLB and ALB were, and still are subject to eradication measures. In the Lombardy region, eradication efforts consisted of removing about 30.000 trees under the supervision of the Plant Protection Service. Since 2004, year of implementation of the contingency plan till now, 20 millions Euros have been spent by the local authorities for tree removal, stump grinding, replantation, survey and monitoring, public awareness campaigns, and scientific research.
In conjunction with the eradication program, biological control studies were initiated in order to find, to identify, and to evaluate the insect parasitoids that could successfully control *A. glabripennis* and *A. chinensis* (Hérard *et al.*,2005). During 2005 through 2008, surveys were made in Lombardy, within and outside the area infested with *A. chinensis*, to find possible new associations between the introduced *Anoplophora* species and natural enemies from the European fauna. Eight local larval parasitoid species belonging to 5 families of Hymenoptera were found attacking both invasive cerambycids. In the major CLB infestation around Parabiago, Italy a Eulophid (Hymenoptera) was also discovered as a gregarious egg parasitoid of *A. chinensis*. It was considered as a new species for science and was described and named *Aprostocetus anoplophorae* by Delvare et al. (2004). The egg parasitoid proved to be strictly specific to *A*. *chinensis*. It is thought that it was introduced as diapausing larvae within some host eggs hidden under the bark of potted living bonsais, or trees for planting, which were supposedly imported from Japan. In order to achieve a better management of the CLB egg parasitoid, specific studies were made in the laboratory to know how to optimize parasitoid rearing, and in the field to determine the geographical distribution of the parasitoid within the current infestation of

the host. The studies commenced in 2009 and 2010. Thanks to the specificity of *Aprostocetus* for CLB, and its high potential as a biological control agent of the pest, it was considered very beneficial to introduce it in the various areas of the CLB infestation where it was still absent. Field experiments to finalize a suitable release technique of the egg parasitoid were planned.

More research studies, still financed by the Lombardy Region, were developed from 2011 till 2014 for their potential positive impact on the suppression of the CLB and ALB populations. They aimed to (1) improve laboratory rearing methods for the potential biological control agent, *A. anoplophorae* and finalize techniques to release and establish the egg parasitoid in sections of the CLB infestations where it was absent; (2) test the efficacy of the sentinel tree technique as a tool for an early detection of the pest; and (3) use baited traps and artificial lures to capture adults of ALB and possibly CLB, during their peaks of emergence and flight in the infested areas, and possibly in other environments where their occurrence was not yet known.

TAXONOMY OF *ANOPLOPHORA* SPP.

Anoplophora chinensis and *A. glabripennis* are members of the recently revised genus *Anoplophora* Hope (Coleoptera, Cerambycidae, Lamiinae, Lamiini) that now consists of 36 species of wood boring beetles that occur throughout Asia (Lingafelter and Hoebeke, 2002). Most species have beautiful colors on the elytra, pronotum, antennae, tarsi and venter. Adults usually have very long antennae (about 1.3-1.6 the body length in males, 1.0-1.5 times in females) and most are large, some over 50 mm. In the larval stage, species in this genus develop in and consume wood of many species of trees. In their revision of the genus *Anoplophora*, Lingafelter and Hoebeke (2002) made 20 synonyms, among which *Anoplophora nobilis* (Ganglbauer) is a new synonym of *A. glabripennis* and *Anoplophora malasiaca* (Thomson) is a new synonym of *A. chinensis*.

Of the 36 species, the biology, habits, and host plants are known for only one-third. The majority of the published papers concern the economically important species: *A. chinensis* including form *malasiaca*, *A. macularia* and *A. glabripennis* including form *nobilis*.

DESCRIPTION OF THE LIFE STAGES OF *ANOPLOPHORA* SPP.

The life stages of *A. chinensis* and *A. glabripennis* are roughly similar in appearance (Fig. 1). **Adults** of both species are glossy black with 10–20 distinct whitish irregular-shaped patches on the elytra, although in rare instances the number of patches ranges from 0 to over 60 (Lingafelter S.W. and Hoebeke E.R. 2002). Patch color is usually white and sometimes yellowish or beige (Lingafelter S.W. and Hoebeke E.R. 2002). Body length usually ranges between 17 and 40 mm (Lieu KOV, 1945; Lingafelter S.W. and Hoebeke E.R. 2002). The most obvious morphological character distinguishing ALB and CLB adults is the presence in CLB of 20–40 small tubercles / granules on the basal quarter of each elytron, while that part of the elytra is smooth in ALB (Fig. 1). Antennae are composed of 11 segments, with a banding pattern in which the basal portion of each segment (antennomere) is pale blue or white and the distal portion is black (Fig. 1). The ratio of antennal length to body length in ALB is about 2 for males and less than 1.5 for females (Hajek AE. *et al.* 2006). In CLB males, antennae are also much longer than body length, and in CLB females either equal or slightly longer than body length.

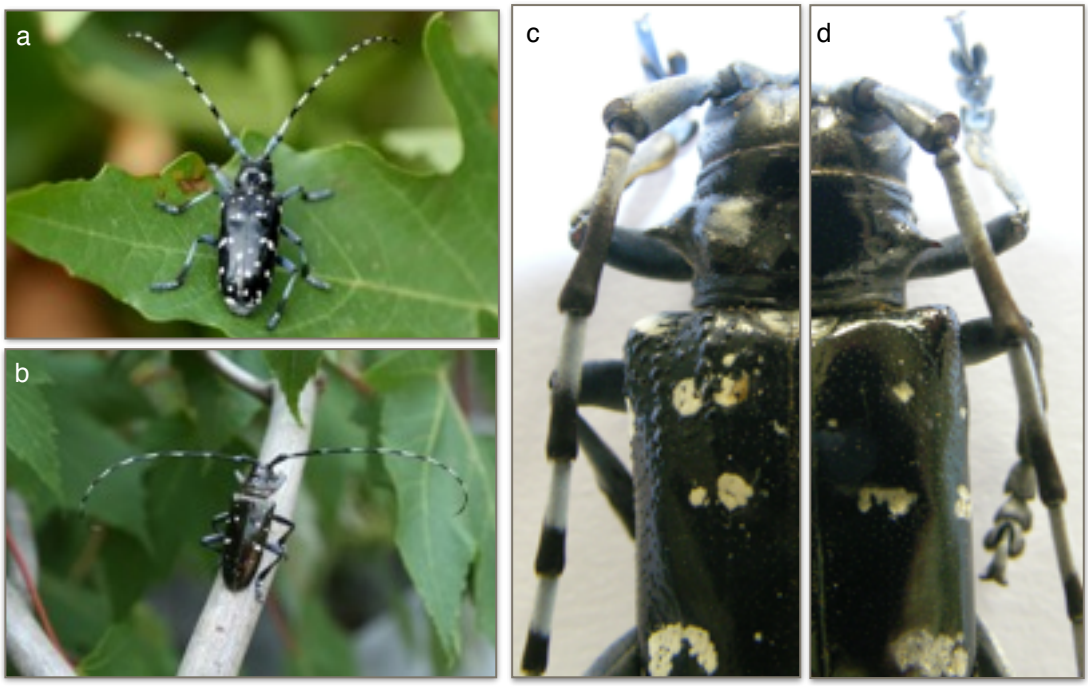


Figure 1
(a) Adult female of *A chinensis* (CLB).
(b) Adult male of *A. glabripennis* ALB).
(c) Grainy surface of the basal portion of the elytra of CLB.
(d) Smooth surface of the basal portion of the elytra of ALB

Eggs of both species are oblong, 5-7 mm long (Figs 2 - 3). At time of deposition, the egg color is initially whitish but during incubation, it can turn to ivory, beige, grey or brown depending on color of the surrounding bark tissues. In CLB and ALB, egg deposition starts with preparation of an incision through the bark, chewed with mandibles. In CLB, the incision is cut in an arc (about 1 cm width) oriented perpendicularly to body axis. Next, the female turns round and insert its protruded ovipositor in the middle of the slit to inject an egg, which is directed to the center of the arc. The egg being injected under a very thin layer of bark tissues near the surface, a median crevice generally opens under the pressure of the ovipositor, giving an inverted T-shape to the slit (the rule in most CLB oviposition incisions). In ALB, the female chews a round small area (1-1.3 cm in diameter) on surface of bark and continues chewing deeper, making a funnel-shaped pit to the cambium layer. This is the rule in most ALB oviposition incisions. The ALB egg is injected under bark, between phloem and xylem. However, in very thin barks some oviposition incisions of ALB show a CLB-pattern. In ALB and CLB, immediately after egg deposition, the hole left by insertion of the ovipositor is plugged with a secretion from the abdomen of the female. Consequently, the eggs are generally hidden in bark, invisible from outside, and we might think they are reasonably well protected against external abiotic factors and natural enemies. If this is mostly true in ALB eggs, in contrast the CLB eggs are more exposed and more vulnerable. The half-open median crevice of the T-shaped slits of CLB is often an easy pathway to egg parasitization. In fact, it is used by a specific gregarious Eulophid to insert its ovipositor to the host egg. In CLB-infested logs, the eggs can be reached by peeling the outer half layer of bark in small pieces around the egg slits. In ALB-infested logs, the eggs can be reached more easily by separating / peeling the bark from the wood. Very often, the plant tissues around the ALB eggs are necrotized and retracted, making a chamber around the egg.

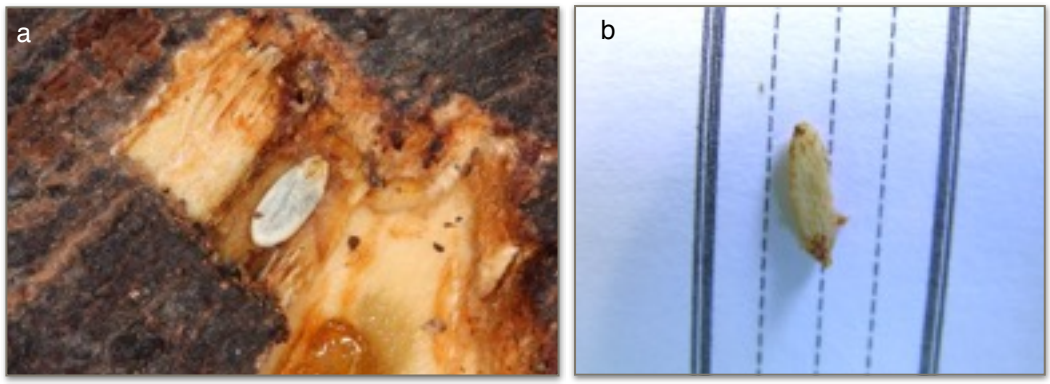


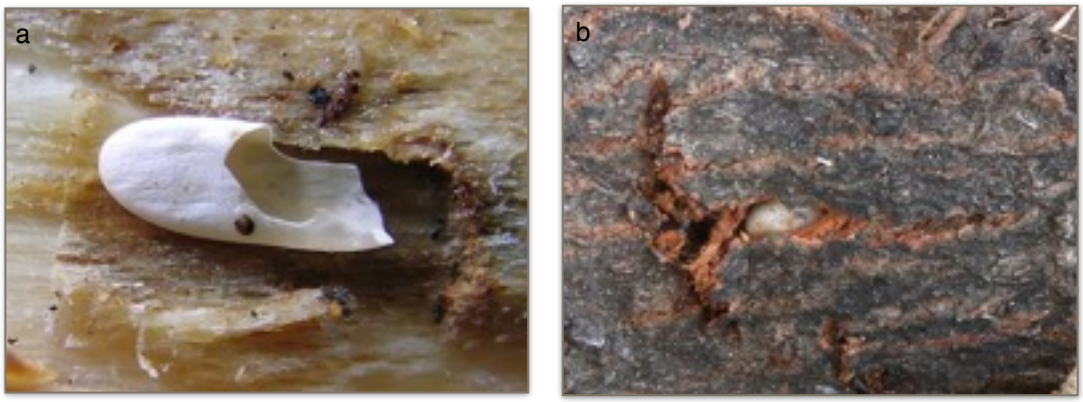
Figure 2
(a) CLB egg within the bark tissue.
(b) ALB egg removed from the bark.

The eggs of CLB are mainly laid at the base of trees around the collar, or on the trunk a few centimeters above ground, or on apparent roots. Very exceptionally, eggs of CLB are deposited higher on trunks. They are laid singly in separate incisions, which can be fairly close from each other. The eggs of ALB are usually laid in tree crowns, on branches 7 cm or more in diameter, on upper part of trunks, and sometimes on the whole trunk when the pest is in high density, or on small trees with too small diameter branches.

In CLB and ALB, at time of egg deposition the chorion is soft and elastic but it hardens considerably during incubation. In the temperatures of late June-early July in northern Italy, development of the embryo takes 10-15 days in CLB, and 15-21 days in ALB. Duration of incubation depends on local temperatures following egg deposition.

Figure 3

(a) Corion of a CLB egg.
(b) Typical CLB T-shaped oviposition slit on *Acer saccharinum.*



(c) CLB T-shaped oviposition slits on a superficial root of *Alnus glutinosa.*
(d) several CLB eggs on a superficial root of *A. saccahrinum.*

(e) debarked collar zone of a maple showing several CLB eggs.
(f) CLB T-shaped oviposition slits on a superficial root of *A. saccharinum*.

(g) CLB female laying eggs on a superficial root of *A. saccharinum.*
(h) Typical ALB oviposition pit.

Larvae of *Anoplophora* spp. are elongated, cylindrical, fleshy, shiny and cream-coloured. The head is prognathous and retracted into the prothorax that is larger than the meso- and methatorax and abdomen. The head has dark brown mouthparts and two black mandibles (Figure 4). The first segment of the thorax (pronotum) is the largest and has a brown sclerotized plate on dorsal side (Fig. 4). Spiracles are visible on the mesothorax and on the abdominal segment VIII. The abdomen has 10 segments (Pennacchio et al, 2012, Ric J. et al, 2007).

Figure 4
(a) Pronotal plate of a CLB larva
(b) Pronotal plate of an ALB larva

ALB and CLB larvae develop in 6 to 11 or more instars. Like in most insects and plants, the duration of each developmental stage of *Anoplophora* spp. is governed by the time necessary to accumulate degree-days above the specific temperature threshold for that stage and reach a sum of degree-days that permits molting to the next stage. Temperature thresholds, and sum of degree-days necessary for each developmental stage were determined in CLB by Adachi (1994), and in ALB by Keena (2006), and Keena & Moore (2010).

In the temperatures of late June - early July of northern Italy, the first two instars of ALB or CLB feed in the rotting cambial region for about 15 days, and the third instar feeds in fresh tissues while boring a gallery partly in inner bark, partly in sapwood for about 11 days (Fig. 5). The early fourth instar of CLB moves deeper, mainly in sapwood. The CLB larva progresses downward to a main root, and can even bore its gallery to a smaller root, sometimes as thin as 13-15 mm in diameter. Frass resulting form the larval feeding activity (a mixture of wood fibers, sawdust, and excrements) is accumulated in the gallery or compacted behind the progressing larva. When the gallery trajectory runs along bark of a root, the larva often open aeration holes that are also used to extrude surplus of frass outside. Substantial piles of frass can be seen on ground. They are evidence of infestation of the tree above.

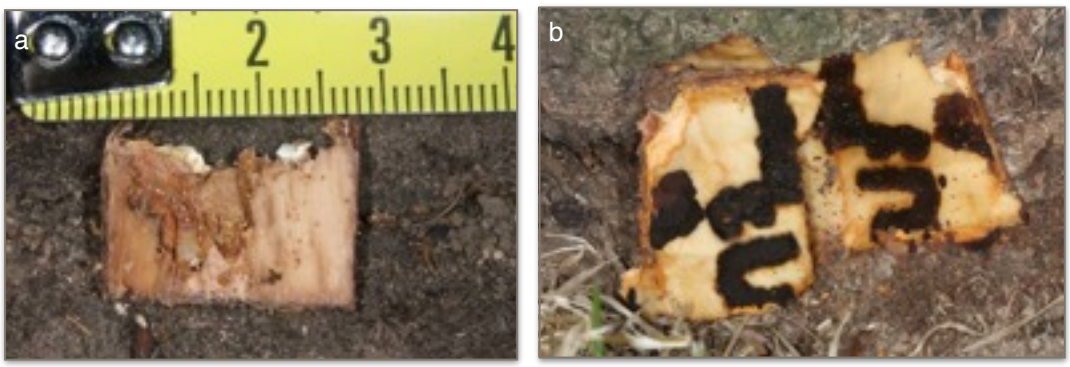


Figure 5
(a) and (b) Young larva feeding in cambial region

When the full-grown CLB larva (Fig. 6) is almost ready to pupate, it turns around at the end of the gallery and goes back upward, either increasing the diameter of its initial gallery or boring a separate new branch of gallery to the stratum around ground level. A site suitable to pupation is generally found in a root visible on ground, or in the lower section of the trunk. The full-grown larva enlarges noticeably the end of its gallery to make a pupation chamber in the outer layers of sapwood, and starts boring an exit tunnel to the inner bark to facilitate emergence of the future adult (Fig. 6). The larva finally covers the inner wall of the pupation chamber with a thick layer of wood fibers; then pupation occurs. The same behavior concerns the ALB full-grown larvae and pupae but pupation occurs in a branch or in the trunk of the host tree and never in a root.

Section area of the gallery increases with size of the larva. The young larva (L1 – L3) is 7 - 20 mm in length and the mature larva can reach 60 mm in length (Fig. 6) (Lingafelter & Hoebeke, 2002; Ric *et al*, 2007). Trajectories of CLB galleries in host plants with multiple attacks are often entangled and gallery length difficult to measure. In large host trees it appears that gallery length can be 5 – 7 dozens centimeters in length. However, CLB is also known to attack small bonsais where they develop successfully. Size of the adults emerging from those bonsais is usually normal. Thus it seems that in some instances CLB larvae are able to show a particularly high efficiency of conversion of digested material to body matter.

Many cerambycid larvae have similar anatomies, and in the same species some variability of certain characters may occur so that a strict identification of the species based on larval anatomy is often problematic. Biomolecular techniques are much preferred as they give a highly reliable identification of the larvae. However, among a very limited number of native and invasive cerambycids, it is possible to define keys of anatomic characters that permit to distinguish those particular species with a reasonable percentage of certainty (Pennacchio et al, 2012; Lingafelter & Hoebeke, 2002).

Although ALB initially attacks the crown of healthy trees in laying eggs in branches with a diameter above 7 cm, it also accepts susceptible stressed trees. It can attack other portions of the tree starting with the upper part of the trunk, to finally colonize the whole trunk during successive years of attack. This is aggravated by the specific behavior of the females, which tend to lay eggs on the tree they emerged from, or on the trees next to it. As ALB larvae bore long tunnels, generally upward, in the inner bark, sapwood, and heartwood, they heavily damage the living tissues that carry water and the organic nutrients to all parts of the plant, and they also affect tree sturdiness as well. This leads quickly to death of branches, and after a few years of repeated infestations, to death of the trees. During the past two decades, in China it happened that planks cut from ALB-infested trees were used to make wood packaging material with a view to export goods to western countries (Fig. 7). As those planks were not properly treated, the pest was transported and unintentionally introduced in the destination countries in North America and Europe. In the introduction areas, ALB larval stages finished developing in the wood packaging material that was often stored in piles outside, and not systematically destroyed. Newly emerged adults attacked susceptible host trees near their site of emergence, creating breeding populations that remained undetected during several years. Thus the pest established and started spreading to local environments, causing damage to the ecosystems, affecting biodiversity, threatening street trees, parks, urban and natural forests, and tree nurseries. When the infestations were detected, and the rules to control the pest determined, substantial and very costly eradication efforts were implemented.

Figure 6

(a) and (b) CLB full-grown larva

(c) numerous CLB larvae collected from an infested maple.
(d) CLB full-grown larva and section of its pupal chamber in a small *Carpinus betulus*.

(e) CLB full-grown larva in the roots of a potted maple *Acer palmatum*.
(f) CLB full-grown larva in a small root of a maple tree.

(g) bonsais of *Malus* spp., with CLB exit hole.
(h) Base of an *Acer pseudoplatanus* showing a CLB larva beneath the bark at the collar zone level.

Figure 7

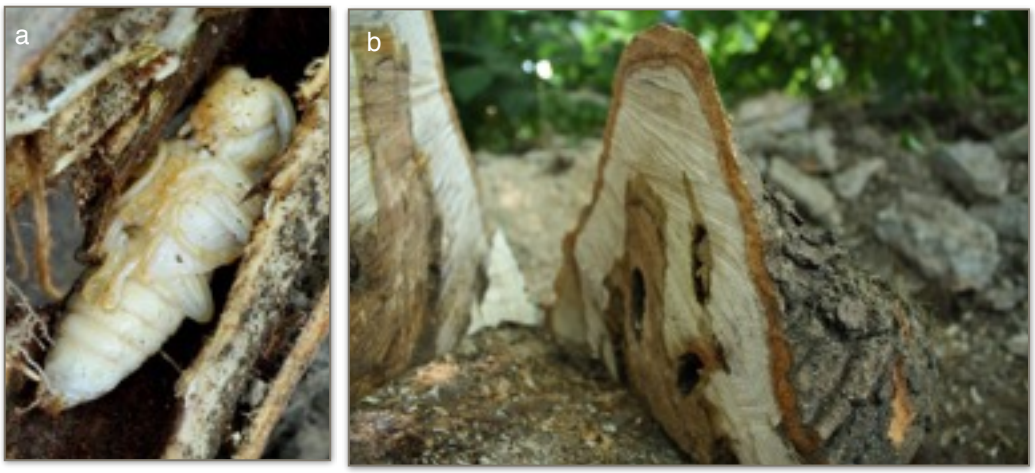
(a) several exit holes due to ALB emergence in the crown of an infested *Acer pseudoplatanus*. (b) several exit holes due to CLB emergence at the base of an infested *Acer saccharinum*.

(c) CLB full-grown larva in the pupal chamber. (d) Chinese company producing WPM (Wood Packaging Material)

Pupae of ALB and CLB are ivory-white with locally pale yellow thickening of the cuticle. Pupae are exarate, showing legs and wings free from the body and a moveable abdomen (Fig. 8). Pupal stage lasts 2 -3 weeks depending on local temperatures. Changes in color of the integument are visible at the end of the pupal stage. This starts with some tanning of the mouthparts and legs, which darken quickly and finally show the colors of the pre-emerging adult.

Figure 8

(a) CLB pupa in a superficial root of an *Acer pseudoplatanus* (b) section of the base of an *Acer saccharinum* showing a CLB pupa into the pupal chamber.



Pre-emerging adults of ALB and CLB have the colors of a normal adult but they stay immobile in the pupal chamber while their cuticle is still soft. Cuticle hardening lasts three or four days. Then, the pre-emerging adult starts moving to finish boring with its mandibles the exit hole that the full-grown larva earlier commenced before pupating. By circular movements of the head, the ALB or CLB adult cuts a perfectly round hole through the outer layer of bark and drags itself out of the gallery. The exit hole is about 10 - 15 mm in diameter, corresponding to the exact width of the elytral shoulders of the emerged adult.

Immediately after emerging at the base of a tree, CLB adults crawl upward on tree trunks to suckers (new shoots growing from the trunks). ALB adults emerged from a trunk or from a branch do the same. They find on suckers their preferred food, the tender bark of new shoots. They can also feed on petioles, midribs and veins of leaves, but rarely on blades. They can move upper into the canopy and feed on bark of new shoots at the distal end of the branches. After emergence, ALB or CLB females usually need to feed for 5-7 days before generating mature eggs. Pairing of adults and mating usually occur while adults get to the feeding sites. A contact pheromone on surface of cuticle of the female triggers in males attempts for mating. The females need several (at least 10) copulations from the same or several males to fill up her spermatheca and subsequently deposit a high percentage of fertile eggs (Keena, 2002). The male often stays in physical contact above a female without mating while she feeds on bark: this behavior has been named bodyguarding.

Figure 9

(a) CLB adult moving up into the crown for maturation feeding.
(b) couple of CLB adults while mating.

(d) CLB female laying eggs on a superficial root of *Acer saccharinum.*
(e) CLB first install larva visible after peeling the bark tissue of a superficial root.

(f) CLB full-grown larva into the pupal chamber in a *Salix caprea*.
(g) CLB emerging beetle.

Life cycle: From egg to adulthood, both species develop in 1 or 2 years, depending on the local climatic conditions, the date of egg deposition, and the number of larval instars of a particular individual (Figure 10). In Northern Italy, it seems that most *Anoplophora* individuals develop in two years.

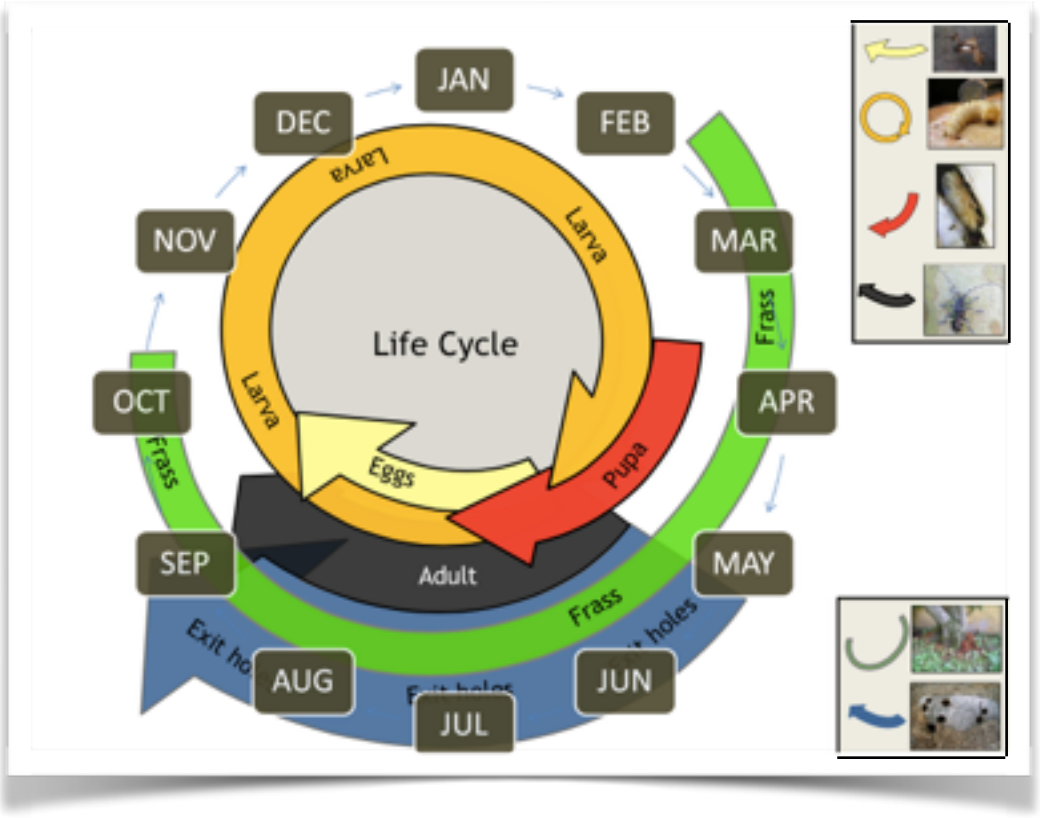


Figure 10
Presence of visible signs of infestation due to CLB and ALB throughout the year.

Development cycles for ALB and CLB in climate of Lombardy region can be summarized as follows:

Option 1. Larvae coming from eggs deposited in June from females emerged in late May, spend enough time in summer temperatures till end of September to develop most larval stages before winter of year n. Those individuals spend winter as full-grown larvae, and pupation can occur in spring of the following year (n+1). Thus, adults emerge in early summer after a one-year cycle.

Option 2. Larvae coming from eggs deposited in mid-summer (late July - early August) do not spend enough time in summer temperatures of the Lombardy region to accomplish most of their larval development before winter of year n. Larval development is resumed in spring and pupation occurs in late summer of the following year (n+1). Adults emerge in September of year n+1. They are late individuals with a still one-year cycle.

Option 3. Individuals coming from eggs deposited in late summer (September), hatched in October, and developing only one or two instars before winter of year n, need the full summer of year n+1 to complete their larval development. They spend a second winter (of year n+1) either as full-grown larvae, or as pupae, and the adults emerge in early summer of year n+2, after a two-year cycle.

HOST PLANTS

ALB and CLB are highly polyphagous (Fig. 11); dozens of tree species from several families (at least 15 families for ALB and 36 for CLB) have been reported as hosts in Asia, Europe, and North America (Hérard F. *et al.* 2006; Lingafelter S. W. and E. R. Hoebeke, 2002; Sawyer A. 2008). However, complete development has not been confirmed on all tree species listed as hosts (Smith M. T. *et al.* 2009).

In its native range, ALB infests trees primarily in the genera *Acer* (Sapindaceae), *Populus* (Salicaceae), *Salix* (Salicaceae), and *Ulmus* (Ulmaceae) (Haack R. A., 2006; Lingafelter S. W. and E. R. Hoebeke, 2002; Wang Z. G., 2004; Williams D. W. *et al.* 2004). Several other genera have been reported as occasional hosts in Asia (CABI 2007; Lingafelter S. W. and E. R. Hoebeke, 2002; Smith M. T. *et al.* 2009; Wang B. *et al.* 2005; Wang Z. G., 2004). In the United States, ALB has completed development on species of *Acer*, *Aesculus* (Sapindaceae), *Albizia* (Fabaceae), *Betula* (Betulaceae), *Cercidiphyllum* (Cercidiphyllaceae), *Fraxinus* (Oleaceae), *Platanus* (Platanaceae), *Populus*, *Salix*, *Sorbus* (Rosaceae), and *Ulmus* (Haack R. A. *et al.* 2006, Sawyer A. 2008). *Acer* was the most commonly infested tree genus in the United States, followed by *Ulmus* and *Salix*. In Canada, complete development has been confirmed only on *Acer*, *Betula*, *Populus*, and *Salix*, although oviposition has occurred on other tree genera. *Acer* was the most commonly infested tree genus in Canada (Turgeon J.J. *et al*. 2007). In Europe, complete development has been recorded on *Acer, Aesculus, Alnus* (Betulaceae), *Betula, Carpinus* (Betulaceae), *Fagus* (Fagaceae), *Fraxinus, Platanus* (Platanaceae), *Populus, Prunus* (Rosaceae), *Salix,* and *Sorbus* (Hérad F. *et al.* 2006; Hérard F. *et al.* 2009). The top five host genera infested in Europe, in decreasing order, were *Acer*, *Betula*, *Salix*, *Aesculus*, and *Populus* (Hérad F. *et al.* 2006). Not all *Populus* species are equally susceptible to ALB attack. For example, in China, *Populus* species in sections *Aigeiros* and *Tacamahaca* are generally more susceptible to ALB than species in section *Leuce* (Wang Z. G., 2004, Yin W. and Lu W. 2005). Whether susceptibility differs greatly in other tree genera has not been reported.

In Asia, CLB has a much broader host range than ALB and may even include conifers in the genera *Cryptomeria* (Cupressaceae) and *Pinus* (Pinaceae) (CABI 2007; Lingafelter S. W. and E. R. Hoebeke, 2002). In Europe, CLB has completed development on species of *Acer, Aesculus, Alnus, Betula, Carpinus, Citrus* (Rutaceae), *Cornus* (Cornaceae), *Corylus* (Betulaceae), *Cotoneaster* (Rosaceae), *Crataegus* (Rosaceae), *Fagus, Lagerstroemia* (Lythraceae), *Liquidambar* (Altingiaceae), *Malus* (Rosaceae), *Platanus, Populus, Prunus, Pyrus* (Rosaceae), *Quercus* (Fagaceae), *Rhododendron* (Ericaceae), *Rosa* (Rosaceae), *Salix, Sorbus,* and *Ulmus* (Hérad F. *et al.* 2006). *Acer* was the most commonly infested tree genus in Europe, followed by *Betula* and *Corylus*. CLB oviposition, and only partial larval development have been recorded on *Acacia* (Fabaceae), *Cryptomeria, Viburnum* (Adoxaceae), *Ficus* (Moraceae), and *Eriobotrya* (Rosaceae) in Europe.

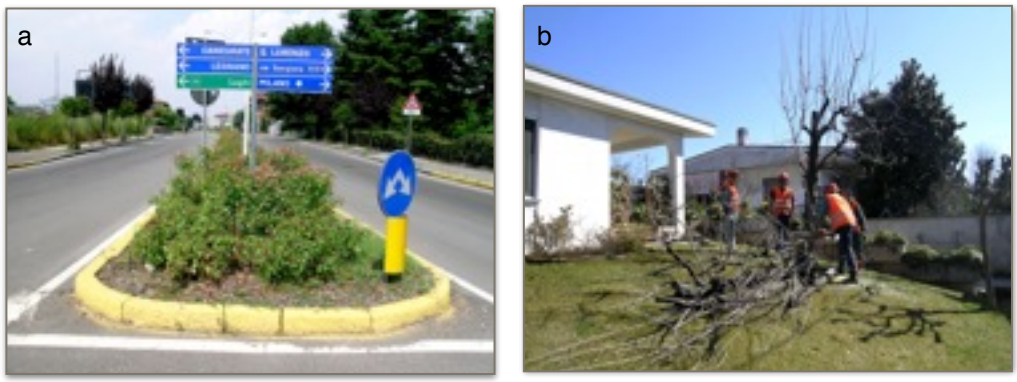


Figure 11
(a) Infested shrubs of *Rosa* spp. in a traffic island at Parabiago, Milan, Italy
(b) Tree felling of an infested maple in a private garden during the eradication campaign.

SIGNS AND SYMPTOMS OF INFESTATION BY *ANOPLOPHORA* SPP.

Infestation of plants by an insect pest is usually discovered, and later monitored by seeking signs and symptoms of its presence in a given habitat. Efficient search by an observer can be made only when a full knowledge of the signs and symptoms of pest presence and their variability has been preliminarily acquired.

A sign of presence of the pest is the insect adult itself found on a host plant, or any physical damage to the host plant caused by the insect at any stage of its developmental cycle. Signs may be observed quickly after the initial attacks by the pest, or they may be identified much later, even several years after the first introduction. Symptoms are host plant's responses to attacks by the phytophage.

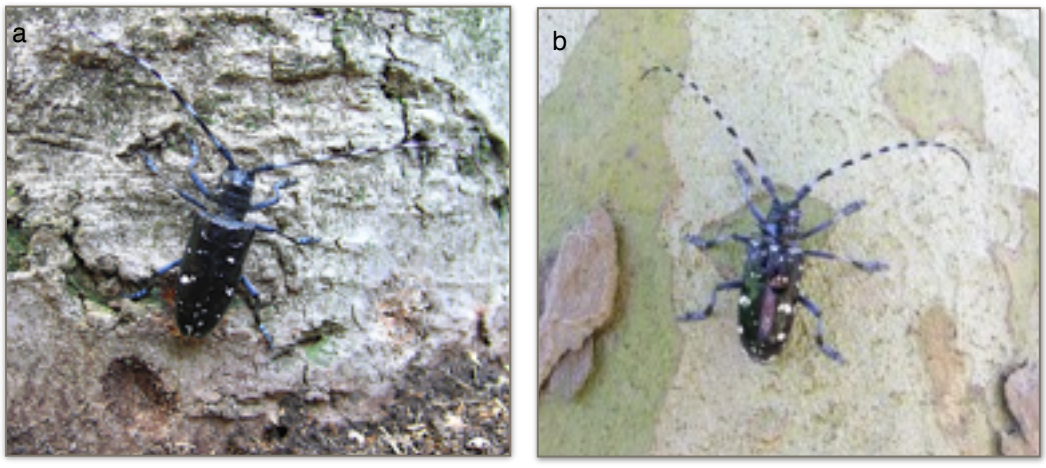
Some signs and/or symptoms may be noticed, but erroneously attributed to a local pest. Often the pest is not noticed before it reaches a fairly high level of infestation. Externally to a host tree, the CLB and ALB major signs that must be sought are : adult specimens of the pests, feeding damage by the adults, oviposition slits or pits, piles of frass and debris from galleries, exit holes of adults. The internal signs are : larval galleries in sapwood (and heartwood), and larvae. Variability of those signs within a single host plant, between host plants of the same species, and between host plants belonging to distinct species must be known to make the best diagnosis. Especially in a situation of low density of the pest, when signs are rare, it is crucial to know the whole range of signs that permit to diagnose with certainty the presence of a given pest, and to distinguish them from signs due to local pests.

External signs

Adult beetles: Historically, the initial detection of an infestation by the alien species ALB or CLB in the introduction areas in Europe and North America was made during summer with the discovery of adult beetles that looked very different from the cerambycid species usually found in the local fauna. Nowadays, a free adult of ALB or CLB that is found between May and October anywhere in Europe on a street tree, in an urban park, a private yard, a garden center, a tree nursery, or an afforested area, is the major sign that an introduction is happening locally, or a breeding population has remained undetected till that day of observation (Fig. 12). ALB adults are usually found on the feeding sites (suckers around trunks, new shoots in canopy near tip of the branches), and on oviposition sites (on branches at least 7 cm in diameter, on trunks). CLB adults are found on the feeding sites (suckers and new shoots), and on oviposition sites mostly around base of the trunks and on visible roots on ground.

Figure 12

(a) ALB adult
(b) CLB adult



Oviposition slits and pits: By inserting its protruded ovipositor through a slit that was previously cut with the mandibles in the bark, the CLB female injects an egg within tissues of the outer the bark (Fig. 13). With its protruded ovipositor, the ALB female injects an egg under bark after having chewed a funnel-like pit to the cambial layer (Fig. 12). The preparation of the oviposition incisions by CLB and ALB females, and their egg deposition behaviors were described above. We add here some details about the variability of shapes of the CLB slits and ALB pits, and some information about the usual locations on host plants of the egg sites.

The incision in an arc, which is cut with the mandibles by a female CLB preparing an oviposition slit, is always perpendicular to her body axis. Thus, the direction on the plant of this part of the slit always depends on the direction of the body of the ovipositing female. On a vertical surface (a tree trunk, or a potted log) the CLB female preparing an oviposition slit generally stays vertically, the head downward to cut the bark; thus, the primary slit is about horizontal. To inject an egg, the female first turns around and stays vertically, her head upward, above the slit (Fig. 13). Moving the tip of her abdomen in the zone where she cut the bark, she blindly searches to direct it to the middle of the slit. Sensilla (chemoreceptors and mechanoreceptors) on tip of the abdomen guide her to find the freshly cut incision and the middle of it (Fig. 13). Under the pressure of the ovipositor the bark often splits, making a crevice that is about perpendicular to the primary slit, resulting in a reverse T-shaped oviposition slit.

In some instances the bark does not split when the ovipositor is inserted and the resulting slit is a simple incision in an arc. That kind of slit is even more difficult to detect among the natural irregularities and crevices of the bark. They are more easily detected on smooth barks.

In a few instances, the bark does not split from the middle of the primary slit, but from one end of it, so that the resulting slit is L-shaped instead of reverse T-shaped. L-shaped slits may be erroneously recognized as normal irregularities of the bark instead of real, atypical CLB egg slits.

Visible roots with a tender bark are often heavily attacked by CLB. On those roots many CLB slits are made along the lines separating the aerial part of the root from the ground. Along these lines, most of the primary slits are cut horizontally. In contrast, many oviposition slits made on the top of the roots are cut perpendicularly to the axis of the roots, and the others are made in varied oblique directions (Fig. 14).

CLB slits are generally difficult to find, even by experienced observers, because they are made around the collar of trees, very often masked by leaves and debris of the litter. The latter has to be removed and the collar zone brushed carefully to remove dirt at the base of the trunks to better reveal the possible presence of the CLB slits. In sandy, or not compacted soils, CLB females often drive their head in soil just under the collar line to make oviposition slits that are particularly well hidden and very difficult to find unless the observer removes a few centimeters of humus in the collar zone before checking. It has been determined that 96% of the CLB slits are made on the base of trunks and on visible roots within 15 cm above ground, and most of them are found within 5 cm above ground (Fig. 15 - BETOTAC report). Very rarely, a few CLB oviposition slits are found much higher on trees, up to 2 or 3 m above ground.

ALB's round pits are mainly found on thick barks. On moderately thick barks, their shape is more oval; and on very thin barks, where a funnel-shaped pit cannot be chewed, they are simple slits similar to CLB oviposition slits. On trunks, ALB females generally stand vertically, the head downward to chew the egg pits. During injection of the egg, the female stands vertically, the head upward, and the egg is injected upward under bark. On oblique

and horizontal branches, body of the ovipositing ALB females is generally oriented parallel to the branch axis.

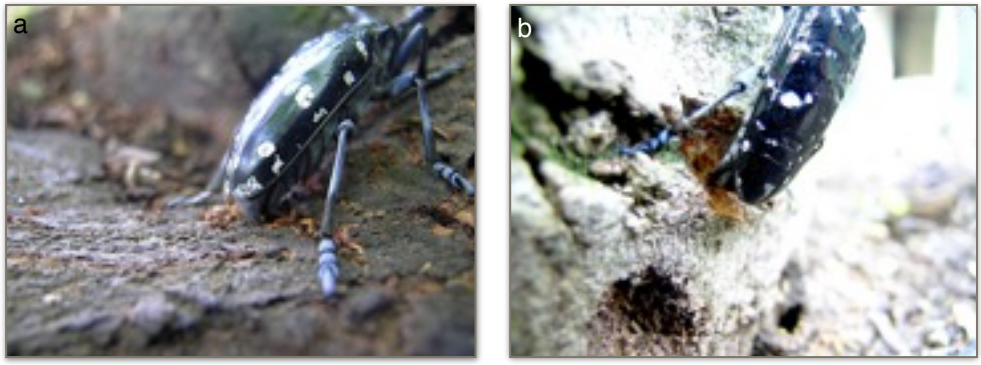
About 20% of the ALB and CLB oviposition pits and slits remain empty. This is because females sometimes renounce injecting an egg in the incision they just prepared. Oviposition pits or slits in which a brown shiny plug is visible in the oviposition hole always contain an egg. However, the plug is not always clearly visible, even in incisions containing an egg.

Freshly cut oviposition pits or slits showing clear bark tissues, in higher contrast with the surrounding external bark colors, are more easily detected than old incisions that darkened, or were masked with dirt. However, being able to detect old CLB incisions during winter is beneficial since they may contain an unhatched host egg with diapausing larvae of a endoparasitoid, the latter being sought for biological control purpose. Detection of oviposition incisions may be sometimes complicated by reactions of the host plant. For example, on young trees with a thin bark, when the cambial layer was damaged by the mandibles of an *Anoplophora* female that cut an oviposition incision, the damaged region often produces a profusion of plant tissues that extrude the insect egg, which dies.

Detection of ALB pits that are high on trees is often difficult even using binoculars; however, on some host plants, sap oozing from the pits either crystallize on bark, or makes a substrate where fumagine develop. Thus, either the presence of a white area of crystallized sap, or a black area of fumagine help detecting attacks by ALB.

Figure 13

(a) CLB female injects an egg within tissues of the outer the bark
(b) ALB female injects an egg under bark after having chewed a funnel-like pit to the cambial layer.



(c) CLB preparing an oviposition slit
(d) CLB female injecting an egg

(e) CLB female moving the tip of her abdomen searching to direct it to the middle of the slit.
(f) sketch of Fig 12 (e)

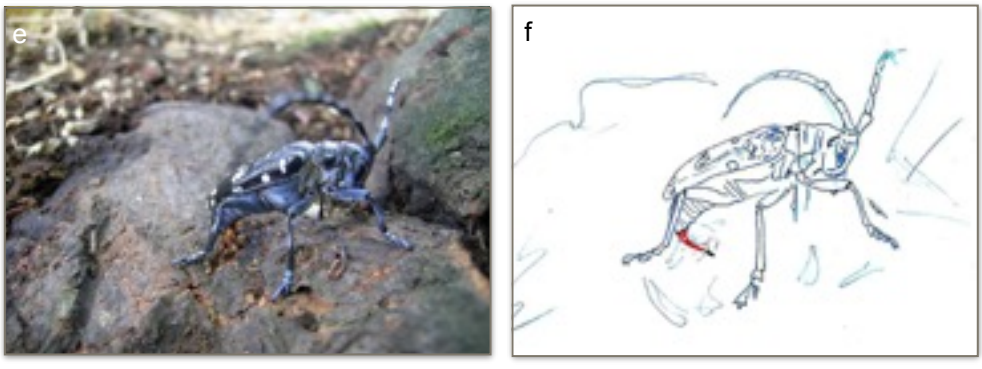
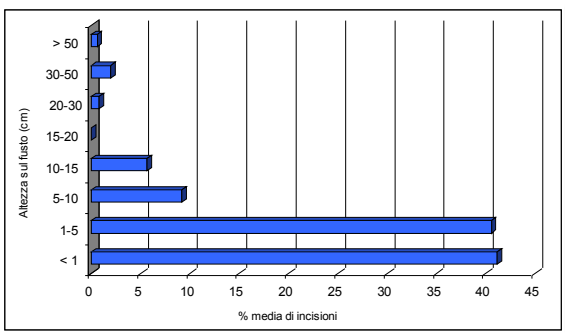
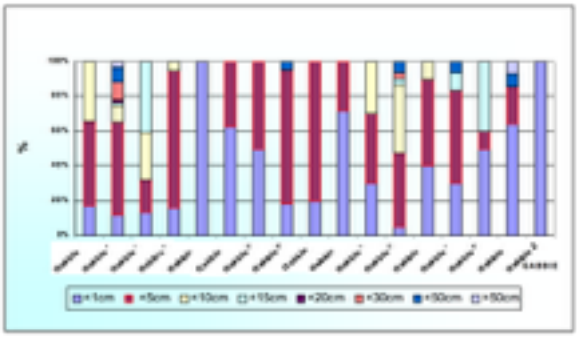


Figure 14 - a debarked T-shaped CLB oviposition slit.

Figure 15 - Data analysis from BETOTAC research project.

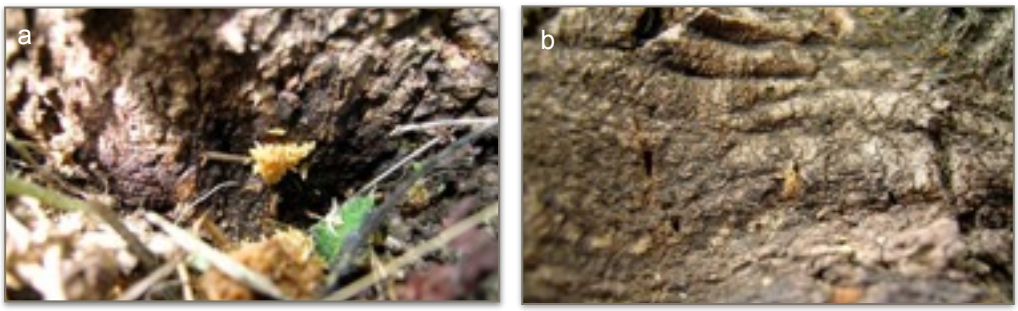




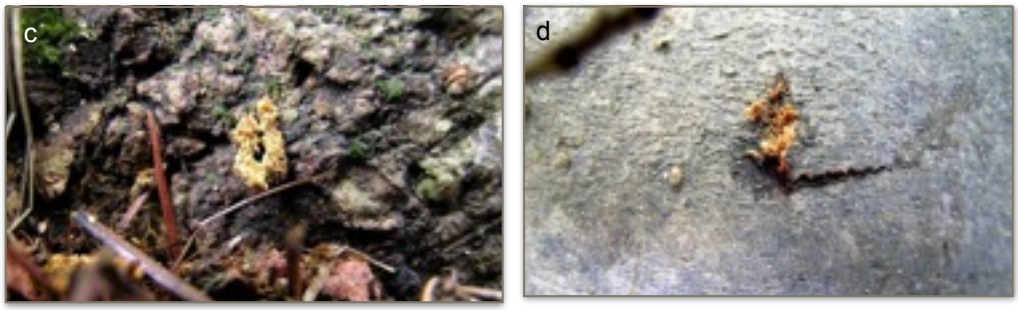
Frass: This is a mixture of excrements, sawdust, and wood fibers resulting from the larval activity. Some frass is stored behind the larva while it bores the gallery. As more frass is produced, it is either heavily compacted in the gallery, or extruded from holes in it. Very early during larval development, some fine frass may be extruded from the initial gallery (Fig. 16), when the L1 and L2 enlarge the egg chamber. Later, the sections of galleries that are tangential to bark are often pierced and the frass extruded in fairly high quantities (Fig. 16 - 17). Size of the elements of frass increases as larvae grow and galleries become wider and longer. In CLB, piles of frass are found on ground, around collar of trees, and along visible roots. ALB frass that is extruded from branches located at more than 3 m above ground is scattered by wind while falling down and is difficult to see on ground. However, when a fork of branches is situated under a branch with an ALB gallery, some frass often accumulates in the fork, which helps detecting pest presence. ALB larvae developing in trunks extrude frass that accumulates around the base of the trunks. Frass is more easily detected in situation of high density of the pest when high quantities are extruded, and hard to detect at the beginning of the infestation. Rate of extrusion of the frass depends on the host plant species, the larval instar, and the environmental conditions. It has been observed occasionally that some infested trees are even free of external frass signs.

Figure 16

(a) (b) (c) frass extruded by CLB L1 - L2 from a maple tree (*Acer negundo*).



(d) freshly cut CLB oviposition slits showing frass extruded by L1



(e) CLB pile of frass at the base of a *Carpinus betulus*
(f) CLB pile of frass at the base of an *Acer saccharinum*

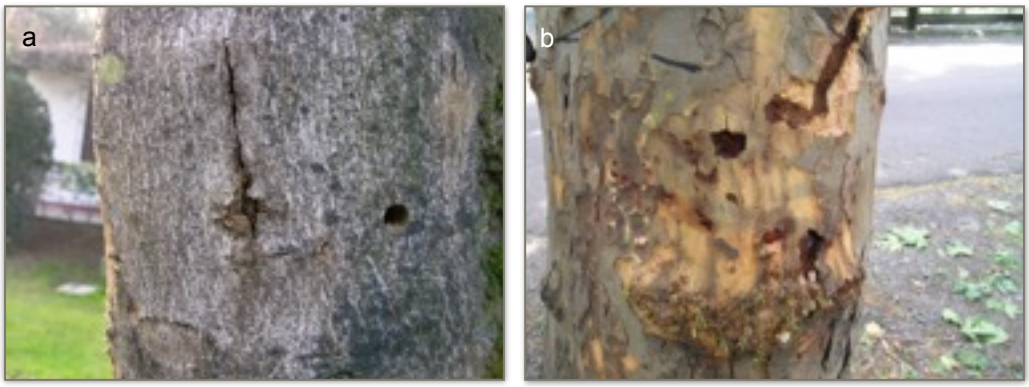
Figure 17

Hundreds of piles of frass at the base of three maples (*Acer saccharinum*)

Hollow bark: This is a large space of coalescent empty wide galleries covered with a thin and concave piece of outer bark that dried out. It is mostly seen on the trunks in old ALB infestations (Fig. 18). It was formed when one or several contiguous wide larval galleries run just underneath the same area of bark. That particular sign shows evidence that the infestation is several years old. In CLB infestations, hollow bark may occur in some roots, but it is only visible on trunks just above the collar of some trees that have been infested and re-infested for years (Fig. 18).

Figure 18

(a) hollow bark and exit hole due to ALB on the trunk a *Acer pseudoplatanus*. (b) hollow bark and exposed galleries due to CLB attack on a *Platanus* spp.



Exposed larval galleries: Years after a hollow bark was formed on the trunk of an ALB-infested tree, healing up of the walls occurred, and the residual outer bark above the area finally fell down, exposing the interior space of the hollow bark (Fig. 19). This is the sign of an ALB infestation that started a few years (or many years) ago. Uprooted sections of CLB-infested roots often show exposed larval galleries. This is due to the small diameter of most roots where several parallel wide galleries were tunneled. The larvae often disrupted the bark of the roots and came to contact with soil. When a piece of dried out outer bark came off from a hollow bark at the base of a trunk infested with CLB (Fig. 19), a cord of very thick, smooth, and tender bark surrounding the old hollow area is often visible. CLB females are particularly attracted to those cords of new bark to lay eggs.

Figure 19

(a) ALB signs of exposed galleries and exit holes on a *Acer pseudoplatanus* (b) base of an *Acer saccharinum* after several years of CLB infestation

Exit holes: The emerging adult of ALB or CLB finishes boring the short gallery that the full-grown larva initiated from the pupal chamber to a place beneath the outer bark. Exit holes are typically perfectly circular, and are 10-15 mm in diameter, but can range from 6 to 20 mm (Lingafelter S. W. and E. R. Hoebeke, 2002; Turgeon J.J. *et al*. 2007; Yan J., Qin X. 1992). In ALB infestations, exit holes are located on branches (usually 7 cm or more in diameter, very rarely smaller), and on trunks (Fig. 20). In Italy, freshly made exit holes may be found during the whole summer since emergence of the adults can occur anytime from June through early October, with a peak of emergence in July. In CLB infestations, exit holes are located at the base of trunks, in a stratum between collar and 50 cm above ground, with most exit holes within the first 15-20 cm (Fig. 20). When trees have roots visible on ground, some exit holes are usually found on those roots. In Italy, new exit holes may be found during the whole summer since emergence of the adults can occur anytime from late May through early October, with a peak of emergence in July.

Most exit holes are visible for several years. However, on many host plants some callus grows around the hole and tends to obstruct it. Some overgrown old exit holes are very difficult to detect.

Figure 20

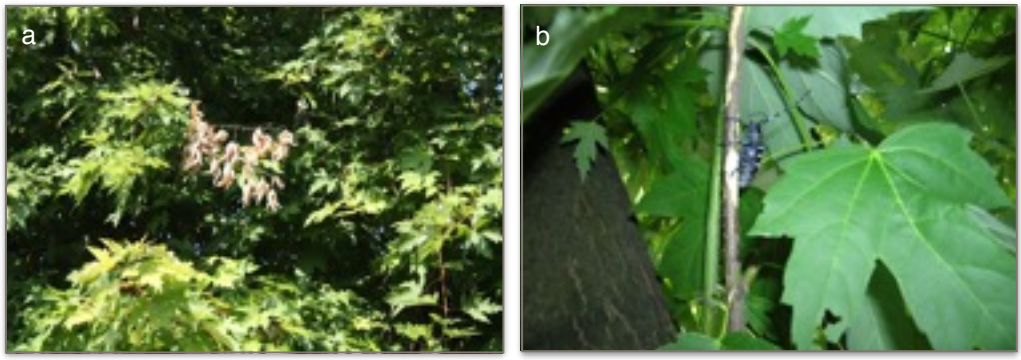
(a) ALB exit holes in the crown of an infested *Acer pseudoplatanus* (b) CLB exit holes at the base of an *Acer saccharinum*

Adult feeding damage: ALB and CLB adults mainly feed on tender bark (Fig. 21) of the new shoots (on suckers growing from the trunk, or on young shoots in the branches), occasionally on petioles and veins of leaves, and rarely on the blades. Fresh damage to bark of the new shoots is symptomatic of the current presence of beetles in the foliage

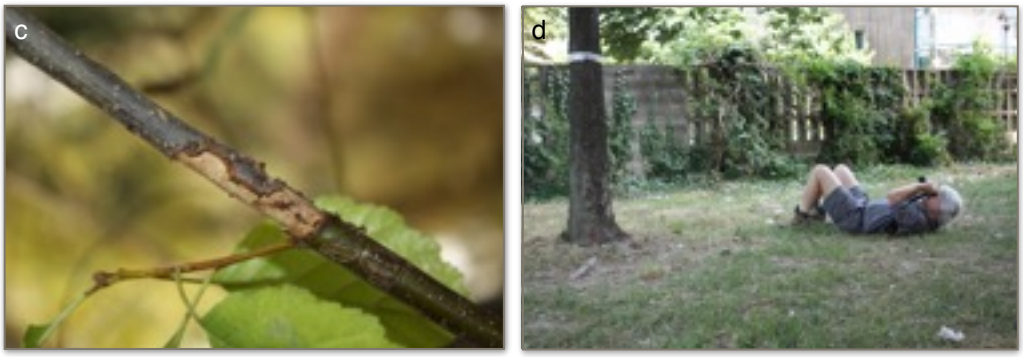
nearby, and is a sign leading to seek for the adults and their ovipostion slits, or pits. Brownish feeding damage, or older scars of feeding damage on twigs is also informative about the history of the colonization of a particular tree by the pest (Fig. 21). Damage to petioles can cause leaves to be severed from their twigs. Leaves fallen down prematurely on ground, and having a chewed petiole are signs of the probable presence of beetles on the trees around. Using binocular helps detecting the feeding damage on suckers and on new shoots in the crown, and finding the beetles.

Figure 21

(a) dried up twig after CLB feeding of the tender bark
(b) CLB female feeding the bark tissue of a twig of an *Acer saccharinum*.



(c) old scar of feeding damage on a twig of *Alnus glutinosa*
(d) Dr Smith M. T. using a binocular to detect beetles in the crown of a maple



Internal signs

If some external signs (except beetles) have been discovered, it is important to look for internal signs and collect some developmental stages that will permit to confirm the identity of the pest species responsible for the infestation.

Eggs and early larval galleries under bark: When oviposition pits or slits are found, it is advisable to open them to collect either an unhatched egg, or the young larva that started boring a gallery (Fig. 22), for later biomolecular analysis. CLB slits on trunk around collar, or on visible roots on ground are easily opened with a penknife by peeling small pieces of outer bark around the slit. Since the egg is located within bark tissues near the surface, there is no need in fact to damage the bark very much to open the egg chamber and reveal the egg inside, or the beginning of a larval gallery containing a 1st or 2nd instar larva. Another technique to collect eggs of CLB or ALB consists of using a carpentry chisel and a hammer to cut a square piece of bark (2 x 2 cm) around the slit, or the pit. The egg of CLB is found between outer and inner bark, and the egg of ALB is found under the inner bark (between inner bark and sapwood). If the egg has already hatched in bark before the sampling date, a young larva (1st, 2nd, or 3rd instar) may be found in its short gallery between bark and sapwood near the oviposition site.

Figure 22

(a) CLB oviposition slits
(b) (c) (d) CLB 1st - 2nd instar larva

Large larval galleries in sapwood and heartwood: Fourth instar- and older larvae bore galleries deeper in sapwood and heartwood, CLB in the lower part of trunk and roots (Fig. 23), and ALB in the trunk and branches. To collect medium size and large larvae and pupae of ALB and CLB, 50 cm portions of plants infested with those pests are cut with a chainsaw, and split with woodcutter hand tools or a powered log splitter. Most CLB larvae being found in roots, the trunk of a CLB-infested tree is cut, about 50 cm above ground, and the aerial parts destroyed. The stump is uprooted and carefully dismantled with appropriate tools to open the larval galleries longitudinally and collect larvae and pupae.

Figure 23

(a) (b) CLB galleries in the heartwood of a trunk of *Salix caprea*

(c) (d) base of infested maples (*Acer pseudoplatanus* and *A. saccharinum)* showing several CLB galleries in the sapwood

Symptoms

Oviposition stain: This is seen as a brownish small area around an oviposition slit or pit (Fig. 24). This is due to a reaction of the plant tissues to some compound injected by the female beetle at the same time than the egg. The plant tissues become brownish, retracted, necrotized. Although it is more an internal symptom, the outer bark also darkens above the egg chamber. This symptom is more pronounced when the bark is thin and smooth. It is also more pronounced on the ALB oviposition sites, than the CLB sites. ALB egg chambers become quickly fairly large after egg deposition because of the strong retraction of the plant tissues around the egg. The newly hatched larva feeds first on the brown and necrotized wall of the egg chamber.

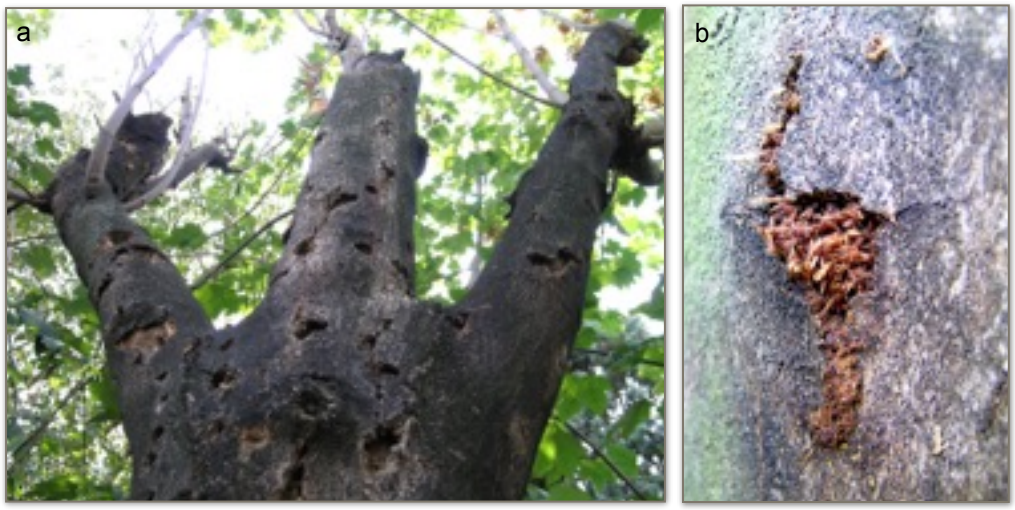
Figure 24

(a) CLB oviposition stain under the bark tissue
(b) several ALB oviposition stains on the outer bark of logs of an infested maple

Crack in the bark: This symptom is seen above some ALB egg chambers when bark is thin and retraction of the plant tissues much pronounced. Outer bark dries out and splits, making a half-open crack (Fig. 25). Larger cracks in bark may be seen when the gallery of a large larva was bored tangentially to the bark surface, in outer sapwood and inner bark, also thinning down some portions of the outer bark, which split. Removing the outer bark around the crack permits the observer to get confirmation about of the presence of a gallery, and to evaluate the approximate age of the damage, freshly made or old.

Figure 25

(a) ALB cracks in the bark of a maple
(b) crack in the bark showing fresh frass extruded from a inner ALB gallery



Missing bark: This may be observed when a large larva bored a gallery tangentially to the bark surface, in outer sapwood and inner bark, also thinning down portions of the outer bark, and subsequently enlarging the gallery within the same area under bark instead of tunneling deeper in sapwood and heartwood. This may result in a large area of hollow bark, covered with a thin outer bark that dries out and finally comes off from the trunk or the branch, leaving a “missing bark” symptom (Fig. 26). At least one-year gap is necessary before this symptom appears.

Figure 26

(a) missing bark at the base of an *Acer sacchainum* infested by CLB
(b) missing bark on the branches of an *Acer pseudoplatanus* infested by ALB

Oozing sap: This symptom is mainly associated with ALB attacks. It appears soon after an ALB pit was chewed in bark. While ALB females chew bark tissues deeply through phloem to the cambium layer, they damage the conducting cells of the phloem, which leads to leaks of phloem sap outside the plant (Fig. 27). Sugar in sap flows sometimes crystallizes on bark, in white areas extending from the oviposition pits, which facilitates detection of the pits. The sap often attracts insects such ants, wasps and flies. It may happen that fumagine develops on surface of sap, thus making it a black area, easily seen too from ground. As most CLB females only chew the outer bark, sap does not ooze from many CLB oviposition slits, and this symptom is of minor importance in detection of that pest.

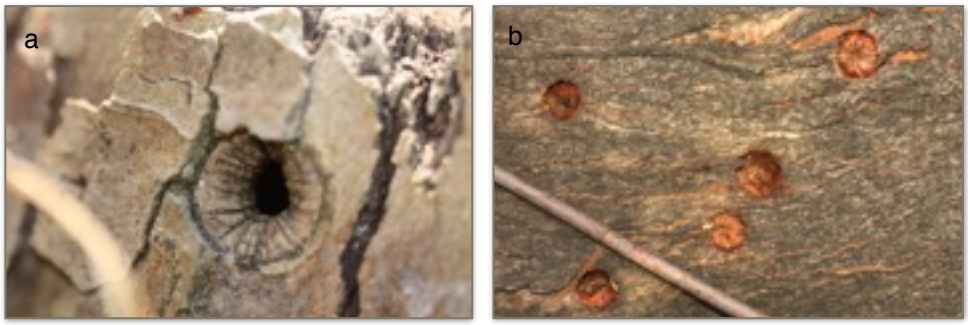
Figure 27

(a) oozing sap due to ALB egg laying on maple
(b) typical symptom of oozing sap on *Platanus* spp. due to CLB larval activity in the cambium layer

Callus tissue around injuries: Most plant tissues that were damaged by larval instars or the adults can form quite visible scars, which either facilitate detection of some damage (around missing barks) or mask others, sometimes totally (overgrown exit holes) (Fig. 27).

Figure 27

(a) CLB exit hole under cicatrization on *Platanus* spp.
(b) several CLB exit holes totally cicatrized on a *Acer saccharinum*



Branch dieback: When trees have been attacked repeatedly, and pest density increased substantially on a plant, heavily disturbing or interrupting the flows of sap to some organs of the plant, branch dieback can appear (Fig. 28 - 29). Occurrence of such a symptom may permit to easily locate a serious infestation, as many trees around a plant showing branch dieback have a high probability to be already affected by the pest.

Figure 28

(a) CLB severely infested *Acer saccahrinum* showing branch dieback
(b) branches dieback due to CLB infestation

Tree death: After years of attack of some host plants by the many beetles of a dense population of the pest, tree death occurs. Like branch dieback, full tree death is a symptom found in an infestation that started more than a couple of years (possibly 10 years) ago (Fig. 29).

Figure 29

(a) branch dieback of an *Acer saccharinum* infested by CLB
(b) full tree death after CLB infestation

CONTROL MEASURES AGAINST *ANOPLOPHORA* SPP. IN LOMBARDY

Because of their high polyphagy on broadleaf tree species and their plasticity to establish in many climatic conditions, the woodborers ALB and CLB have a very high potential to colonize new environments worldwide, and are considered as serious threats to street trees, urban parks, urban forests, tree nurseries, tree stands for timber production, threats also to natural forests and their biodiversity, to conservation of the environments, and threats to the industries exploiting some of those natural resources, as maple syrup, forest ecotourism, etc…

The introduction pathway of CLB is through the importation of living trees and ornamentals (including bonsais) from Asian countries, and the introduction pathway of ALB is through the use of infested wood packaging material while importing goods from Asian countries. In Lombardy region, beetles of the pests emerging from some imported material have created breeding populations in several sites, and for CLB, pest stages were disseminated to many other sites through movements of plants, or firewood, very likely more than through natural spread. For many years the pest developed substantial populations that remained undetected. Similar situations occurred in many other sites in Europe and North America.

In Lombardy region, CLB was first detected in 2000. For more than a decade the Plant Protection Organization (PPO) of Lombardy developed a high level of expertise and substantial efforts to contain the pest effectively, and even have its geographical distribution area moved back. During those efforts, the PPO of Lombardy insisted in conducting successive high impact campaigns to improve public awareness, using all available media. Thanks to it, in 2006, a new serious infestation of CLB was discovered in Brescia Province, 150 km away from the first CLB infestation detected in 2000 at Parabiago, near Milano. In 2007, as a result of those information campaigns, and during surveys focused on CLB in Milano province, the first occurrence of another *Anoplophora* pest (ALB) was discovered in Italy. Municipalities concerned by the pest(s) were involved in the information efforts. Leaflets and posters about CLB were distributed to the public. A substantial campaign of public awareness was also made in Milan subway system, with deployment of almost 200 posters in 82 subway stations. Photos of the beetle and a stump with many exit holes were shown. Each year from 2008, posters were deployed on walls of the subway stations for one month, June through July during the major emergence period of the beetle.

A voice-mail and an e-mail address were also activated by ERSAF (the organism responsible of the eradication efforts against CLB and ALB in Lombardy), and an officer in charge was designated to treat all the mails and alerts. In 2008, 500 notifications were treated, and this number increased during the following years: 1069 in 2009, 1447 in 2010, 835 in 2011, and 1338 in 2012. Information meetings with technicians of the municipalities and the public were organized, press articles were released in newspapers and magazines, radio and TV spots were broadcasted, and expert articles were published in scientific journals (Cavagna B. *et al.* 2013).

Good information to the public was the most effective method to permit numerous notifications from people, and a high rate of detection of the pest in a large variety of habitats, private gardens in urban area and countryside, and in areas other than the municipalities with already known infestations. Today (March 2015), the area monitored in Lombardy region covers almost 45.000 hectares, distributed in the three provinces of Milano, Varese and Brescia, including 73 municipalities. CLB is present in 34 of them, and

the buffer zone includes 39 municipalities. The PPO of Lombardy demarcated the infested zone, and a buffer zone. The first contingency plan was initiated in 2004 and was followed by two other 3-year plans and one 2-year plan that will end in 2015 (Lombardia Verde n.4, 10-11, 2014). The application cost of those plans from 2001 to 2013, consisting in survey, tree felling, replantation, research and information campaign was estimated 18 millions Euros.

Figure 30

(a) Poster “Tarlo asiatico” the common name assigned to the beetle for the public awareness campaign
(b) (c) Milano underground during the period when the information campaign is held

RESEARCH

In their introduction areas in Europe and North America, CLB and ALB are officially considered by decrees from the national and regional authorities, as quarantine pests that are subject to specific contingency plans heading towards their eradication. Efforts to eradicate the pests include the exhaustive detection of the infested trees, and their subsequent destruction. Successful eradication of the pests in a few infestations in North America and Europe has been achieved after considerable and very costly efforts. However, as nobody yet knows if eradication can be ultimately achieved especially in the most serious and extended current infestations, research on alternative methods of control, some being dedicated to containment of the pests, are under way. For about ten years, studies to evaluate the potential of Biological Control methods against CLB and ALB were conducted in Lombardy, and some are reported below.
During the past eradication efforts in several countries affected by ALB and/or CLB, it has been shown that a high probability of eradication exists in the small infestations, which have been discovered soon after a breeding population established. Obviously, the early detection of the pest in a new area is the key towards an effective control of it. In contrast, in the largest infestations, many years of intense monitoring and tree felling permitted to achieve some containment of the pests, but did not yet permit their eradication. As the residual populations of the pests represent persisting threats to the surrounding environments, and can be the source of new satellite infestations, effective early detection methods are necessary to better monitor the pest within the delineated infested area, and in the buffer area. Two early detection methods are presented below.

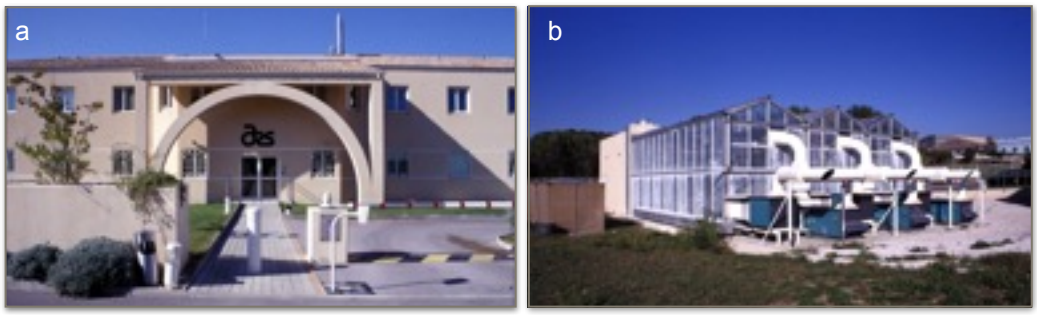
SUMMARY OF RESEARCH:
Biological control: In 2002, preliminary field studies were made in Lombardy in collaboration with the European Biological Control Laboratory, Montferrier-sur-Lez, France, which is a research laboratory of the United States Department of Agriculture, Agricultural Research Service. An exploration work started in the CLB infestation at Parabiago, MI with the first objective to make an inventory of the local natural enemies that would accept the alien pest as a host. Two techniques were used: 1) direct search and collection of developmental stages of CLB in the natural infestation, followed by laboratory rearing of the sampled specimens to determine if some natural enemies were associated with the pest; 2) exposure in the field at Parabiago of healthy host stages (eggs and early larvae of ALB and CLB) in potted sentinel logs to attract parasitoids. Initially, it was assumed that possible new associations between the alien pests and some parasitoids from the local fauna would permit us to identify potential biological control agents.

Very early during the winter 2001-2002, unhatched CLB eggs that were directly collected in the natural infestation were found parasitized by gregarious larvae of a Eulophid (Hymenoptera) that seemed to be in diapause at time of collection. The identification to the family level was made using biomolecular techniques. From the samples of larvae collected in February 2002, and maintained in the laboratory till the end of diapause, adults of the eulophid emerged in June 2002. During attempts of identification of the eulophid, it appeared to be a species new to science, and it was described as *Aprostocetus anoplophorae* (Delvare et al. 2004). Every year since that discovery, the parasitoid was found in CLB eggs in Parabiago area, and a high rate of parasitization was observed locally. Its specificity to the eggs of CLB was shown in laboratory experiments and in field observations, and thus *A. anoplophorae* appeared to be a potentially very promising candidate for biological control of CLB. Further laboratory studies in quarantine at the USDA/ARS/EBCL were carried out to develop rearing techniques for *Aprostocetus*,

try to establish a laboratory colony, and study the major life history traits and host attack behavior of the eulophid. Field work aimed to: 1) determine the geographical distribution of the parasitoid throughout the CLB infestations near Milan; 2) finalize release techniques to try to establish the egg parasitoid in sections of the CLB infestation where the parasitoid is still absent.

Figure 31

(a) EBCL - European Biological Control Laboratory headquarter at Montpellier, France
(b) Quarantine greenhouse at EBCL



Early detection: Two techniques were tested in the field to try to capture the first emerging adults of the pest using sentinel trees, and semiochemical-baited traps. 1) The sentinel tree method was based on deployment of highly attractive trees (e.g. *Acer saccharinum*) in areas where CLB was known to occur, and in areas where it occurred in the past. Attraction of beetles to the plants was combined with use of a killing agent (contact insecticide) sprayed on the 50 cm lower section of the trunks where the adults are known to mate and deposit eggs. The objective was to kill the beetles before they lay eggs. The combination of the attraction method and poisoning of the attracted beetles gives this strategy its name: attract-and-kill technique. 2) Use of traps for beetles, baited with specific artificial lures during the period of emergence and flight of the beetles is known as another effective tool to detect the presence of the pest in the surrounding habitats. The bait consisted of a blend of the ALB male pheromone and some plant volatiles. Initially finalized to attract ALB beetles, the semiochemical was also effective in attracting CLB beetles.

Biological Control Research (2002 through 2011):

During 2002 through 2008, surveys were made in Lombardy, within and outside the area infested with CLB to find possible new associations between the alien pest and natural enemies from the European fauna. In 2005, the Lombardy region financed a two-year project “BETOTAC”, and in 2007 a second two-year project “ANOCHI” in two agreements between Lombardy Region and USDA/ARS/EBCL (through Minoprio Foundation).
The objectives of the research were:
1) Determine, during field explorations in CLB-infested areas, the native natural enemies that may have accepted the alien invasive species as a host;
2) Finalize rearing techniques to maintain colonies of the major natural enemies in the laboratory;
3) Determine host specificity, life history traits, and foraging behavior of the major natural enemies of CLB/ALB, as well as any insect parameter that would permit to evaluate them as promising candidates for biological control.

1) **Field explorations in CLB-infested areas to discover the native natural enemies that may have accepted the alien invasive species as a host.**

Some of the exploration work consisted of sampling life stages of CLB directly in the infestation naturally occurring in Lombardy, and rearing them until emergence of the parasitoid adults. CLB eggs parasitized by *Aprostocetus* were regularly found every year around Parabiago.

None larval parasitoid was found on any instars of CLB collected in the natural infestation but this was probably due to the small numbers of infested host plants that were available for sampling.

Figure 32

(a) Dr Hérard F. and Dr Cocquempot C. desmantelling an infested stump to collect CLB early stages.
(b) first CLB parasitized egg found in 2002.

(d) Dr Hérard F. searching for CLB parasitized eggs at the base of a maple
(e) (f) dissection of an infested stump at Parabiago (MI)

(g) (h) eradication attempts carried out by the Lombardy Region PPS and data collection by researchers

Because of the high difficulties in obtaining the authorizations to sample CLB larvae directly on infested trees (in the private or public domains), which required cutting, uprooting and destroying trees, most of the exploration work was finally made indirectly by exposing CLB- and ALB-infested sentinel plant material (potted logs) in sites selected in protected properties. Potted logs were preliminarily infested in EBCL quarantine with early stages (eggs, L1, and L2) of the hosts CLB and ALB, and transported to the exposure sites. The sentinel plants were exposed in the field for 2 weeks, and returned to the EBCL

quarantine for dissection and rearing of the parasitized stages until emergence of adults of the parasitoids. The latter were submitted for identification to a taxonomist.

The egg parasitoid, *Aprostocetus anoplophorae* was attracted to CLB eggs within the sentinel plants but was never found in ALB eggs. This result was a very valuable indication of its probable specificity for CLB. Not known from the European fauna, the eulophid was thought to be originating from eastern Asia, and was very likely transported in host eggs under bark in the bonsais and plants for planting that have been imported during the prior 15 years from Japan or China into Italy.

Eight larval ectoparasitoid species were found regularly on the first two larval stages of CLB and ALB in the sentinel plants: *Spathius erythrocephalus* Wesmael (Hym.: Braconidae), *Eurytoma melanoneura* Walker, *Eurytoma morio* Boheman (Hym.: Eurytomidae), *Calosota agrili* Nikol'skaya, *Eupelmus aloysii* Russo (Hym.: Eupelmidae), *Cleonymus brevis* Boucek (Hym.: Pteromalidae, Cleonyminae), *Trigonoderus princeps* (Hym.: Pteromalidae, Pteromalinae), and *Sclerodermus* sp. (Hym.: Bethylidae). These larval parasitoids from the European fauna are known to occur on varied xylophagous insects. Their occurrence on both exotic hosts represents new host-parasitoid associations (Hérard *et al.* 2013). *S. erythrocephalus* and *T. princeps* were the parasitoids the most frequently observed on CLB and ALB in most locations where the susceptible life stages of CLB and ALB were exposed in Lombardy. Search for new parasitoid species does not seemed crucial any longer and was discontinued during the following years.

However, more studies were needed in the laboratory to finalize the *Aprostocetus* rearing technique, and in the field to better know the geographical distribution of the parasitoid within the distribution area of the host. Both subjects were studied in 2009, and are presented here briefly (Hérard et al. - Internal report - P.O. 2009).

Surveys were made in many sites within the CLB infested area in Lombardy, but *Aprostocetus* was only collected in Parabiago area. It was suspected that the artificial spreading of the pest by people moving plants and firewood was quicker than the natural spreading of the parasitoid from its initial point of introduction. Therefore, given the potential importance of *Aprostocetus* in the control of the pest, it was thought that its spreading should be artificially accelerated by inoculation of colonies released in the sections of the CLB infestation where the parasitoid was not yet present.

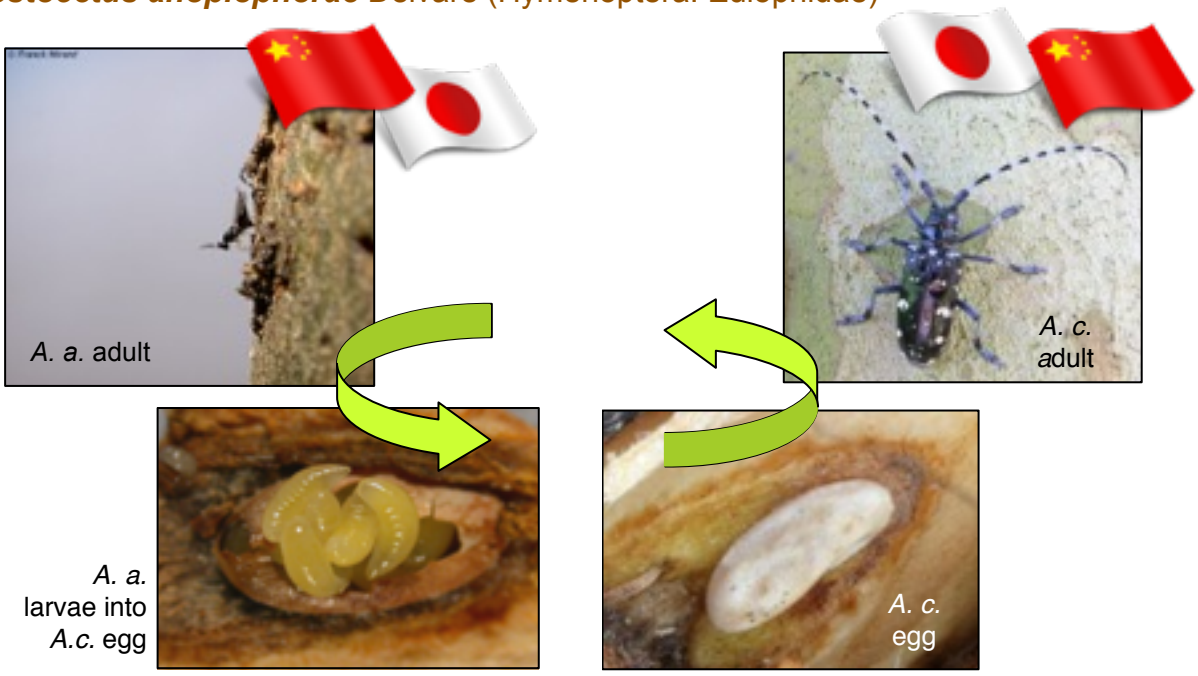
Figure 33

(a) potted logs of *Salix* spp. in the quarantine at EBL
(b) transportation of the *Salix* spp. from EBCL (Fr) to Parabiago (It) for field exposure

(c) cage for the 2 weeks exposure of the infested *Salix* spp. at Parabiago (It)
(d) logs after exposure in Italy were replaced in the EBCL quarantine for further evaluation

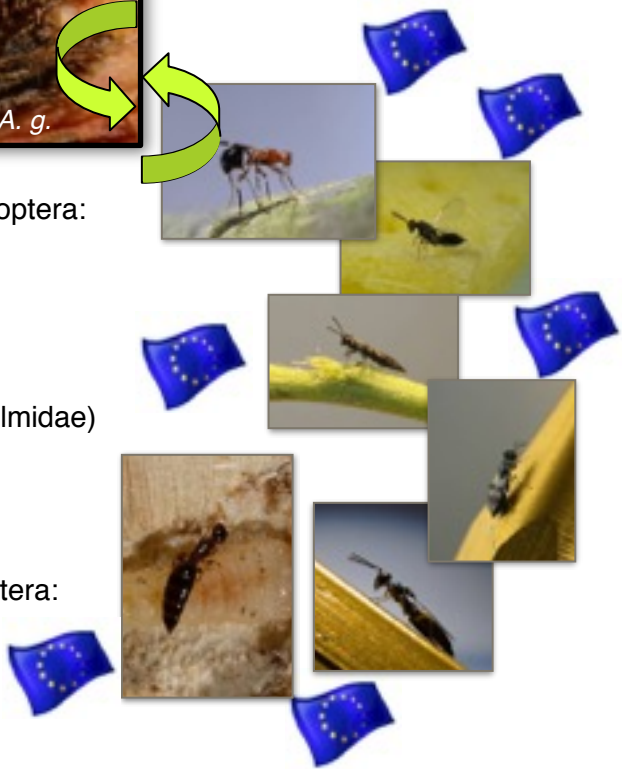
Figure 34 - Early stages parasitoids of *A. chinensis* in Lombardy (Italy)

Aprostocetus anoplophorae Delvare (Hymenoptera: Eulophidae)



L1 and L2 indigenous larval parasitoid of *A. chinensis* and *A. glabripennis*

New association
Exotic pest – *Indigenous parasitoid*



Spathius erythrocephalus Wesmael (Hymenoptera: Braconidae)

Eurytoma melanoneura Walker (Hymenoptera: Eurytomidae)

Calosota vernalis Curtis (Hymenoptera: Eupelmidae)

Cleonymus brevis Boucek (Hymenoptera: Pteromalidae, Cleonyminae)

Trigonoderus princeps Westwood (Hymenoptera: Pteromalidae, Pteromalinae)

Sclerodermus *sp.* (Hymenoptera: Bethylidae)

Photos: F. Hérard

Evaluation of *Aprostocetus anoplophorae* in the natural infestation around Parabiago:

As a following to its initial discovery in 2002, complementary explorations and collections of CLB eggs in 2005-2006 in the Parabiago area aimed at evaluating the distribution and impact of the egg parasitoid. Eggs of CLB parasitized by *A. anoplophorae* were collected at Parabiago and at San Vittore Olona, a village close by. From 49 trees that were removed for CLB eradication purpose, 17 trees contained CLB-parasitized eggs. Those trees belonged to the following species : *Acer saccharinum*, *Fagus silvatica*, *Aesculus hippocastanum*, *Prunus laurocerasus*, and *Betula pendula*. The egg parasitoid was present in 34.7% of the CLB-susceptible trees in that area. From the 46 parasitized eggs collected, 529 adults of *A. anoplophorae* emerged [sex ratio (♂:♀) 1:5.5]. *A. anoplophorae* is a gregarious species; the observed secondary clutch size varied between 1 – 34 (counted as adults emerged per host egg) (Hérard *et al.* - BETOTAC and ANOCHI Internal report).

In **2008**, a field study of the impact of *Aprostocetus* on CLB eggs was carried out at Canegrate near Parabiago, in a CLB-infested woodlot of mixed deciduous tree species (*Acer campestre, Acer pseudoplatanus, Corylus avellana, Malus domestica, Prunus avium, Ulmus pumila*). It showed that the egg parasitoid was well established in that area and its impact was quite significant. The area is considered as the starting point of the first CLB infestation in Northern Italy. A sample of 60 stumps from the woodlot in Canegrate showed that 72% of the 136 CLB eggs deposited in the site were attacked by the parasitoid. From the group of 136 parasitized eggs of CLB, 1124 adults of *Aprostocetus* emerged (213♂ and 911♀) [with a sex ratio (♂:♀) 1:4.3].

Other studies have shown the strict specificity of *A. anoplophorae* for *A. chinensis,* the long period of occurrence of the parasitoid adults duration summer (late June through late September), the good synchronization of development of the host and its parasitoid, the occurrence of 2 generations per year, and the gregariousness of the parasitoid, which is potentially favorable to optimize its rearing (Hérard *et al.* - Internal report - P.O. 2009).

Surveys were made in many sites within the CLB infested area in Lombardy, but *A. anoplophorae* was only collected near Parabiago. It was suspected that the artificial spreading of the pest by humans was much more rapid than the natural spreading of the parasitoid from its initial point of introduction. Therefore, given the importance of this parasitoid in the control of the pest, it was believed that its artificial spreading by humans is absolutely necessary to combat the pest where the parasitoid is not present yet.

On the other hand, the knowledge of the exact delimitation of the current distribution area of *A. anoplophorae* was desirable. One of the envisioned work plans was consisted of sampling CLB-infested trees along 4 crossed transects centered on Parabiago and orientated towards 8 directions. This allowed to identify 8 points on the border of the current parasitoid distribution area, and to obtain a sufficient number of individuals to inoculate other CLB-infested sites still exempt of this parasitoid. In the course of the tree sampling, attention was payed to the host plant species wearing the parasitized eggs, in order to determine if certain CLB-susceptible host plants are more attractive for the parasitoid.

Exploration during **2009** of the *Anoplophora chinensis*-infested area around Parabiago (MI) were carried out in order to study the geographical distribution of its gregarious egg parasitoid, *Aprostocetus anoplophorae* (Hym.: Eulophidae). A grid of about 2 x 2 km square cells (Fig. 36 – yellow lines), covering the map of the known distribution of CLB around Parabiago (Fig. 36 – white thick line), was used to organize the exploration of that area to determine the presence of *A. anoplophorae*. The 9 cells that were visited in June - July 2009 are orange-colored on the map in Fig.36.

Figure 35

(a) sampling the base of a *Platanus* spp. with a chainsaw (b) plates of bark removed from the collar zone of an *Acer pseudoplatanus*

Fifteen sites were selected in the 9 cells, out of 30 cells that were included in the overall distribution area of CLB around Parabiago. The sampling technique consisted of *1.* selecting host trees where signs of CLB presence could be observed (either CLB exit holes, or larval frass, or oviposition slits), and cutting with a chainsaw at the base of trees, within the portion of trunk between collar and 50 cm above ground, larges plates of bark (about 30 x 20 cm) attached to a few centimeters of the subjacent wood, and placing each sample individually in a very fine mesh plastic screen bag until emergence of the parasitoid adults in the EBCL quarantine laboratory; *2.* seeking oviposition slits on trees, cutting bark around them with a wood chisel on a 4 – 5 cm^2 area to remove it with the CLB egg inserted in it, and placing the samples individually in small aerated transparent plastic containers, until dissection or until emergence of adults of the parasitoid.

Figure 36 - map of the area around Parabiago (MI)

Aprostocetus anoplophorae was found in 11 out of the 15 sites where CLB eggs were collected (red spots in Fig. 36). So far, the presence of the parasitoid was confirmed in 8 out of the 9 cells of the grid superimposed on the map of the CLB distribution area around Parabiago where exploration was made (Fig. 36). Although most visited sites where the parasitoid was found belonged to the central area of the CLB distribution, it was also found at Inveruno, which has a rather off-center situation within the host distribution area.

A first attempt to establish *A. anoplophorae* in a site where it was still absent was made in summer 2008, in a CLB-infestation at Assago. Large alder trees *Alnus glutinosa*, boring a

heavy population of CLB, were selected as release points of several batches of adults of *A. anoplophorae*. The result of this release was checked during spring 2009 but apparently it was failed.

In the year following (**2010**-**2011**), in order to detect presence or absence of the egg parasitoid within the CLB infestations of Northern Italy **(Parabiago area, Assago near Milan, Parco delle Cave in Milan, Montichiari and Gussago near Brescia)** a distinct protocol was designed, which was implemented in sites where exploration was not made earlier.

The protocol consisted of preparing CLB-infested sentinel trees in the quarantine laboratory at Montpellier (France), and exposing them in selected sites to attract and capture *A. anoplophorae* where it occurred. Sentinel tree exposure lasted 2 weeks and took place from early June through late August (see the dates of exposure in each site in Figure 39). The sentinel trees were potted maple trees *Acer pseudoplatanus,* with a trunk of 3 – 4 cm in diameter at collar level, and 2.5 – 3.5 m high (Fig. 37). Bark thickness of these young trees was about 2 mm or less.

Figure 37

(a) Young *Acer pseudoplatanus* before infestation with eggs of *Anoplophora chinensis* (CLB)
(b) Potted *Acer pseudoplatanus* freshly infested with eggs of CLB, and exposed within a CLB-infestation in Italy to attract and capture the egg parasitoid *Aprostocetus anoplophorae*

(c) CLB egg poorly inserted in thin bark
(d) CLB eggs extruded by the profusion of plant tissues (callus) of a young *Acer pseudoplatanus* when ovipositing CLB females damaged the cambium layer

CLB females readily accepted these plants as oviposition sites but the thinness of the bark was an obstacle to obtain a high rate of survival of the host eggs and the parasitoid larvae. In normal situations, CLB oviposition slits are chewed at the base of big trees where the bark is thick, and the eggs are inserted within the bark tissues (1 egg per slit). On young host trees with a thin bark, the CLB females chewing oviposition slits often damaged the cambium layer under bark, and the eggs were rarely properly inserted within the bark tissues (Fig. 37). In addition, the damaged cambium often produced a profusion of plant tissues that finally extruded the CLB eggs (Fig. 37).

Among the eggs that were not extruded, many of them remained widely exposed to external conditions, rain or sun, or were subject to predation. An abnormal proportion of the non parasitized CLB eggs died, and among the parasitized eggs, survival of the parasitoid larvae was rather low. During 2010, an overall number of 84 sentinel trees were exposed in the16 sites listed in Table 1. At each date, 2 trees were exposed in the sites which were known to house *A. anoplophorae* populations. The objective was to get enough specimens to finalize a laboratory rearing technique for the parasitoid, and to study the conditions of its diapause. Only one sentinel tree was placed in the other sites of exposure at each date.

Following exposure, the sentinel plants were stored in a quarantine greenhouse and the lower portion of the trunk was wrapped in a sleeve cage of organza where the emerging adults of the parasitoid were collected (Fig. 38). After a month, if no emergence of either a CLB larva or parasitoid adults occurred, the eggs were dissected to confirm their status (non embryonated, embryonated and dead, parasitized egg either with living parasitoid larvae or with dead parasitoid larvae).

Figure 38

Sleeve cages of organza around the portions of trunks with CLB oviposition slits, following 2 week-exposure in the field in Northern Italy to capture the parasitoid *Aprostocetus anoplophorae*.

Figure 39 - sites and dates of sentinel trees exposure during 2010

Table 1: Sites where sentinel trees infested with CLB eggs were exposed to capture *Aprostocetus anoplophorae* in 2010

Site	Locality	GPS North	GPS East
A	Legnano	45° 35.82	8° 56.03
C	Canegrate 1	45° 34.54	8° 56.19
D	Parabiago	45° 32.01	8° 57.31
E	Canegrate 2	45° 34.47	8° 56.47
F	Assago (City)	45° 23.86	9° 08.00
G	Assago (countryside)	45° 22.77	9° 07.75
H	Castellanza	45° 36.47	8° 54.61
I	Villa Cortese	45° 33.71	8°53.07
J	Pogliano	45° 32.25	8° 59.17
K	Nerviano	45° 32.91	8° 58.92
L	Garbatola-Grancia	45° 33.37	9° 00.76
M	Casorezzo	45° 31.47	8° 54.75
N	Mesero	45° 30.21	8° 51.41
O	Milano (Parco delle Cave)	45° 27.98	9° 06.02
P	Brescia area (Montichiari)	45° 25.44	10° 23.57
Q	Brescia area (Gussago)	45° 35.75	10° 09.85

Table 2: Dates of exposure in 2010, in selected sites of Northern Italy, of CLB eggs inserted in potted sentinel trees (*Acer pseudoplatanus*)

Site	Locality	Exposure 1	Exposure 2	Exposure 3	Exposure 4	Exposure 5
		8 - 25 Jun	25 Jun - 6 Jul	Jul 6 - 20	Jul 20 - Aug 3	Aug 3 - 24
A	Legnano	✓	✓	✓	✓	✓
C	Canegrate 1	✓	✓	✓	✓	✓
D	Parabiago	✓	✓	✓	✓	✓
E	Canegrate 2		✓	✓	✓	✓
F	Assago (City)		✓		✓	✓
G	Assago (countryside)		✓			
H	Castellanza		✓	✓	✓	✓
I	Villa Cortese		✓	✓	✓	✓
J	Pogliano		✓	✓	✓	
K	Nerviano		✓	✓	✓	✓
L	Garbatola-Grancia		✓	✓	✓	✓
M	Casorezzo		✓	✓		
N	Mesero		✓	✓	✓	✓
O	Milano (Parco delle Cave)		✓	✓	✓	✓
P	Brescia area (Montichiari)			✓	✓	✓
Q	Brescia area (Gussago)			✓		

Figure 40

Geographical distribution of *Aprostocetus anoplophorae* (Hym.: Eulophidae) within the distribution area of *Anoplophora chinensis* around Parabiago (MI), Italy, in 2010

From the CLB-infested material collected during 2009, *A. anoplophorae* was found in 11 sites (appearing in red spots in Fig.40) including ten sites in the central portion of the CLB infestation around Parabiago, and one site in Inveruno, which is located 10 km south-west of Parabiago. During 2010, presence of the parasitoid was detected using sentinel trees that were exposed within the distribution area of the pest in Parabiago area. Potted trees were placed in 4 sites of the previously known distribution area of the parasitoid, and in 7 sites outside it (the blue spots in Fig. 40). The objective was to figure out if the current geographical distribution of *A. anoplophorae* was larger than the one determined in 2009.

Among the exposure sites selected in 2010, the 4 sites A, C, D, and E were already known as sites belonging to the geographical distribution of *A. anoplophorae,* and this was confirmed again in 2010. In our field work in 2010, we experienced some drawbacks related to bark thinness of the sentinel host plants that we used. Although over 80% of the deposited eggs were susceptible to parasitism, egg mortality was fairly high (24.1% of the susceptible eggs) because the CLB eggs were not properly protected by a sufficiently thick bark against sun heating, rain, and predation. Furthermore, 14.8% of the susceptible CLB eggs were chewed by predators because they were partly exposed outside the bark of their host plants. In future studies, we would recommend using sentinel trees with a sufficiently thick bark (3 mm minimum). This would allow CLB females to prepare oviposition slits without damaging the cambium layer of the host plants, which would prevent profusion of plant tissues and subsequent extrusion of the eggs. In addition, a better protection against predation and hostile environmental conditions would be ensured, and a higher survival rate of the host and its parasitoid would be obtained.
The CLB eggs in the sentinel trees exposed in Assago area, were not parasitized. In 2010, it appears that the parasitoid does not occur yet within the CLB infestation around Assago.
The CLB eggs in the sentinel trees exposed in Parco delle Cave, Milan, were not parasitized. In 2010, it appears that the parasitoid does not occur yet within the CLB infestation in that part of Milan.
The CLB eggs in the sentinel trees exposed in Montichiari, 21 km South East of Brescia, were not parasitized. In 2010, it appears that the parasitoid does not occur yet within the CLB infestation in that part of Brescia area.

The CLB eggs in the sentinel trees exposed in Gussago, 12 km North West of Brescia, were not parasitized. In 2010, it appears that the parasitoid does not occur yet within the CLB infestation in that part of Brescia area.
Thus, the distribution of the egg parasitoid *A. anoplophorae* appeared to be restricted to the central portion of the largest CLB infestation around Parabiago. It is still absent from the other known CLB infestations in Northern Italy. Given the high impact the egg parasitoid can exhibit within the pest population where it established by itself (like in Canegrate), we strongly recommended manipulating the other pest populations by releasing the parasitoid in every other known CLB infestations of northern Italy. Inoculative releases were suggested by exposing sentinel trees with parasitized eggs of CLB containing diapausing larvae of *A. anoplophorae* (Hérard *et al.* - Internal report - P.O. 2010).

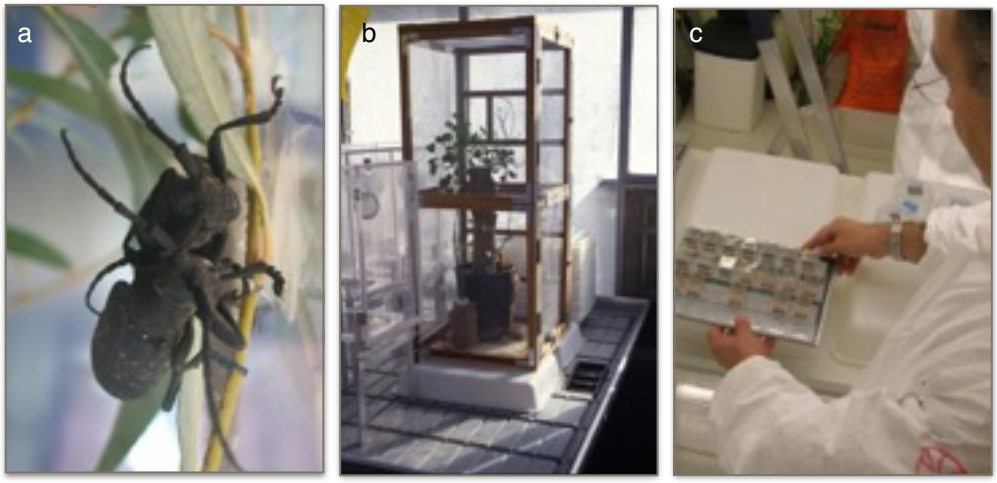
2) Laboratory tests aimed at finalizing rearing techniques to maintain cultures of the potential natural enemies

Laboratory studies were carried out at EBCL, Montpellier, France to finalize rearing techniques to maintain cultures of the potential natural enemies, to determine host specificity, life history traits, foraging behaviour, as well as any insect parameter permitting to evaluate them as possible candidates for biological control. Laboratory tests showed that the two major larval parasitoids, *Spathius erythrocephalus* (Hym.: Braconidae) and *Trigonoderus princeps* (Hym.: Pteromalidae) have some features of good biological control agents but they can attack a variety of xylophagous hosts. Unfortunately, this polyphagy may prevent them to be highly effective against the two invasive target pests.
A particular attention was devoted to *A. anoplophorae,* the specific egg parasitoid of CLB, and especially to its impact in the field, in the core of the infestation near Parabiago.

Laboratory rearing techniques: In quarantine laboratory at EBCL, continuous rearing of the target hosts CLB and ALB, and of some alternative hosts, *Lamia textor* (L.) and *Morimus asper* (Sulzer) (Coleoptera, Cerambycidae) was conducted on host plants and on artificial diet. Rearing techniques were developed to successfully maintain laboratory cultures of the two major larval parasitoids *S. erythrocephalus* and *T. princeps*. The egg parasitoid of CLB, *A. anoplophorae* was also successfully reared in the laboratory, but the technique used so far should be improved and optimized if we want to establish continuous rearing of a large colony.

Figure 41

(a) alternative hosts of CLB and ALB parasitoids
(b) cage of rearing inside the EBCL quarantine greenhouse
(c) cages of emergence of parasitoids



Evaluation of *Spathius erythrocephalus* and *Trigonoderus princeps:* In the laboratory, life-history traits of the larval parasitoids *S. erythrocephalus* (Figure 42) and *T. princeps* were studied to evaluate these insects as potential biological control agents. Egg-laying behavior, longevity, fecundity, larval and pupal development, natural mortality, effect of host size on parasitization, parthogenesis, and sex ratio in the progeny, were measured using several cerambycid hosts. The results of these studies showed that *S. erythrocephalus* and *T. princeps* are long living insects with potentially high performances. However, we suggested several orientations for further studies: (1) optimize rearing conditions; (2) investigate the effects of host age, host size, and host patch size, on primary and secondary clutch size of *S. erythrocephalus*, on sex allocation by both parasitoids, and on sex ratio in their progeny; (3) compare parasitoids performances on varied hosts at the same time in similar conditions, in order to make valid scoring and comparisons between more or less susceptible hosts, including *Anoplophora* spp., and determine the potential host range of the parasitoids (the first tests of host specificity showed that *S. erythrocephalus* and *T. princeps* seem to be rather polyphagous); (4) conduct tests using varied host plants, preferably living potted plants, and not only freshly cut bolts, associated with various hosts, to determine the most attractive associations for the parasitoids, and to study the so defined tritrophic interrelationships; (5) carry out studies of their foraging behavior, measuring search rates, deciphering the mechanisms of host attack with the aid of video recording (video data were collected already with *T. princeps*, but similar studies still have to be made with *S. erythrocephalus*); (6) in the laboratory, study the role of olfaction in the detection from a distance of a potential target, represented by a plant-host complex placed in a wind-tunnel; (7) conduct field experiments including parasitoids releases to try to better understand insect spreading and attraction to exposed target hosts, and measure efficiency of the parasitoids to find and attack those hosts in the field (Hérard *et al.* - Internal report - P.O. 2010).

Figure 42

(a) Adult of *Spathius erythrocephalus*

3) Laboratory studies to determine host specificity, life history traits, foraging behavior of the major natural enemies of CLB/ALB, as well as any insect parameter that would permit to evaluate them as promising candidates for biological control.

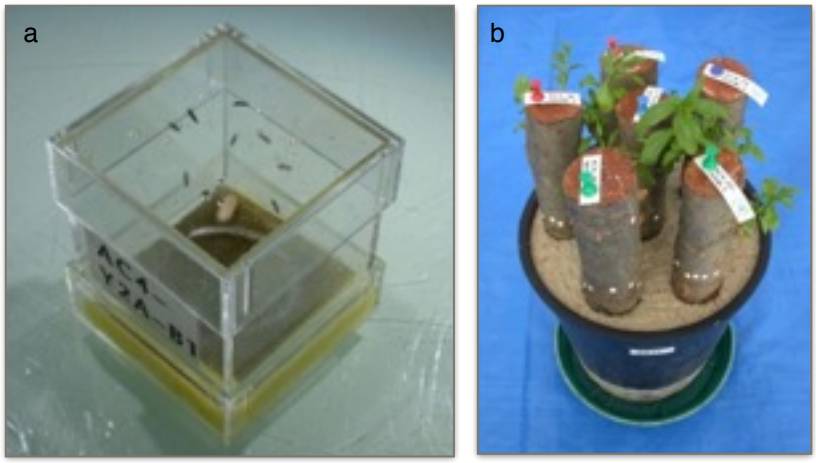
***Laboratory rearing of A. anoplophorae*:** Previous attempts to rear *A. anoplophorae* in the laboratory using small bolts infested with eggs of CLB were not satisfactory. The parasitized host eggs had to be removed from the dessicating bolts and were placed separately in emergence cages. A fairly low rate of survival was recorded among this material. It was assumed that the freshly laid CLB host eggs have to be preferably presented to the ovipositing female parasitoids in living plants in pots. It was also assumed

that the diapausing parasitoids in their host eggs would be better preserved in living host plants until the following year. It also seems that a better yield of the *A. anoplophorae* rearing would be obtained if we knew exactly what particular age of host egg is preferred by the ovipositing parasitoid female, and at what age the parasitoid female is more efficient in its egg laying behavior.

In the laboratory, potted host plants of *A. chinensis*, infested with eggs of the pest, were exposed to ovipositing females of the gregarious egg parasitoid *Aprostocetus anoplophorae* (Hym.: Eulophidae) in order to establish a colony during summer 2009. As most of the progeny in host eggs entered diapause, artificial hibernating conditions had to be determined to allow a good survival rate of the colony until emergence of the adults in summer 2010. The host plants of CLB that were used in our attempts to rear *A. anplophorae* were young *Prunus laurocerasus*, *Corylus avellana,* and rooted cuttings of *Salix alba* planted in 6.5 liter pots with compost. The host plants were exposed to the ovipositing CLB females for a short time (1 – 3 days) to get a few (1 – 5) eggs per plant (Fig. 43). A very small number of hosts per plant was preferred to better know the age of the host eggs and to avoid cannibalism among the CLB larvae later emerging from unparasitized eggs. To obtain the parasitization of the host eggs, the CLB-infested plants were caged individually with 2 females of *A. anoplophorae* for 1 – 3 days. Varied age host eggs were exposed to varied age parasitoid females to try to figure out what combinations of ages of the host and the parasitoid led to a better rearing yield. After removing the ovipositing *Aprostocetus* females from the host plants, the plants were inspected periodically to determine if some of the host eggs were unparasitized. When some fine frass was expelled out a gallery entrance the young larva was killed by pushing a cutter blade through the bark. In this way, parasitized eggs next to newly hatched larvae were saved from cannibalism.

Figure 43

(a) Cage of emergence of *Aprostocetus anoplophorae* from CLB eggs separated from the host plant. (b) *Salix* infested with eggs of CLB ovipositing females of *Aprostocetus anoplophorae*



In many parasitized CLB eggs, no adult of the parasitoid had emerged within a month after parasitization of the host. Dissection of a few of these eggs showed that the parasitoid larvae were still alive in the host eggs, but they entered diapause. Artificial hibernation conditions were created by gradually lowering the temperature of the rearing room where this material was stored. To save space, the living plants containing parasitized CLB eggs were uprooted and replanted all together in humid compost in large containers. From October 22nd, 2009 they were placed in a dark room at 11°C. Survival of the diapausing parasitoid larvae within host chorions under bark of the living plants was expected and assessed in spring-summer 2010. To facilitate achievement of the larval development of the parasitoid, the plant-insect material was placed in April 2010 in a greenhouse at 22°C with natural lighting. At that point, the plants were replanted individually and then covered with a sleeve cage of organza to get the emerging adults of the parasitoid.

In the quarantine laboratory, 740 CLB eggs in 133 host plants were exposed to ovipositing *A. anoplophorae* females. Parasitized eggs were found in 78 host plants out of the 133 host plants used. The total number of parasitized eggs was 335. A few parasitoid adults (31 females and 7 males) emerged as a second summer generation from 5 CLB eggs, on August, 15th, 28th, and September 10th, and 14th, 2009.
It appeared that all the other parasitoid larvae entered diapause until spring 2010. Twelve host eggs that did not hatch were separated from their host plants and were dissected to assess their “parasitized” status. Parasitism was positive.
Previous studies had shown: - the strict specificity of *A. anoplophorae* for *A. chinensis*; - the long period (June through late August) of the parasitoid activity; - the synchronism of host and parasitoid developments, - the occurrence of 2 generations per year in the parasitoid; - its gregariousness, which is an advantageous feature for its rearing.
However, several difficulties arose during our previous efforts to rear the egg parasitoid in the laboratory. It seemed that variability observed in rate of parasitism of the hosts could depend on age of the host eggs and/or age of the ovipositing parasitoid females at moment of host attack. Testing the possible effect of both parameters on rearing success turned out to be necessary. Variability of survival rate of the parasitoid larvae within host eggs in plants appeared to be tied to some drying out of host plant material, or to some desiccation of host eggs when they were separated from the host plants until emergence of the parasitoid adults. In addition, optimal laboratory conditions during diapause (temperature, humidity, dark : light cycle, preferred host plants) were not clearly known. Thus, the egg parasitoid *A. anoplophorae* needed more investigations: 1. in the laboratory, to finalize its rearing and to get large colonies of it; 2. in the field, to better know its geographical distribution within the current distribution area of the host. Both questions were specifically studied during 2009 and 2010.

Survival of the diapausing parasitoid larvae within host eggs under bark of the living plants was expected and was assessed in 2010. To facilitate achievement of the larval development of the parasitoid, the plant-insect material was placed in April 2010 in a greenhouse at 22°C with natural lighting, in sleeve cages of organza. At that period, as no emergence of *Aprostocetus* adults was obtained, the plants and the parasitized CLB eggs were dissected to assess the status of the post hibernating material: all the parasitoid larvae were found dead in the host eggs, showing evidence that the conditions of the host plants, and/or the temperature and humidity conditions during diapause, or following diapause, were not appropriate to survival of the parasitoid. In 2010, the living host plants containing parasitized eggs with diapausing larvae of *Aprostocetus* were stored in a walk-in chamber at 11°C, with a 14:10 hour dark-light cycle. The root system of each plant was kept undisturbed in its pot, and the pots will be lightly watered regularly during the whole hibernation period. Dark-light cycle will be modified with a longer lighting from March through June 2011 (Hérard *et al.* - Internal report - P.O. 2010).

BIOLOGICAL CONTROL RESEARCH (2011 THROUGH 2014):

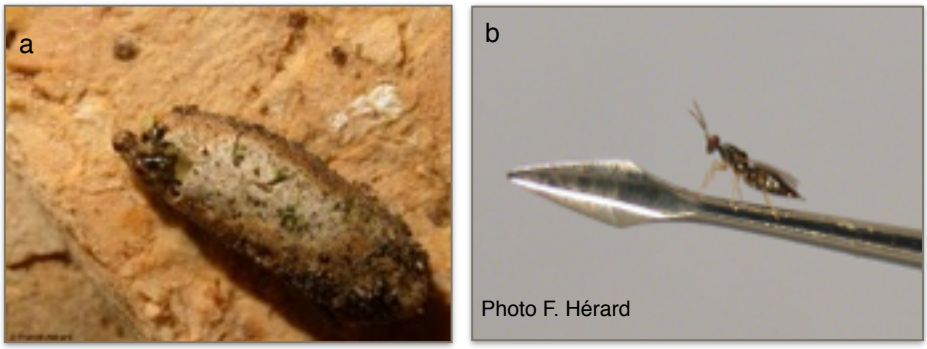
PREFACE

For the first time on February 20th, 2002 at Parabiago, Italy, 2 eggs of *A. chinensis* were found parasitized by a gregarious egg parasitoid, the larvae of which were hibernating inside the host chorions. One larva was genetically characterized using DNA-based markers, and the larvae from the other host egg were reared to adulthood. The molecular analysis, using sequence data of the second expansion segment (D2) of the 28S ribosomal subunit revealed that the taxon might be assigned to the genus *Asprostocetus*. When adult specimens of this taxon emerged, their assignment to the genus *Aprostocetus* was confirmed by comparative morphology analysis. Sequencing data which were also performed on this sample of adults corroborated the above referenced result. Based upon morphological characters, the new species was named and described as *Aprostocetus anoplophorae* n. sp. (Hymenoptera: Eulophidae) Delvare *et al.* (2004).

Given the host specificity of *A. anoplophorae* for CLB, the high rate of parasitism observed in some sites, and our current knowledge of the geographical distribution of the parasitoid, which is firmly established in the core of the infestation around Parabiago, it appears that the egg parasitoid is the best candidate for biological control of the pest. Although continuation of pest eradication remains a high priority, releases of *A. anoplophorae* throughout the areas where it does not occur yet should be made to help contain the pest as much as possible during the eradication effort.

Figure 44

(a) CLB parasitized egg
(b) Adult of *Aprostocetus anoplophorae*



Following this important discover, many research topics were developed in order to conduct laboratory studies in the USDA/ARS/EBCL quarantine in France, to determine an appropriate rearing method of the parasitoid, to study its biology and behaviour, as well as conducting field studies with the aim of establishing the parasitoid in areas of the CLB infestations in Italy where it does not occur yet (Assago area, Parco delle Cave and Bosco in Città, Montichiari and Gussago).

Success of establishment of the egg parasitoid is tied to good synchronicity between emergence of the host and the parasitoid at the beginning of summer. To better ensure this synchronicity, it is assumed that diapause of the parasitoid should happen in the exact weather conditions the hosts are exposed to. Diapause/hibernation of the parasitoid occur in its larval stage in the host eggs.

Parasitoid release consisted of exposing CLB-infested sentinel trees containing parasitized eggs, with the parasitoids being in diapausing larval stage, into selected sites within CLB infestations of northern Italy. To do this, in early spring of 2012, sentinel trees (*Acer pseudoplatanus*) with trunk diameter 10 cm and more at collar level were planted in Italy, in the selected study sites within CLB infestations. The base of trunks was protected

by iron mesh screen sleeve cages to avoid unexpected infestation by the surrounding population of CLB. During fall of the same year, a first sample of sentinel trees was experimentally infested with CLB eggs laid by females caged around the base of the trunks. Then *Aprostocetus* females were caged around the freshly laid eggs of CLB to trigger parasitization. In the weather conditions of that period of the year, the full-grown larvae of *Aprostocetus* were supposed to enter diapause in the host eggs, and spend winter and spring 2013 in that stage. At several dates in June and July 2013, we used the 10 trees of circle 1 and circle 2 to create a local population of freshly laid CLB eggs as candidates for parasitization by the *Aprostocetus* individuals emerging from the 5 trees in the center.

Materials and Methods

The research on biological control was initiated each year with two crucial actions:

- in Italy, Lombardy region, it was mandatory the recruitment of CLB infested wood within the known geographical distribution area of *Aprostocetus anoplophorae.* This was done in the period autumn-spring, in conjunction with the eradication attempts. The collected material was then transported to the quarantine at EBCL-USDA, ARS, where emerging adult of the parasitoid were used as a starter to reactivate the laboratory rearing;
- in France, Montpellier, in the same period, a rearing of potted susceptible trees belonging to the genera *Acer pseudoplatanus* was started, to be used during the whole summer in the Italian infested area. These trees were sentinels to study, monitoring, release and capture *Aprosptocetus* in the natural field conditions.

As for the bio-ethology of these insects, the research program was developed on a three years consecutive study. We were in fact dealing with three living entities:

- the xylophagous pest, *Anoplophora chinensis;*
- the parasitoid *Aprostocetus anoplophorae*;
- the tree, were the life cycles of the two species were occurring.

1ST YEAR RESEARCH 2011-2012

In February 2012, as soon as the weather conditions were favorable for the beginning of the eradication program, during the trees removal carried out by ERSAF, stumps of infested trees located into the first outbreak of *A. chinensis*, were uprooted.
Recruitment of CLB infested stumps/superficial roots was necessary to reactivate the rearing in the quarantine at EBCL.
The selection of this infested material, to be collected and transported in France, was done in accordance with the following parameters:

- stumps/superficial roots were collected into the known distribution area of *Aprostocetus*, around Parabiago (Mi);
- stumps/superficial roots were showing a good level of CLB parasitization that was meaning 1) presence of exit holes 2) abundant frass and mainly 3) a high number of slits (slits due to *A. chinensis* egg laying might improve the number of parasitized eggs in it);
- stumps/superficial roots were of a size that would allow us the uprooting and storage into aluminum trunks (Mod- SIAT D 140 - mm 902x495x397H) that were used for the delivery at EBCL. In case the volume was too big, this material was peeled to get at least the bark (showing slits).

On the base of the over mentioned needs, some potential and interesting stumps were observed in Canegrate, a municipality close to Parabiago. The recruitment of good material for our purpose was time consuming and took several days of field work and observation (Figure 45).
Two areas of particular interest were found during the survey and following ERSAF eradication attempts. The first one was located in a small urban forest, were a huge number of infested maples (*Acer saccharinum*) and Carpinus (*Carpinus betulus*) were found (Canegrate 1). The second place, always in Canegrate municipality, was a rough of seedling maples (*Acer pseudoplatanus*) along the border of a private fence, in a rural area of the village (Canegrate 2).
As shown in the map, both areas were located close to some known areas were *Aprostocetus* was found in previous years (Vivaio La Fornace, Parco del Castello).
In these two areas, 30 stumps (25 *Acer pseudoplatanus* and 5 *Carpinus betulus*) has been uprooted (Figure 45).
At the end of March-beginning of April, the material was transported to EBCL in the quarantine laboratory for the following collection of emerging adults of *Aprostocetus*.

Figure 45

(a) aerial map of Canegrate and points of stump uprooting
(b) row of seedling maples

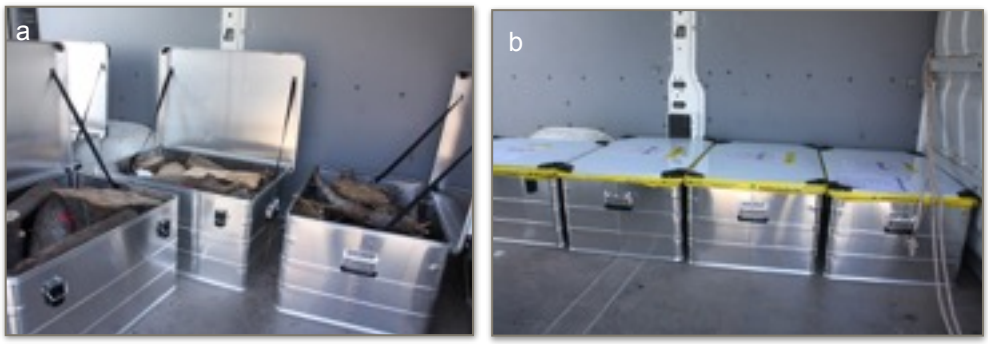
(c) tree removal of infested stumps in the urban forestry area at Canegrate
(d) urban forestry area Canegrate

(e) (f) stumps cleaned and reduced in volume before transportation to EBCL

For the delivery of the material to EBL, wood was placed into aluminum trunks that were hermetically close and labelled with the PPS plastic band. On each trunk, a copy of the LOA and one of the declaration “A QUI DE DROIT” was sticked (Attachment 1 and 2), to ensure all the formal and technical request, compulsory for any movement of infested quarantine material.
Trunks were transported with a Fiat *Ducato maxi*.

Figure 46

(a) metallic trunks loaded with the infested stumps
(b) trunks hermetically closed before transportation from Italy to France (at EBCL quarantine lab)



In the field, parasitoid release took place by exposing CLB-infested sentinel trees containing parasitized eggs, with the parasitoids being in diapausing larval stage, into selected sites within CLB infested zones in northern Italy.

To do this, in early spring of 2012, sentinel trees (*Acer pseudoplatanus*) were planted in Italy, in the selected study sites nearby CLB infested areas (Fig. 47). The base of trunks was protected by iron mesh screen sleeve cages to avoid unexpected infestation by the surrounding population of CLB. During fall, a first sample of sentinel trees (ST1) was experimentally infested with CLB eggs laid by females caged around the base of the trunks. Then *Aprostocetus* females were caged around the freshly laid eggs of CLB to trigger parasitization.

Figure 47

(a)(b) *Acer pseudoplatanus* to be used as sentinel trees during the field research

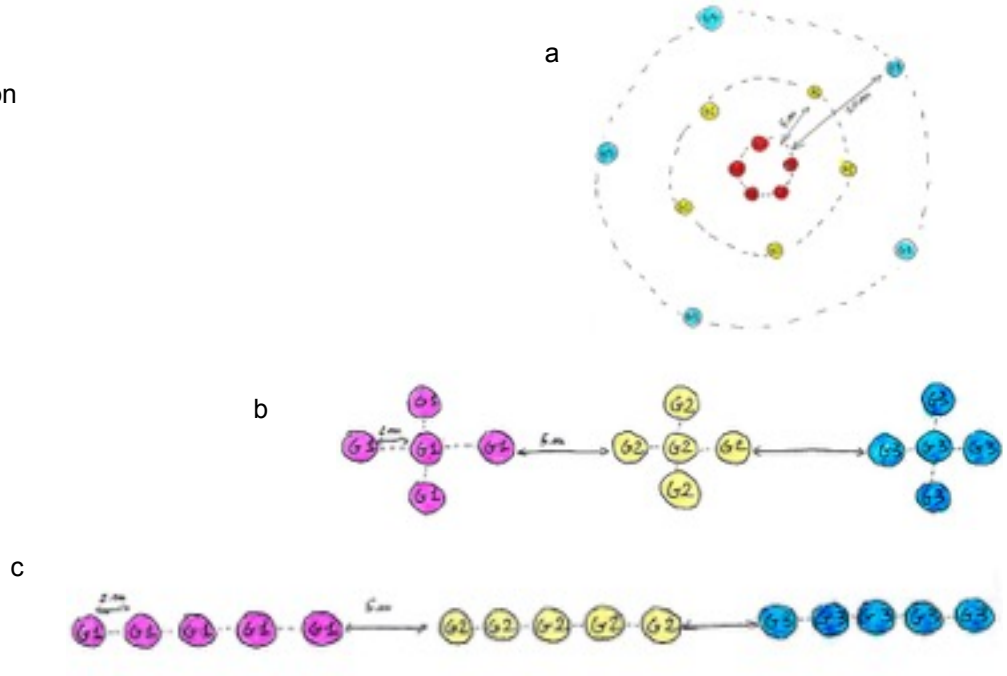
(c) (d) tree plantation of the maples according to the experimental design

In March-April, 2012, **90** (+ 20 as a reserve) maples (*Acer pseudoplatanus*) with a diameter at the base of 8-10 cm, were planted in 6 sites (15 trees per site within the same area) of the CLB infestations in Lombardy where *Aprostocetus anoplophorae* does not occurred yet.
The experimental design of each plot consisted in a central release point containing 5 trees planted fairly close together and 2 concentric circles of 5 trees each. A first circle of 5 trees planted at 5 m from the trees in the release point, and a second circle of 5 trees at 10m from it.

In relation to the accessibility in the property/land, the experimental design was changed by placing the trees in a rough as shown below:

Figure 48

(a) (b) (c) tree disposition according to different experimental designs



(d) experimental plot at Bosco in Città - Parco di Trenno
(e) experimental plot at Assago - Cascina Santa Marta

The 6 experimental plots were located at: 1) **Montichiari**, 2) **Gussago**, 3) **Assago** – **Loc. Az. Quadrio**, 4) **Assago** – **Cascina S. Marta**, 5) **Parco delle Cave** – **Ass. Il Bersagliere** and 6) **Bosco in Città/Parco di Trenno** (Fig. 49).

Figure 49 - *Google maps* of the six releasing points:

1) Montichiari (BS)

2) Gussago (BS)

3) Assago – Loc. Az. Quadrio (MI)

4) Assago – Cascina S. Marta (MI)

5) Parco delle Cave – Ass. Il Bersagliere (MI)

6) Bosco in Città/Parco di Trenno (MI)

During the whole summer, watering was guarantee even if a certain number of these trees was lost during the hot and dry summer.
For this reason, a few trees were replanted in November 2012.

In **September 2012, 10 - 16**, the 5 trees in the center of each experimental site, received CLB eggs from females caged in wire mesh screen sleeve cages around the trunk. Then the CLB eggs were exposed to *Aprostocetus females*, so that the CLB eggs were assumed to get parasitized. The weather conditions, with cool nights during September, were supposed to induce diapause of the parasitoid larvae in the parasitized CLB eggs. They were supposed to complete development and emerge in early summer 2013.

Figure 50

(a) Dr Hérard releasing adults of CLB to lay eggs on the sentinel tree

(b) CLB female laying egg
(c) particular of the exposure cage
(d) (e) CLB successfully done oviposition slits

More specifically, 3 adults of *A. chinensis* (2-3 ♀ + 1 ♂) were placed inside a wire mesh cage, allowing them to mate and lay eggs for a period of two days (Fig. 50). Each cage was built 10-20 cm up to the collar of the sentinel tree. It was composed of a Nylon tissue of around 30-40 cm high, closed up and down with a metallic wire. In the lower part of the cage obtained, sand was placed to create a new collar zone were the female beetle of *A. chinensis* should have laid eggs. The sand was watered to create an appropriate microfilm for the beetle. Twigs of *Acer negundo* were also introduced in the cage, to feed the beetles during the few days expected for the egg laying. Clips on the side of the cage were used to close a split created in the tissue to handle the beetles. An additional wire mesh cage was than built around the previous one, to make sure of the preservation of the cage during the time of beetles exposure.
In total, during the experimentation were used 135 adult beetles obtained from the artificial rearing at EBCL and 24 female adults of *Aprostocetus anoplophorae.* CLB eggs were exposed to *Aprostocetus* females by replacing the metallic cages by Nylon mesh sleeve cages, with very fine mesh (200 microns) and a female parasitoid, so that the CLB eggs get parasitized. In the weather conditions of that period of the year, the full-grown larvae of *Aprostocetus* have entered diapause in the host eggs, and will spend winter and spring

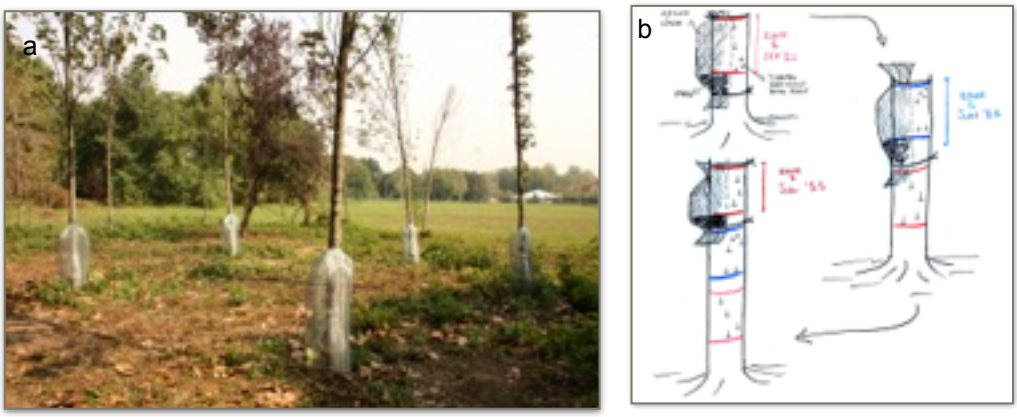
2013 in that stage. Release/spread of the parasitoid was assumed to occur by itself when the parasitoid adults emerged in June-July of 2013.
At several dates in June and July 2013, we used the 5 trees of circle 1 to create a local population of freshly laid CLB eggs as candidates for parasitization by the *Aprostocetus* individuals emerging from the 5 trees in the center. To get this, we placed CLB females in wire mesh sleeve cages on the trees of circle 1. Each cohort of CLB eggs was suitable hosts during at least 10 days for the parasitoid adults emerging from the 5 trees in the center. In this way, we determined if *Aprostocetus* successfully went through diapauses in these experimental conditions, and if it discovered hosts at 5m or 10m distance from point of emergence.

During summer 2013, establishment of the parasitoid was assessed experimentally as follows: among the sentinel trees in the study sites, a second sample of them (ST2), which have been protected since time of plantation from CLB attacks, was experimentally infested in June – July of 2013 with CLB eggs by caging CLB females around trunk of the trees. They should have attracted the *Aprostocetus* females emerging from the parasitized CLB eggs in the first sample of sentinel trees (ST1).

The CLB eggs in ST1 and ST2 were checked for parasitism by removing the sentinel trees and carefully peeling the bark to look for the level of eggs parasitization (Fig.51).

Figure 51

(a) Example of releasing site at Bosco in Città
(b) sketch of the experimental infestation process



2ND YEAR RESEARCH 2012-2013

In February, March and April, 2013, during the tree felling activities developed by ERSAF, stumps and superficial roots infested by the wood borer have been uprooted in the know distribution area of the parasitoid (fig. 52 - 53).
In total were collected:

- n° 44 sumps of *Prunus laurocerasus;*
- n° 8 stumps of *Acer pseudoplatanus;*
- n° 14 stumps of *Rosa* spp.
- n° 1 stump of *Cotoneaster*

Figure 52

(a) uprooting *Prunus laurocerasus*
(b) stumps collected and cleaned

The infested material was initially stored in a greenhouse located at Villanstanza, Milan. Since the recruitment of the stumps was time consuming, this work took several days.
Once the quantity of stumps reached a certain number, they were transported to Montpellier, to be stored into the quarantine facility for further research use.
This time the transport was done directly by the EBCL staff (Dr Hérard) through a Toyota Land Cruise which was used to move the aluminum trunks at final destination. The mission took place on April 9 to 10, 2013.
The movement of the infested material was, according to the protocol, supported by the related phytosanitary documentation (LOA).
From this infested wood, adults of *Aprostoccetus* were supposed to be collected in the laboratory, during the following moths.
Stumps and superficial roots were cleaned and reduced in size before transportation to EBCL. Each portion of wood was geo-referenced and labelled with the tree species and the date of collection.

Figure 53
(a) map show the places of stump collections during 2013

In the quarantine lab of the EBCL, each portion of wood was caged in Nylon bags to collect adults of *Aprostocetus* that were supposed to emerge in the following months.

In the filed, during summer 2013, the establishment of the parasitoid was assessed experimentally as follows: on the same sentinel trees (ST1) in the study sites, additional portion of the trunk were artificially infested in June - July of 2013 with CLB eggs by caging CLB females around the trees trunk. The aim was attracting the *Aprostocetus* females emerging from the parasitized CLB eggs in the first portion of the sentinel trees parasitized in September 2012.

More specifically, the field work timetable was the following:
June 17 – 21, 2013, before the parasitoid adults emerge:

(1) We got newly laid CLB eggs on the same sentinel trees (ST1) as targets for the emerging parasitoids. To make this, 30 mated CLB females from the laboratory colony of EBCL (Montpellier) were used, and installed in nylon mesh sleeves during 3 days (18 – 20 June). The sleeves around the eggs were removed on June 21st.
(2) We also removed the sleeves of September 2012 to allow the emerging parasitoid adults to spread either to CLB eggs in naturally infested trees, or to the CLB eggs just laid by females released on the same trees.

July 1 – 5, 2013:

(1) 30 other mated CLB females from the laboratory colony of EBCL were installed in nylon mesh sleeves during 3 days (2 – 4 July). The sleeves around the eggs were removed on July 5th. These newly laid host eggs served as targets for the *Aprostocetus* adults emerging in early July.

(2) In addition, the experimental plan included the use of 10 big sentinel trees in pots, preliminarily infested in the quarantine facilities of EBCL with CLB eggs. These trees were exposed within the known geographical distribution area of *Aprostocetus* (Parabiago area, Milan surroundings). They were placed in 10 distinct sites (1 tree per site). The aim was obtaining samples of *Aprostocetus* DNA from various origins within the distribution area of the parasitoid in Italy.

July 15 – 19, 2013:

(1) We got newly laid CLB eggs on the same trees (ST1) served as targets for the emerging parasitoids. To make this, 30 mated CLB females from the laboratory colony of EBCL (Montpellier) were used, and installed in nylon mesh sleeves during 3 days (16 – 18 June). The sleeves around the eggs were removed on June 19th.

(2) The 10 sentinel trees in pots (exposed from early July) were collected and transported back to EBCL quarantine to separate *Aprostocetus* stages in ethanol 95% for later DNA analyses. COI marker (mitochondrial) was used to assess possible belonging to distinct lineages, and measure percentage of divergence among samples.

August 19 – 23, **2013**: the sentinel tree (ST1) used in the study sites, were cut and transported to the quarantine facilities at EBCL for subsequent dissection and assessment of parasitization.

September 9 – 13, 2013: 30 CLB females from the laboratory colony in EBCL were placed in nylon mesh sleeves during 3 days on a new group of sentinel trees (ST2), to get newly deposited host eggs used as targets for the *Aprostocetus* adults of the summer generation, emerging in late August 2013. At that time of the year, their progeny should entered diapause.

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During 2014 the study has continued following the activities developed throughout 2012 and 2013.

In April 2014, in sites included in the distribution area of *Aprostocetus* in Italy, we collected stumps bearing CLB egg slits and stored them in a quarantine laboratory until emergence of parasitoid adults in late June – early July 2014 (Fig. 54).

So, that, on appropriate time, we reactivated rearing of the previously chilled larvae from the laboratory colony of CLB reared in quarantine to get enough numbers of CLB adults.

Our rearing in the laboratory was focused on using thick bark host plants to rear *Aprostocetus* year round. This was assessed by uprooting medium size healthy stumps, keeping a section of trunk, about 1m height and at least 20 cm in diameter at collar level, and transplant them from the field into large plastic pots. The transplanted stumps were transported to a quarantine laboratory, and covered with fine mesh white plastic cages to be infested with CLB eggs, and expose to *Aprostocetus* females for parasitization. The diapausing material in the potted living stumps is hibernated outside in an insect-proof quarantine tent. The objective is to confirm that using host plants with thick bark permits us to get a continuous rearing of the parasitoid, including a high rate of survival among the diapausing *Aprostocetus* larvae.

Once introduced into the quarantine greenhouse of the EBCL in Montpellier, the trunks were opened and each portion of wood was packed into an organza bag, then placed in a cell at 25 C° and 50% of humidity (Fig. 54).

Figure 54

(a) (b) (c) stumps collected and cleaned during 2014

(d) delivery of the stumps at EBCL (Fr)
(e) (f) (g) stumps in Nylon bags, stored under controlled conditions

In total were stored under controlled conditions a number of 57 portion of wood. The *Aprostocetus* emergence was daily recorded and starting from the end of June, beginning of July the firsts imago were collected into the Nylon bags. These adults were used to reactivate the parasitoid colony at EBCL.

The first occurrence of these insects started on June 26th. The following one were recorded as follow: 30/06 - 2/07 - 3/07 - 7/07 - 10/07 – 30/07 (Attachment 3).

A number of 101 adults of *Aprostocetus* were collected from 10 of the 57 bags. The sex ratio was 88 females (♀) and 13 males (♂).

The recruitment of these parasitoids was need to:

- improve the knowledge related to biology and ethology of this insect, having a stable population in the rearing at EBCL;
- demonstrate the validity of the rearing and release protocol based on the use of stumps with a thick bark.

On the base of the knowledge developed in the previous years, the *Aprostocetus* rearing was focused on the use of stumps with a thick bark, to avoid egg crashing during the tree growing.
This kind of bark is typically present in trees with a diameter of at least 20 cm at the base.
These stumps were uprooted from the field in France, during one day of work on the 22nd of July. In a natural environment, at Aniane, along the river, 5 big stumps (2 *Populus* spp., 1 *Acer negundo* and 2 *Salix* spp.) were uprooted (Fig. 55).
Then, the material was transplanted in big pots to be used in the quarantine for further studies related to CLB and *Aprostocetus*.

The transplanted stumps were transported to a quarantine laboratory, and covered with fine mesh white plastic cages to be infested with CLB eggs, and exposed to *Aprostocetus* females for parasitization. The stumps were maintained alive in the large heavy pots, which were however still movable across the laboratory on individual rolling carts. The diapausing material in the potted living stumps was hibernated outside in an insect-proof quarantine tent. The objective is to confirm that using host plants with thick bark permits us to get a continuous rearing of the parasitoid, including a high rate of survival among the diapausing *Aprostocetus* larvae.

The exposure of the stumps to *A. chinensis* female took place from July 29th to August 1st (Fig. 56)

Figure 55

(a) collection of stumps with thick bark at Aniane (Fr)
(b) potted stumps infested with CLB eggs and exposed to *Aprostocetus*

Figure 56 - Data of exposure of *A. chinensis* and *Aprostocetus* on stumps with thick bark

	A	B	C	D	E	F	G	H	I	J	K	L
1	***Aprostocetus anoplophorae Italy* _ test thick bark _ 2014/2015**											
2	Host plant #	CLB egg type	Host CLB egg #	Date CLB eggs laid	Date CLB eggs exposed w/ A.A	Nb of A.A ♀ / ♂ per sleeve & ref. number	Date A.A emergence	Date A.A removal or death (D)	Egg in diapause (Nov. 2014-May 2015)	Date of emergence of progeny	No. Of A.A progeny (♀ / ♂)	
3											♀	♂
4	Trunk # 1	Malasiaca	unknown	29/07-01/08/14	01/08/14	1 ♀	30/07/14(N°51)	unknown				
5	Populus				04/08/14	1 ♀	07/07/14 (N°21)					
6	Trunk # 2	Malasiaca	unknown	29/07-01/08/14	01/08/14	1 ♀	30/07/14(N°51)	unknown				
7	Populus				04/08/14	1 ♀	07/07/14 (N°21)					
8	Trunk # 3	Malasiaca	unknown	29/07-01/08/14	01/08/14	1 ♀	30/07/14(N°51)	unknown				
9	Salix				04/08/14	1 ♀	07/07/14 (N°21)					
10	Trunk # 4	Malasiaca	unknown	29/07-01/08/14	01/08/14	1 ♀	30/07/14(N°51)	unknown				
11	Salix				04/08/14	1 ♀	30/07/14 (N°51)					
12	Trunk # 5	Malasiaca	unknown	29/07-01/08/14	01/08/14	1 ♀	30/07/14(N°51)	unknown				
13	A. negundo				04/08/14	1 ♀	30/07/14 (N°51)					

An additional field work was developed at Assago (Milano province), where 5 sentinel trees were exposed from September 9th to September 26th, 2014 to get parasitization of CLB eggs.

Figure 57

(a) potted sentinel tree exposed at Assago
(b) view of the site of exposure

Given the host specificity of *Aprostocetus anoplophorae* for CLB, the high rate of parasitism observed in some sites, and our current knowledge of the geographical distribution of the parasitoid, which seems to be widely distributed and firmly established throughout the major infestation around Parabiago, it appears that the egg parasitoid is the best candidate for biological control of the pest. In the laboratory at Montpellier, studies on the optimal rearing conditions to have continuously parasitoid individuals available were essential to evaluate the most important biology traits and the behavior of the parasitoid. Furthermore, finalizing rearing methods to establish a large laboratory colony of *A. anoplophorae* is crucial to get sufficient numbers of individuals for subsequent field releases.

RESULTS

In middle August 2013, the 30 sentinel trees (5 in each area) in the six areas of releasing were checked by cutting them, transported to the quarantine laboratory at EBCL and carefully debarked.

Data results of the field experiments about *Aprostocetus* release-capture 2012 and 2013 showed that the percentage of slits containing a CLB egg was particularly low on most sentinel trees.

The overall percentages of slits (all sites together) containing a CLB egg were 23.7% in September 2012, 46.6% in June 2013, and 35.2% in July 2013. That means we have to improve considerably the experimental conditions to allow the CLB females to lay eggs on the sentinel trees.

The percentage of CLB eggs that were killed, apparently because of insect predators, was high: 81.3% in Sep 2012, 54.5% in June 2013, and 43.9% in July 2013. As we cannot prevent some predation by insect predators, this is why we need to get many more CLB eggs on each tree.

Only one egg among those exposed in Sep 2012 (it was in Montichiari) was attacked by the parasitoids released in the sleeves. Unfortunately, the diapausing larvae of the parasitoid in that host egg died during diapause That means we also have to improve considerably the experimental conditions to allow the *Aprostocetus* females to lay eggs in the hosts on the sentinel trees.

Considering the special case of Assago**:** 3 CLB eggs exposed in end of June 2013, and 2 CLB eggs exposed in early July 2013 were attacked by *Aprostocetus*. As the parasitoids involved in these attacks cannot come from our release in September 2012, they must

come from a local population of *Aprostocetus* pre-occurring in that site. This means that establishment of the parasitoid in that site was already obtained.

These mostly negative results were very informative:

- They showed that another type of host plants, with thick bark at collar level should be used. In such host plants CLB eggs that are laid within tissues of the outer bark, an inert microhabitat, won't be threatened by any reaction of the plant.
- A sufficiently long exposure of the plants to the egg-laying CLB females would permit us to get more host eggs per plant.
- A higher number of ovipositing parasitoid females per cage should be used to increase the percentage of parasitized host eggs.
- Because they are available in higher numbers in early summer, the *Aprostocetus* adults emerging as a first cohort in late June–early July from post-hibernating hosts should be preferred, over the second cohort of *Aprostocetus* adults emerging in August from the summer generation, to attack new hosts on the sentinel trees. We know that most of their progeny enter diapause at the end of the larval development and thus do not develop a summer generation. Emergence as adults of the most numerous diapausing individuals is expected in early summer 2015. Their occurrence will be assessed by exposing new CLB eggs on the same trees during that period. Those without a diapause, emerging in late August, 2014 will escape in the surrounding CLB infestation and will start colonizing the site at the end of summer 2014.

Considering the field exposure at Assago, in 2014, the 5 sentinel trees that were exposed from September 9th to September 26th, 2014 to get parasitization of CLB eggs, we get confirmation that the egg parasitoid is present at Assago, and it is still very active during late summer.

By separation of all eggs from the exposed trees, and dissection of some of those eggs, we determined that parasitized eggs were present in the 5 trees.

A total of 67 CLB eggs were exposed in the 5 trees. We checked presence of the parasitoid in 17 of them, which makes 25% intermediary parasitism (Attachment 4).

But a certain number of the eggs that were not dissected did not yet hatch to give a CLB larva. So, they must be parasitized. However, it will be checked later, in next spring, at the end of diapause. We need to study the conditions for a successful diapause.

So, at the end of diapauses, the potential total rate of parasitism could be in fact 58%.

In July 2015, the sentinel trees exposition will be done again in Assago, to try to determine, what percentage of the population of the parasitoids enter diapause immediately in July, and what proportion gives some progeny in late August

Planned research (2014-2015):

Field work: Release of *Aprostocetus anoplophorae* to insure a high rate of survival of the diapausing parasitoid larvae using the following new protocol.

In April 2014, in sites included in the distribution area of *Aprostocetus* in Italy, we plan to collect stumps bearing CLB egg slits and store them in a quarantine laboratory until emergence of parasitoid adults expected in late June – early July 2014.

On appropriate time, we plan to reactive rearing of the previously chilled larvae from our laboratory colony of CLB reared in quarantine to get enough numbers of CLB adults.

In 6 CLB-infested sites where the parasitoid did not yet pop-up, we will select 30 still healthy CLB-susceptible trees (5 trees per site) with thick bark at collar level, thus trees with > 20 cm diameter trunks. In late June - early July, 2014 when we get *Aprostocetus* adults emerging from stumps collected in April, 2014 we plan to start the field experiment by stapling fine mesh white plastic cages at the base of the trees. We will place a pair of CLB adults in each cage for about a week to get sufficient numbers of eggs in thick bark.

Two or three *Aprostocetus* mated females will be introduced in the cages to parasitize the CLB eggs. Most of the larvae in the parasitized eggs will enter diapause until spring 2015. The not diapausing individuals will develop during summer 2014 in about 55 days. Thus 40 days after parasitization of the eggs, the multiple fine mesh white plastic cages will be replaced with one large cage of iron fine mesh that will permit a first cohort of *Aprostocetus* adults to escape in late summer throughout the infestation. The large iron cage will also exclude predation by birds and some insects. It will cover the whole bottom of trunk and visible roots where CLB adults could emerge from, during summer 2015. *Aprostocetus* adults emerging in July, 2015 will be able to escape and continue colonizing the site. In late June – early July, 2015 pairs of beetles will be placed in the iron mesh to get new sets of freshly laid eggs that will be attacked by the emerging adults of *Aprostocetus*, assessing their survival during diapause. Collection and dissection of some of these eggs will permit us to confirm parasitoid activity in early summer 2015.

EARLY DETECTION:

1. THE SENTINEL TREES METHOD

PREFACE

Another research topic was the use of the evaluation of the efficacy of the Sentinel trees techniques. This method is based on the possibility of using highly attractive tree species (e.g. *Acer saccharinum*) in a way of attract and kill the adult beetles before oviposition in areas where infestations are known to occur. This includes areas that are difficult to monitor, such as natural woodlands. The primary objective is to attract beetle and kill them by spraying an insecticide on the trunk surface. The pesticide/insecticide was applied to the lower 50cm of the trunk where adult ALB/CLB lay eggs. Killing beetles that are attracted to and land on the sentinel trees gives this strategy its name, **Attract-and-Kill**.
Mark-release-recapture experiments revealed that adults of both species can disperse 1–3 km during their life span, although most remain near the tree where they emerged (ALB: Bancroft JS and Smith MT, 2005; Sawyer A, 2006; Smith MT et al. 2001; Smith MT *et al.*, 2004; USDA APHIS 2008; Williams DW et al., 2004 ; CLB: Adachi I, 1990; Zhou J et al., 1984).
In general, the potential objectives of the sentinel trees include:
Detection

1. Detection of ALB/CLB within areas where no beetles are known to occur, specifically high risk areas:
    a. Ports of entry (risk of introduction). The objective is 'interception';
    b. Areas known or suspected of importing plants from the countries of origin of CLB, e.g. nurseries;
    c. Areas, such as parks where campers may bring infested wood;
    d. Areas, such as where horticultural companies dispose plant debris;
    e. Other
2. Detection of ALB/CLB within areas where adult beetles and/or infested trees have previously been found. The objective is to detect 'pockets' of infested plants, or what may be referred to as incipient populations that are below the detection threshold using the current visual inspection methods.
3. Detection of ALB/CLB surrounding areas where adult beetles and/or infested trees are currently and/or previously been found. The objective is to delimit the outermost boundary of an infestation. In other words, the objective is to find small 'satellite' infestations outside the current Regulated Zone.

4. Detection of ALB/CLB within areas within an infestation where it is difficult to survey, e.g. areas that are difficult to access; areas with host plants that are themselves difficult to inspect e.g. ground growing juniper that have a dense canopy; plants with thorns; etc. The objective is to detect infested plants.

Attract and Kill (CONTROL)

The focused objective of this strategy is to control the beetles in areas where infestations are known to currently harbor or previously harbored beetles or infested trees. This includes areas that are difficult to monitor (monitor is different from survey). Simply stated, the primary objective in not to determine if a population currently or previous existed, but is to prevent attack of more plants or any potential movement. Therefore, sentinel trees could be treated as follows:

1. An insecticide could be sprayed on the entire sentinel tree, including the canopy and trunk or just on the base of the truck in case of a CLB control;
2. An insecticide band could be attached to the sentinel trees;
3. A fungal band could be attached to the sentinel trees;
4. Other.

Killing beetles that are attracted to and land on the sentinel trees gives this strategy its name, Attract-and-Kill.

One caveat that may complement the Attract-and-Kill strategy is to apply a killing agent to non-infested host plants that are in close proximity to the sentinel trees. The killing agent would simply be applied to the lower 50cm of the trunk where adult CLB lay eggs. The objective would be to kill beetles prior to oviposition. This should contend with the fact that not all beetles attracted to the sentinel trees may in fact land on the sentinel tree. The same thing was seen when using baited traps, and hence it was plan to apply/attach a killing agent to the host trees in close proximity to baited trap. In the ALB eradication program in the U.S.A., the non-infested host trees in some areas are treated with a systemic insecticide as a prophylactic treatment. These areas are typically of considerable size. As such, sentinel trees placed within such areas would have the added advantage of killing beetles that land on these systemically treated host trees.

In Lombardy we stated that sentinel trees were eventually be placed:

1. Along the established outer boundary of the Regulated Zone. The Regulated Zone boundary is an estimate.
2. In areas within the infestation where host trees have been treated with insecticide.
3. Along the edges of areas within the infestation where only infested trees were removed and/or where all host trees were removed.
4. In high risk areas beyond the estimated Regulated Zone, including
    a. Risk due to potential movement by people: populated areas, e.g. villages, where people might inadvertently carry beetles
    b. Risk due to potential natural movement or spread by the beetle itself. Considering that ‘Distance’ is only one of several important considerations, the placement of sentinel trees depends upon a combination of ‘Distance’ from an infestation and the host trees in close proximity to these distances.
        (1) Distance: Place the sentinel trees at incremental distances from the hypothetical perimeter of a given infestation:
            - ➢ At ca. 100m
            - ➢ At ca. 500m
            - ➢ At ca. 1,000m

- ➢ At ca. 5,000m
- ➢ At ca. 10,000m

(2) Types of landscapes:

(a) Based on Plant Composition – potentially plant sentinel trees in at least four types of landscapes: 1. areas with only host plants; 2. areas with only non-host plants; 3. areas with a mix of host and non-host plants; and 4. areas with no plants (e.g. agricultural fields). All four types of landscapes are suitable.

(b) Based on Spatial Structure – potentially plant sentinel trees in: 1. closed canopy landscapes (e.g. forests, woodlots, parks, the willows along the water's edge); and 2. open canopy landscapes. Both are suitable.

Material and Methods

Given the above circumstances, our field work was developed in a way to consider both the possibilities of detected and control the citrus longhorn beetle, *A. chinensis* in two outbreaks located in Brescia Province, at Montichiari and Gussago. Two rows of 50 and 38 trees respectively, of *A. saccharinum,* were planted in March 2012. The tree size was around 3m in height and almost 6-8cm of diameter at the base, in the collar zone. The distance between each other was 1,20m.

Figure 58 - Aerial maps of the sentinel trees plantation

1) **Gussago**

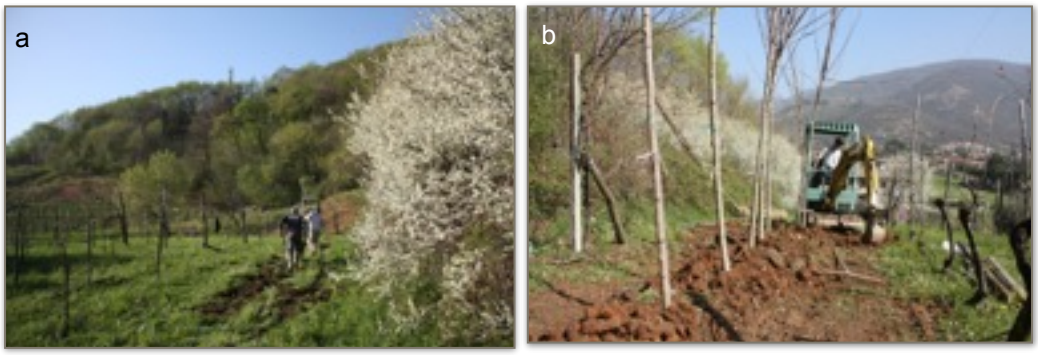
2) **Montichiari**

1) At Gussago the row of sentinel trees was placed in proximity of a hill were in spring 2010, severe tree felling were carried out as for the eradication attempts (Fig. 59). Hazelnuts trees (*Corylus avellana*) were removed in the woodlot where the sentinel trees were planted. On the hill surrounding the woodlot were still present maples (*Acer pseudoplatanus* and *A. campestre*) as well as, *Corylus avellana*, *Carpinus betulus, Alnus glutinosa* and *Ostrya carpinifolia*. All these are susceptible to CLB infestation but the survey already don at that date didn't show any infested tree.

In this kind of environment the survey was quite difficult as for the orography of the hill, in some places with slopes, and the density of the vegetation which increased the difficulties of the visual inspections.

Figure 59

(a) (b) tree plantation at Gussago



Even if the conventional survey made by the technicians of the Lombardy Region PPS did not showed further signs or symptoms of infestation, the sentinel trees would allow us to:

- evaluate the eventual **higher attractivity** of the sugar maple, *Acer saccharinum*, in a place where there is abundance of equally attractive tree species;
- implement the conventional survey with the eventual possibility of **capture adults** beetle;
- **reduce the beetle population** through the use of a chemical of the sentinel trees.

2) At Montichiari the row of sentinel trees was also placed in the middle of an infested hill where in the previous years, repeated felling of infested tees were occurred (Fig. 60). In this place too, the huge surface of the area and vegetation density were a limiting factor for a successful and proper survey.

Figure 60

(a) (b) tree plantation at Montichiari

In both areas, the base of each tree was sprayed with a micro-encapsulate insecticide (KARATE® Zeon - Lambda-cyhalothrin) two times during the flying period of the beetle (June and July), with the aim to verify the efficacy of the technique.A backpack mechanical pump (Mod. Volpi 15 lt) with a full cone nozzle was used to spray the insecticide.
Weekly visual inspections were performed from June to September, looking for 1) **adults**, 2) **feeding activity** of imago on the twigs, 3) **frass** at the base of the trees or, **egg slits**.

Tesis	Commercial product	Composition	Dose (ml,g p.c./hl)	Dose (ml,g s.a./hl)	Distribution method
1	Karate Zeon	Lambda-cialotrina 9.48 %	330	35	Spray on the bark's trunk (lower 50cm)

RESULTS

Even if this method looked promising, in our field work the outcome was not satisfactory. In fact signs and symptoms of the beetle presence were not recorded during the first season (2012) and neither in the following one (2013). The main reason was probably related to the trees attractiveness, which was apparently very limited as for the difficulties in creating a good canopy.
The hot summer registered in 2012 and the difficulty in watering the plants had a negative effect on plants growth, affecting the results of the trial.

EARLY DETECTION:

2. SEMIOCHEMICAL-BAITED TRAPS

PREFACE

The success of eradication efforts may depend on early detection of introduced populations; however, usual detection methods has been limited to identification of tree damage (oviposition pits, exit holes, piles of frass), and the serendipitous collection of adults, often by members of the public.

A key point of the last years research was the development, deployment, and evaluation of semiochemical-baited traps. Surveys for infested trees and signs of beetle presence are worldwide basically conducted by ground-surveyors in the case of CLB infestation and from the grown by using binoculars or through tree climbers, in ALB. These methods are costly and time consuming. In addition, for ALB, ground surveys are estimated to be only 30% effective at spotting infested trees when only oviposition sites are present, while even tree climbers are only about 60-75% effective (USDA-APHIS, 2013b) while for CLB, no statistical information related to the efficacy of the survey are available.

Consequently, the development of more efficient and cost-effective methods for the detection of infestations and for monitoring and verification of eradication programs is a top priority (Neheme *et al.*, 2010). ALB has been described as a somewhat sedentary species, which often re-infests the same host tree until it is exhausted as a resource (Williams *et al.* 2004). Mate finding and copulation appear to involve a complex series of behaviors, including responses to chemical and visual cues. Males produce a volatile pheromone (Zhang *et al.* 2002) that, when perceived in combination with certain plant-derived volatile compounds, attracts primarily virgin females (Nehme *et al.* 2010). Once males and females are in close proximity, mate finding appears to include additional visual and chemical cues, including a female-produced sex trail pheromone (laid down by the female as she walks across the host) that is attractive only to males (Hoover *et al.* 2014) and a female-produced contact pheromone that stimulates males to initiate mating (Zhang *et al.* 2003). In earlier field studies conducted in China, reported that a mixture of the plant volatiles linalool, linalool oxide, *cis*-3-hexen-1-ol, *trans*-pinocarveol and *trans*-caryophyllene, presented in combination with the male pheromone—a 1:1 mixture of 4-(*n*-heptyloxy)butan-1-ol and 4-(*n*-heptyloxy)butanal—significantly increased trap catches of females, of which 85% were found to be virgins (Neheme *et al.*,2010).

Based on the above researches developed in Massachusetts, a seasonal trapping study with the same lures and some additional ones from Russia, were initiated in Lombardy in 2013, within the ALB and CLB infested areas.

Traps were deployed throughout the Lombardy area of infestation (Milano and Brescia) with the primary goal to determine whether semiochemical-based traps can successfully capture beetles of ALB and eventually CLB.

MATERIAL AND METHODS

The trapping area is under both regional and state quarantine and the mission of the Plant Protection Service program is eradication. This meant that the number of trapped beetles would be expected to decrease as the eradication program removed beetle infested trees over time.

Trap Design and Deployment: woodborer panel traps (ChemTica, Costa Rica) retrofitted with locking twist off collection vessels and multi funnel traps (Econex, Spain) were used during 2013. Traps were coated with teflon spray to increase the slipperiness of the trap surface.

In 2013, a technicians from Fondazione Minoprio and ERSAF installed each trap by selecting a limb in the tree canopy. The top of the trap was attached to the limb into the tree trunk at least ~2 m above ground. This approach was intended to minimize public access to the traps. Deployed traps did not directly contact foliage or other limbs. Where possible, susceptible open-growing or forest-edge trees were selected for trapping so that beetles could easily fly into the traps and because the beetle is known to inhabit riparian edges in forests in its native habitat (Williams et al. 2004). Non-host tree species near *Acer* spp. were sometimes used when no other option was available in a given location. Trap height, tree species and latitude/longitude coordinates were recorded for each tree.

Figure 61

(a) traps distribution in Milano area
(b) traps distribution in Brescia area

(c) truck with platform used to hang up traps
(d) MFT

(e) ChemTica emitters
(f) CVT

Traps were regularly checked. All asian beetles caught in the traps were collected, placed in plastic bags and were frozen until assessed. Damaged specimens were counted, identified where possible and then discarded. Specimens in good shape were counted, and stored in alcohol or pinned for further analyses.

Trap Distribution: a spatial overview of the distribution of traps within the ALB and CLB infested areas of Lombardy Region, at Corbetta (Milan), Parabiago (Milan) and Brescia (Gussago and Montichiari) (Fig. 61) In 2013, 50 traps were deployed across the regulated area during May and August and removed during September. Traps were placed in areas where infested trees had previously been detected and, removed in surveyed areas.

All traps were removed during the last week of September.
Of the 50 traps, 35 of these were “Cross Vane Panel Traps” – CVT – and 15 “Multi-Funnel Traps” - MFT.

Traps were placed in three different dates: on May 24th (15 in ALB area and 1 in CLB area of Milan), on June 27th, (21 in ALB area and 3 in CLB area of Milan) and August 8th (9 in CLB area in Gussago – Brescia).
The medium average of the height from the ground level was 3,82 m.
The 50 trees on which the traps were hanged on were belonging to 9 genera, 2 these non host of the longhorn beetle.
The 64 % of the traps were placed on trees belonging to the maple's genera (*Acer* spp.)

Lures: The lures used for these studies included various mixtures of the plant volatiles linalool, linalool oxide, *cis*-3-hexen-1-ol, *trans*-pinocarveol, δ-3-carene, and *trans*-caryophyllene presented alone or in combination with the ALB male-produced pheromone, which is a 1:1 mixture of 4-(*n*-heptyloxy)butan-1-ol and 4-(*n*-heptyloxy)butanal (Zhang *et al.* 2002). The lure formulations and emitters were prepared by either ChemTica (ChemTica Internacional S.A., Heredia, Costa Rica) or Russian Dep. of Forest Quarantine (All-Russian Center of Plant Health Qaurantine, Bykovo, Moscow Region) and respectively supplied to us by the Penn State University (USA) and the University of Padua (Italy).
ChemTica emitters consisted of plastic pouches with color-coding for each chemical, and Russian emitters consisted of plastic bubbles caps.
Both lures were primarily produced to capture ALB but their efficacy was, for the first time, tested on CLB too.

RESULTS
In 2013, a total of 32 beetles were captured (Attachment 5).
A number of 28 CLB beetles were captured during the season (23 ♂ and 5 ♀). Captures were recorded in July (n° 11 specimens), August (n° 7) and September (n° 10). All the positive traps were located Legnano municipality (23 captures) and Gussago (5 captures).
Capture of adults were occurred in traps placed on *Acer*, *Platanus*, *Salix* and *Ostrya*.
A number of 4 ALB beetles were captured during the season (all the specimen were ♂) and occurred during July. These traps were located in Corbetta municipality (1 capture) and Vittuone (3 captures).
Encouraging results were obtained from this cooperative study that showed the efficacy of these traps in capturing CLB. This has been not known yet since the lures were initially patented to capture ALB. Studies to implement the efficacy of these lures are focused on the aldehyde component of the pheromone lure that has been stabilized (this component oxidizes rapidly, cross-linking and dropping rapidly in release rate after just a few weeks in the field). An increase of the release rate of the kairomones was also addressed.

Of the 50 traps, 9 of these have captured.
Of the 4 *A. glabripennis* captured, all were attracted with the ChemTica kairomone (100% delle catture).
21 *A. chinensis*, were captured with ChemTica kairomone. 7 were captured with the Russian lure.
Concerning the trap's type:
- 1 capture of *A. glabripennis* has been occurred with CVT;
- 9 captures of *A. chinensis* has been occurred with CVT;
- 0 captures of *A. glabripennis* has been occurred with MFT;
- 22 captures of *A. chinensis* has been occurred with MFT.

The most important data obtained from the study is the attractivity of these lures for CLB.

DISCUSSION

Given the host specificity of *A. anoplophorae* (Him: *Eulophidae*) for *Anoplophora chinensis* (CLB), the high rate of parasitism observed in some locations in Lombardy, and our current knowledge of the geographical distribution of the parasitoid, (firmly established in the core of the infestation around Parabiago - Milan) , it appears that the egg parasitoid is the best candidate for the biological control of the pest. Although continuation of pest eradication remains a high priority, releases of *A. anoplophorae* in the areas where it does not occur yet, should be done to help contain the pest as much as possible during the eradication effort.

Four major orientations are recommended for future work on evaluation of *A. anoplophorae* as a biological control agent against CLB:

1. study the development cycle and life history traits of the egg parasitoid;
2. study CLB - *A. anoplophorae* host-parasitoid relationship;
3. develop an *A. anoplophorae* rearing technique;
4. studies about the geographical distribution of *A. anoplophorae* around Parabiago, Italy.

The research plan for 2015 once more financed by the Lombardy Region and concerning biological control has the following specific objectives:

- Objective 1: assessing the increasing impact of the egg parasitoid in the area where it first established. To do so, in July 2015 in selected places, sentinel trees previously infested in laboratory with freshly laid CLB egg, will be exposed. Seven years after observations (in 2008 at Canegrate) of the impressive impact of the parasitoid, we would like to get data showing the current occurrence of *Aprostocetus*, in those sites, and the proportion of attacked hosts on sentinel trees.
- Objective 2: in July 2015, releases of the parasitoid will be done in areas where the beneficial organism does not occur yet, by means of exposing potted sentinel trees containing parasitized CLB eggs. We know that from the sentinel trees, some non-diapausing individuals of the parasitoid will emerge as adults in August 2015, and will attack host eggs in the surrounding natural infestation of CLB. We also know that in some of the parasitized CLB eggs on the sentinel trees, larvae of the parasitoid will enter diapause in July 2015 and the adults will emerge from July 2016. So, at least two cohorts of emerging *Aprostocetus* adults are expected (in August 2015, and July 2016) from the sentinel trees exposed in July 2015, and will contribute towards parasitoid establishment.
- Objective 3: In selected sites within the geographical distribution area of *Aprostocetus* around Parabiago, stumps of CLB infested trees will be collected during spring 2015, and stored in EBCL quarantine laboratory till the emergence of adults of the parasitoid, which will be used to attack freshly laid eggs of CLB and prepare the sentinel trees used for objective 2.

The use of sentinel tree technique will be not considered anymore in further trials but it might be implemented by the Plant Protection Service of Lombardy Region as an additional tool for detecting the beetle presence. In this case, it will be necessary to consider the costs of tree plantation and maintenance which require weekly watering to guarantee a good development of the crown and a certain attractiveness. In addition, trained and knowledgeable technicians will also be necessary to look daily for signs and symptoms of the beetle presence.

The number of captures recorded with baited traps has shown a high effectiveness of these tools in detecting beetles within invaded landscape. Moreover, the unexpected capability of capturing CLB, not only ALB, has increased the interest in their mass use

within Lombardy areas of infestation. In fact, in 2014 the system was implemented by the Lombardy Region Plant Protection Service which added 50 additional traps.

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Attachment 1 - Example of LOA issued in 2014 (from France to Italy)

Ministero delle politiche agricole alimentari e forestali

[illegible]

Roma

Al Servizio fitosanitario regionale
Via Pola, 12-14
20124 - MILANO

Oggetto: importazione dalla Francia di adulti di *Anoplophora chinensis* e di *Aprostocetus anoplophorae*.

Alla Fondazione Minoprio
Viale Raimondi, 54
22070 Vertemate con Minoprio (CO)

Si fa riferimento alla richiesta avanzata dalla Fondazione Minoprio di Vertemate con Minoprio (CO), per l'importazione nel territorio italiano di n. 200 adulti di *Anoplophora chinensis* e n. 200 adulti di *Aprostocetus anoplophorae* dalla Francia – European Biological Control Laboratory, USDA, ARS,Campus International de Baillarguet CS90013 Montferrier sur Lez 34988 Saint-Gély-du-Fesc Cedex, per scopo di sperimentazione e ricerca scientifica.

Al riguardo, acquisito il parere favorevole del Servizio fitosanitario della Regione Lombardia, si comunica l'approvazione, a norma degli articoli 45 e 46 del decreto legislativo 19 agosto 2005, n. 214, delle attività di ricerca in questione che saranno effettuate presso le strutture site all'indirizzo del richiedente.

Codesto Servizio fitosanitario, in considerazione dei rischi fitosanitari, deve verificare nella fase di sperimentazione la rispondenza del materiale alle condizioni previste nell'allegato XV del decreto leg.vo suddetto.

Inoltre, il suddetto Servizio deve inviare una relazione dettagliata alla fine della sperimentazione.

Il Responsabile del Servizio Fitosanitario Centrale
Dr. Bruno Caio Faraglia

IL DIRETTORE GENERALE
Dr. [illegible]

LETTERA DI AUTORIZZAZIONE

1. [illegible] European Biological Control Laboratory, USDA, ARS,Campus International de Baillarguet CS90013 Montferrier sur Lez 34988 Saint-Gély-du-Fesc Cedex	Lettera di autorizzazione [illegible]
2. [illegible] Dr. Matteo Maspero - Dr. Giovanni D'Angelo Lab. Fitopatologico C/o Fondazione Minoprio Centro MIRT – Fondazione Minoprio Viale Raimondi, 54 22070 Vertemate con Minoprio (CO)	3. [illegible] Servizio Fitosanitario Centrale - ITALIA
4. [illegible] Lab. Fitopatologico C/o Fondazione Minoprio Centro MIRT – Fondazione Minoprio Viale Raimondi, 54 22070 Vertemate con Minoprio (CO)	5. [illegible] Francia
7. [illegible]	[illegible]
8. [illegible] *Anoplophora chinensis* *Aprostocetus anoplophorae*	9. [illegible] n. 400
10. Tipo materiale Insetti adulti di *Anoplophora chinensis* e insetti adulti di *Aprostocetus anoplophorae*	
11. Dichiarazione supplementare Il presente materiale è trasportato nella Comunità ai sensi della direttiva 2008/61/CE	
12. Informazioni supplementari	
13. [illegible] Luogo del visto: Montferrier Data: 28 juillet 2014 Nome e firma del funzionario autorizzato: C. Pueyo	14. [illegible] Luogo del rilascio: Roma Data: Nome e firma del funzionario autorizzato: IL DIRETTORE GENERALE Dr. [illegible]

Example of LOA issued in 2014 (from Italy to France)

Communautés Européennes Lettre officielle d'autorisation n°2014/LR/LOA/00086

Nom, adresse de l'expéditeur/ organisation de protection des végétaux du pays d'origine **Dr Matteo MASPERO** **Centro MIRT Ricerca** **Fondazione Minoprio** **V. le Raimondi, 54 22070, Vertenate con Minoprio (CO)** **ITALIA** **Tél : 39 348 28 24 819**	**Lettre officielle d'autorisation** **pour l'importation des organismes nuisibles, des végétaux, des produits végétaux et autres objets pour des travaux à des fins d'essai ou à des fins scientifiques et pour des travaux sur les sélections végétales** ***(Délivrée conformément à la directive 2008/61/CE)***
2 Nom et adresse de la personne responsable des activités autorisées Dr Lincoln SMITH Directeur EBCL D. COUTINOT Responsable quarantaine European Biological Control Laboratory Campus International de Baillarguet CS 90013 Montferrier sur Lez 34988 St. Gely du Fesc France	**Nom de l'organisme officiel responsable de l'Etat membre de délivrance** **Direction Régionale de l'Alimentation, de l'Agriculture et de la Forêt. SRAL** **Maison de l'Agriculture Place Antoine Chaptal** **CS 70039 34 060 Montpellier Cedex 02**
Adresse et description du ou des sites spécifiques de maintien en quarantaine Quarantaine EBCL EBCL-USDA-ARS Campus International de Baillarguet 34980 Montferrier-sur-Lez Tel.04 99 62 30 42 (30 00) - Fax. 04 99 62 30 49 dcoutinot@ars-ebcl.org	**5. Lieu d'origine (avec, jointe, la preuve documentaire)** **Italie**
7. Point d'entrée déclaré pour le matériel introduit d'un pays tiers Roissy (PEC) ou autre point d'entrée communautaire déterminé par le transporteur	**6. Numéro du certificat phytosanitaire**
8. Nom(s) scientifique(s) du matériel, y compris les organismes nuisibles concernés *Anoplophora chinensis* (Forster) (Coleoptera : Cerambycidae)	**9. Quantité de matériel** 500 œufs 500 larves 500 nymphes 500 adultes
10. Type de matériel Œufs, larves, nymphes, adultes d'*Anoplophora chinensis* ses parasitoïdes, prédateurs et entomopathogènes	
11. Déclaration supplémentaire **Ce matériel est importé dans la Communauté conformément à la directive 2008/61/CE** **Le Laboratoire EBCL bénéficie d'un agrément pour son activité de quarantaine** **Matériel destiné à des travaux scientifiques dans le domaine phytosanitaire**	
12. Information supplémentaire Introductions prévues du 1er juillet 2014 au 30 juin 2015. Tous les bois et végétaux importés, ainsi que ceux utilisés en quarantaine subiront un passage à l'autoclave et seront ensuite incinérés après leur sortie de la quarantaine. Personnel impliqué : F. HERARD & N. RAMUALDE (EBCL) ; M. SMITH (BIIRL, USDA-ARS, Newark, DE)	
13. Endossement par l'organisme officiel responsable de l'Etat membre d'origine du matériel **Lieu d'endossement :** **Date :** 19 SET. 2014 **Nom et signature du fonctionnaire autorisé :** Beniamino Cavagna SERVIZIO FITOSANITARIO NAZIONALE – SERVIZIO FITOSANITARIO REGIONALE – REGIONE LOMBARDIA – ITALIA	**14. Cachet de l'organisme officiel responsable de délivrance** **Lieu de délivrance : Montpellier** **Date : 30 Juillet 2014** **Nom et signature du fonctionnaire autorisé :** **C. PUEYO** RÉPUBLIQUE FRANÇAISE – SERVICE RÉGIONAL DE L'ALIMENTATION – MINISTÈRE DE L'AGRICULTURE

Cette autorisation est valable du 1er juillet 2014 au 30 juin 2015

Attachment 2 - Example of “A Quit de droit”

A QUI DE DROIT

Dr. Matteo Maspero, Chercheur Entomologiste de Fondazione Minoprio, V.la Raimondi, 54 – 22070, Vertemate con Minoprio (CO) Italie, tél. +39031900224 (int. 242), conduit ce véhicule transportant des souches d'arbres conditionnées dans des malles hermétiquement closes et cadenassées pour empêcher la dispersion des organismes vivants contenus dans ces souches.
Les organismes vivants sont des larves d'insectes creusant des galeries dans le bois. Le ravageur est une espèce de Coléoptère Longicorne, espèce invasive d'origine asiatique *Anoplophora chinensis* (espèce inscrite à l'annexe 1 de la directive 2000/29/CE). Sont également présents dans l'écorce, des œufs du ravageur parasités par l'auxiliaire Eulophidae, *Aprostocetus anoplophorae*.

Adulte *Anoplophora chinensis* — Larve *Anoplophora chinensis* — Œuf *A. chinensis*

Ce matériel vivant circule accompagné d'une lettre officielle d'autorisation (LOA) délivrée par le Service de Protection des Plantes de la Région Languedoc-Roussillon, endossée par le Service de Protection des Plantes de Lombardie, Italie (LOA 12LR005).

Ce matériel vivant circule conformément à la directive 2008/9/CE et est destiné au Laboratoire EBCL qui bénéficie d'un agrément Quarantaine végétale (arrêté préfectoral n°090473 du 22 juillet 2009 valable cinq ans).

Ces malles ne peuvent être ouvertes en dehors d'un espace de quarantaine, elles seront ouvertes dans la quarantaine du Laboratoire EBCL et les organismes vivants étudiés en conditions de quarantaine.

Pour tout problème éventuel ou demande d'information complémentaire, merci de bien vouloir contacter : M. Franck Hérard, Responsable du projet *Anoplophora*, EBCL, au 0620436340 ou M. Dominique Coutinot, Responsable de l'activité quarantaine, EBCL au 0609380131.

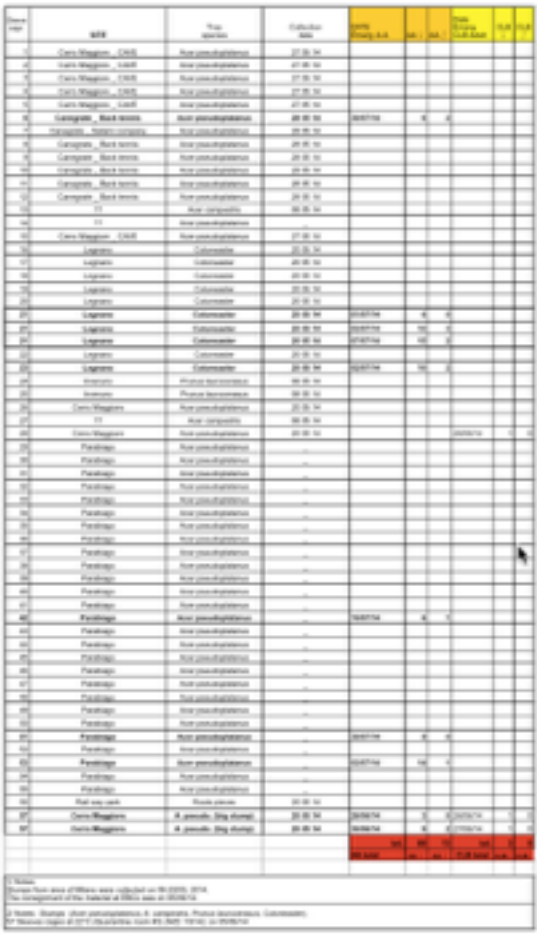
Ces espèces sont sans danger pour la santé humaine et animale.

Cette importation est conforme à la Convention sur le Commerce International des Espèces de Faune et de Flore Sauvages menacées d'extinction, Washington, 3 mars 1973 (CITES) ; amendée à Bonn le 22 juin 1979. Ces espèces ne sont pas inscrites sur les annexes de la dite Convention.

Le 13 mars, 2012

European Biological Control Laboratory (EBCL-USDA-ARS)
Campus International de Baillarguet
34980 Montferrier-sur-Lez
Tel: 0499623000 Fax 0499623049

Attachment 3:



Attachment 4:

FIELD EXPERIMENT ON RELEASE - CAPTURE OF *Aprostocetus anoplophorae* ON SENTINEL TREES IN CLB-INFESTED AREAS IN ITALY (2012-2013) (DATA PER SITE)

SITE	ZONE 1 (14- Sep, 2012)				ZONE 2 (18 - 30 Jun, 2013)				ZONE 3 (2 - 15 Jul, 2013)				OFF zones			
		CLB eggs				CLB eggs				CLB eggs				CLB eggs		
	% CLB slits with an egg	% predated	% parasitized	% hatched	% CLB slits with an egg	% predated	% parasitized	% hatched	% CLB slits with an egg	% predated	% parasitized	% hatched	% CLB slits with an egg	% predated	% parasitized	% hatched
BIC	46,6	100	0	0	67,5	66,6	0	33,3	36,5	26,6	0	73,3				
PDC	6,4	100	0	0	28	57,1	0	42,8	15	66,6	0	33,3	0			
ASQ	31,5	83,3	0	16,6	56	42,8	10,7	46,4	60,7	35,2	11,7	52,9				
ASM	17,1	83,3	0	16,6	44,4	100	0	0	0				0			
MON	30,4	57,1	14,2	28,5	52,9	55,5	0	44,4	60	45,8	0	54,1	52,3	90,9	0	9
GUS	18,6	62,5	0	37,5	27,5	25	0	75	12,5	100	0	0	97,3	13,5	0	86,4

Attachment 5:

ALB and CLB monitoring trap- 2013

CVT	Municipality	Street	Tree species	h from ground level (m)	Exposure 24/5/2013	Check 26/06/13	Lure Replacement 26/06/13	Exposure 27/06/13	Check 16/07/13	Lure Replacement 01/08/13	Check 01/08/13	Exposure 06/08/13	Check 03/09/13
ALB area													
A1	Vittuone	Zara	*A. pseudoplatanus*	4	x	negative	√		negative	√	negative		negative
A2	Vittuone	Gramsci	*A. pseudoplatanus*	3,5	x	negative	√		negative	√	negative		negative
A3	Vittuone	Trento	*A. saccharinum*	3,5	x	negative	√		negative	√	negative		negative
A4	Sedriano	Gagari	*A. pseudoplatanus*	3,5	x	negative	√		negative	√	negative		negative
A5	Sedriano	Colombo	*A. saccharinum*	3	x	negative	√		negative	√	negative		negative
A6 TNT	Vittuone	Zara	*A. saccharinum*	4				x	negative		negative		negative
A7	Vittuone	Zara	*A. pseudoplatanus*	3	x	negative	√		negative	√	negative		negative
A8	Vittuone	Restelli	*P. nigra italica*	2,5	x	negative	√		negative	√	negative		negative
A9	Vittuone	Gandhi	*P. nigra italica*	2,5	x	negative	√		negative	√	negative		negative
A10 TNT	Vittuone	Gandhi	*A. negundo*	4				x	negative		negative		negative
A11	Vittuone	Monviso	*B. pendula*	4	x	negative	√		negative	√	negative		negative
A12	Vittuone	Cavour	*A. pseudoplatanus*	4	x	negative	√		negative	√	negative		negative
A13	Vittuone	Fiume	*P. nigra italica*	5,5	x	negative	√		negative	√	negative		negative
A14	Vittuone	Pascoli	*U. pumila*	2,5	x	negative	√		negative	√	negative		negative
A15 TNT	Vittuone	Pascoli	*U. pumila*	2,5				x	negative		negative		negative
A16 V1	Vittuone	Pascoli	*U. pumila*	3,5				x	negative		negative		negative
A17 V3	Vittuone	IV Novembre	*A. pseudoplatanus*	3				x	negative		negative		negative
A18 V3	Vittuone	Marzorata	*A. negundo*	3				x	negative		negative		negative
A19	Vittuone	Casa Portaluppi	*A. pseudoplatanus*	5,5	x	negative	√		negative	√	negative		negative
A20	Corbetta	Casa Oldani	*A. pseudoplatanus*	3	x	negative	√		Positive 1 ↓	√	negative		negative
A21 V1	Corbetta	Casa Oldani	*S. alba*	2				x	negative		negative		negative
A22 V2	Corbetta	Zanella	*A. saccharinum*	4				x	negative		negative		negative
A23 V4	Vittuone	Monte Bianco	*A. pseudoplatanus*	4				x	negative		negative		negative
A24	Corbetta	Zara	*M. nigra*	3	x	negative	√		negative	√	negative		negative
A25	Corbetta	Paganini	*A. pseudoplatanus*	3,5	x	negative	√		negative	√	negative		negative
CLB area													
A26	Legnano	Molini	*Platanus x acerifolia*	6	x	negative	√		negative	√	Positive 2 ↓		Positive 1
A27 V2	Legnano	Molini	*A. pseudoplatanus*	6				x	Positive 1 ↓		negative		negative
A28	Gussago	Cudula Campo	*A. campestre*	4								x	negative
A29	Gussago	Manica	*Ostrya carpinifolia*	4,5								x	Positive 5
A30	Gussago	sentiero Val Volpara	*A. campestre*	3,5								x	negative
A31	Gussago	Fontana	*A. campestre*	4								x	negative
A32	Gussago	vicolo Mincio	*A. campestre*	3								x	negative
A33	Gussago	sentiero Strada Sentissimo	*Ulmus minor*	5								x	negative
A34	Gussago	Favoni	*A. pseudoplatanus*	4								x	negative
A35	Rodengo Saiano	Montello	*U. minor*	4								x	negative

MFT	Municipality	Street	Tree species	h from ground level (m)	Exposure 27/06/13	Check 16/07/13	Lure Replacement 01/08/13	Check 01/08/13	Exposure 06/08/13	Check 03/09/13
ALB area										
B1 V1	Sedriano	Gagari	*A. negundo*	3,5	x	negative		negative		negative
B2 V3	Sedriano	Colombo	*A. saccharinum*	3	x	negative		negative		negative
B3 TNT	Sedriano	Gagari	*A. pseudoplatanus*	3,5	x	negative		negative		negative
B4 V1	Vittuone	Zara	*A. pseudoplatanus*	2,5	x	negative		negative		negative
B5 TNT	Sedriano	Zara	*A. pseudoplatanus*	3	x	negative		negative		negative
B6 V3	Vittuone	Restelli	*P. nigra italica*	3,5	x	negative		negative		negative
B7 V1	Vittuone	Gandhi	*A. negundo*	4	x	negative		negative		negative
B8 AM	Vittuone	De Amicis	*S. alba*	4	x	Positive 1 ↓	√	negative		negative
B9 AM	Vittuone	Carducci	*A. pseudoplatanus*	5,5	x	Positive 1 ↓	√	negative		negative
B10 AM	Vittuone	Casa Portaluppi	*A. negundo*	7	x	Positive 1 ↓	√	negative		negative
B11 V3	Corbetta	A. Giussano	*A. saccharinum*	3	x	negative		negative		negative
B12 TNT	Vittuone	Zanella	*A. saccharinum*	4	x	negative		negative		negative
CLB area										
B13 AM	Legnano	Molini	*Platanus x acerifolia*	6	x	Positive 5 ↓		Positive 5 ↓		Positive 3 ↓
B14 V4	Legnano	Molini	*Platanus x acerifolia*	6	x	Positive 6 ↓		negative		negative
B15 AM	Rodengo Saiano	Montello	*Corylus avellana*	4					x	negative