1.5 Accidental Introduction into Italy and Establishment of *Aprostocetus fukutai* (Hymenoptera: Eulophidae) in Citrus Longhorned Beetle Infestations

F. Hérard[1], M. Maspero[2] and M.C. Bon[1]

[1]USDA-ARS European Biological Control Laboratory, Montferrier-sur-Lez, Hérault, FRANCE, fherard@ars-ebcl.org, mcbon@ars-ebcl.org, [2]Fondazione Minoprio, Vertemate con Minoprio, Como, ITALY, m.maspero@fondazioneminoprio.it

Citrus longhorned beetle (CLB), *Anoplophora chinensis* (Förster) (Coleoptera: Cerambycidae), was accidentally introduced from Asia into 11 countries of Europe and neighbouring states, including Italy, France, the Netherlands, Switzerland, England, Croatia, Germany, Guernsey, Lithuania, Denmark, and Turkey, putting at risk a wide range of broadleaf trees. The destruction of the entire infested trees containing the damaging larval stages of CLB is the preferred method for eradicating the pest. Eradication efforts are mandatory and have been successful in the localities where early detection and rapid action were possible. As of 2017, eradication of CLB has not been achieved in Croatia and Italy. Since 2004, the Plant Protection Service of Lombardy, Italy removed thousands of infested trees. However, in 2015 small residual pest populations were found again in the Parabiago area and the eradication efforts are continuing.

The gregarious egg parasitoid *Aprostocetus fukutai* Miwa & Sonan (Hymenoptera: Eulophidae), which is native to Asia was discovered in CLB infestations near Parabiago, Italy in 2002. Initially, the egg parasitoid was thought to be a new Asian species of Aprostocetus, which was described as Aprostocetus anoplophorae (Delvare *et al.,* 2004). Recent collections of the CLB egg parasitoids in China and Japan, new morphological studies and comparisons with the individuals from Italy, and biomolecular data showed evidence of the synonymy between *A. anoplophorae* Delvare and *A. fukutai*, and Japan was found to be the country of origin of the population established in Italy (Bon *et al.*, unpublished data).

Geographical distribution of *A. fukutai*: In Italy, the parasitoid is not present in all CLB infestations of Lombardy. Its geographical distribution was determined by sampling CLB eggs in the field populations, and by exposing in the field potted sentinel trees containing CLB eggs that had been laid in the laboratory, to attract the parasitoid. In 2010, *A. fukutai* was established in the central area of the CLB infestations around Parabiago, and absent from the other infestations of Lombardy.

Development cycle: *A. fukutai* is a gregarious egg parasitoid of CLB that spends winter in diapause as a full-grown larva in the closed host egg shell. Depending on year (2003-2015), emergence of parasitoid adults from the host eggs collected in the field during diapause started in June or early July and extended for one or two months, reaching 50% of the cumulative emergence between late June and late July, which is in synchrony with the peak of egg deposition of its host.

In laboratory rearings of *A. fukutai*, among the CLB eggs that were parasitized in early summer of year 'n', the parasitoid larvae entered diapause in 83.2 ± 2.9% (mean ± SE) of the hosts. Diapause termination took place in late spring of year 'n+1', and the adults emerged in June-July. In the remaining 16.8 ± 2.9% of the host eggs parasitized in early summer of year 'n', the parasitoid larvae did not enter diapause, and a summer generation developed in 49.8 ± 0.6 days (mean ± SE), with adults emerging in late August to early September of the same year. In the field, the presence in early fall of an active population of adult parasitoids was revealed during the exposure of potted sentinel trees baited with CLB eggs: for instance in 2014 at Assago, 47.8 % of the exposed eggs were attacked by *A. fukutai*. In the field and in the laboratory, in all host eggs attacked in September, the parasitoid larvae entered diapause until early summer of year 'n+1'. Thus, both cohorts of parasitoid larvae could enter diapause, which started in mid summer and fall, respectively. There was a statistically significant difference ($P < 0.001$) in the mean duration of development from egg to adult (including diapause) between the two cohorts, which developed in 347.2 ± 0.8 days, and 284.1 ± 1.9 days (mean ± SE), respectively. The main effect of this difference was the synchronization of emergence in early summer of the first *A. fukutai* adults from both cohorts.

Gregariousness and sex ratio: *A. fukutai* females lay a cluster of eggs within a host egg and the parasitoid larvae develop gregariously. The mean number of parasitoids per host egg, calculated from the adults emerged in samples of eggs attacked in the field in Italy was 10.34 ± 0.29 (min = 1; max = 34). The immature stages in a given host egg develop synchronously, and the adults emerge altogether on the same day thru a single exit hole. The mean sex-ratio calculated from the adults that emerged from samples of eggs attacked in the field in Italy was 1 : 5.00 ± 0.29 (1♂ : ♀ ± SE).

Impact of *A. fukutai* on CLB in the field in Italy: In early spring 2008 (during dormancy of immature CLB and diapause of the *A. fukutai* larvae), a sample of 60 stumps of CLB-susceptible trees were collected in a woodlot at Canegrate, and stored individually in emergence bags in quarantine. From the adult beetles and parasitoid adults that emerged in summer 2008, we determined that 38 trees had been infested in 2007 with an overall number of 136 CLB eggs, among which 38 eggs were not parasitized and developed to adult stage, and 98 eggs (72%) were parasitized and produced 1,124 adults of *A. fukutai*. This showed that, in an introduction area, a coadapted Asian egg parasitoid may have a significant impact on an *Anoplophora* species. For comparison, in 2014 in the native range, in an abandoned orchard of pomelos at Meizhou, Guangdong, China, the rate of parasitism of CLB eggs by *A. fukutai* was 79.6%.

Following the substantial CLB eradication efforts by the Italian authorities, the pest population has been heavily depressed, and this has also affected the parasitoid populations. In 2015, exposing potted sentinel trees baited with CLB eggs, and collecting small samples of CLB-infested trees in the historical geographical distribution area of *A. fukutai*, we have shown the persistence of a low level population of the parasitoid in 50% of the sites (4 out of 8 sites) where *A. fukutai* had been abundant until 2010. Thus, *A. fukutai* is still present to attack the pest in the host trees which have yet remained undetected, and it can also serve as an indicator of the presence of residual populations of CLB, where and when the parasitoid is captured in CLB egg-baited sentinel trees.

Host specificity of *A. fukutai*: In 2003, in the laboratory, preliminary no-choice tests were made using logs infested with either CLB eggs, or ALB eggs (Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambycidae)). The logs were caged individually with 3 females of *A. fukutai*. Five CLB eggs were parasitized, while ALB eggs were not attacked. During summer 2003, in a CLB infestation at Parabiago where *A. fukutai* was established, choice tests consisted of exposing, during 2 weeks (4 times from mi-July thru early September), CLB- and ALB-infested logs together in the same plot to attract parasitoids. In 21 logs containing 114 CLB eggs, 13 CLB eggs were parasitized by *A. fukutai*, while in the 29 logs containing 113 ALB eggs, none egg was attacked. During summer 2004 in the same site new choice tests were carried out from June 1st thru mid July: in 41 logs containing 229 CLB eggs, 17 CLB eggs were parasitized, and in 10 logs containing 62 ALB eggs, one ALB egg was attacked. This egg contained 2 parasitoid larvae that reached pupal stage. As one pupa died, and the second did not molt as an adult, a molecular genetic analysis was made before its death. The analysis confirmed strict similarity with the CLB egg parasitoid. However, we considered this unique attack of an ALB egg by *A. fukutai* as a fluke. During summers 2005 and 2006, in the same site new choice tests were made: while some CLB eggs were found parasitized, no other ALB egg was attacked. In 2012, in the laboratory, no-choice tests were made to test *A. fukutai* individuals that emerged from CLB eggs collected in a citrus orchard in Yancun, Guangdong, China. Thirty four *Salix* (Salicaceae) logs were used in these tests, and the target hosts were eggs of CLB, ALB, and *Lamia textor* (L.) (Coleoptera: Cerambycidae) (LT). The logs were caged individually with one female *A. fukutai* for 10-14 days. Twelve logs contained an overall 99 CLB eggs, 11 logs contained 127 ALB eggs, and 11 logs contained 42 LT eggs. *A. fukutai* parasitized 32 CLB eggs, but none of the ALB or LT eggs were attacked. We think the conjunction of these results argue for a strict specificity of *A. fukutai* for CLB.

Moderate dispersal ability of *A. fukutai*, and release technique to spread the parasitoid in isolated pest populations: The moderate individual flight and dispersal abilities of *A. fukutai* seem to be governed by a few endogenous and exogenous factors: the host and the parasitoid females show a similar search pattern for oviposition sites, in that they tend to remain in the same site (one tree or a small group of trees) as long as the resource for oviposition sites is sufficient. The successive generations of CLB females lay eggs in the collar zone of trees that have been previously attacked by their parents, often until no more space with adequate quality bark is available to make oviposition slits. As the *A. fukutai* adults emerge from host eggs that are often located a few centimeters from freshly made egg slits, they do not need to fly a long distance to find hosts. Thanks to its gregariousness and to its apparent sedentary nature, the parasitoid has a high potential to quickly increase its population, and control the pest locally. However, as the dispersal ability of CLB widely exceeds that of the egg parasitoid, the geographical distribution of both insect species tends to be a mosaic of isolated plots with and without presence of the parasitoid. In urban environments, the patchy distribution of the parasitoid is aggravated by exogenous factors like the presence of extended blocks of buildings, industrial plants without a host tree, zones of unsuitable host plants, or other obstacles that affect more the movements of *A. fukutai* than those of the CLB females. In such a situation, intentional introductions of the parasitoid to isolated pest populations could make up for the parasitoid lagging behind its host. The strict specificity of the parasitoid to CLB, and perfect fit with the biology of its host make *A. fukutai* a potentially excellent candidate for classical biological control of the pest where it has been introduced. In the parasitoid-host (*A. fukutai*-CLB) system, which involves a strictly specific parasitoid, the accidental introduction of the parasitoid in Italy was an advantage, but it obviously was insufficient to control the pest, in particular because urban environments create many artificial obstacles to the natural spread of the parasitoid.

We started testing a release technique to spread the parasitoid in isolated CLB populations, using potted sentinel trees infested with parasitized CLB eggs. These were set up in the release sites for the entire diapause of the parasitoid, and thus were subjected to the same climatic conditions as the hosts in the hope to obtain a better synchronization of their emergence. In addition, we recommend to let 2-3 CLB larvae develop in the release tree, which is securely covered with a closed cylinder of strong wire cage with fine mesh (3-5 mm) to retain the emerging CLB adults, and let the parasitoid adults fly away and establish in the surrounding local host populations. On the release tree, the emerged CLB adults feed on the new shoots, mate, and deposit eggs in the collar zone. In this way, some of the newly emerged parasitoids can attack the freshly laid CLB eggs on the release tree, ensuring the occurrence of a second generation of *A. fukutai* including the two cohorts, one not diapausing to release new parasitoids in late summer, and the other diapausing to release new parasitoids in early summer of the next year.

References

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