

# First record and characterization of *Aganaspis daci* (Weld, 1951) (Hymenoptera, Figitidae, Eucoilinae), a parasitoid of fruit flies, from Italy

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## Abstract

*Aganaspis daci*, a larval-pupal parasitoid of several tephritid species, was unexpectedly recovered in the Campania region (Southern Italy), where it had not been intentionally released. An integrative approach was used to conduct a comprehensive characterization of this parasitoid, confirming its identification through a comparison with specimens obtained from laboratory rearing. While *A. daci* emerged from puparia of *Ceratitis capitata* during this study, its original association was recorded with *Bactrocera dorsalis*. The presence of *A. daci* in Italy highlights its successful establishment, possibly facilitated by the recent invasive process of its host, *B. dorsalis*, offering promising prospects for future tephritids control strategies. It is intriguing to note that the mt-haplotypes found in Italy were only partially shared with those observed in specimens originating from a Spanish rearing, suggesting likely distinct origins for at least part of the Italian population.

## Keywords

*Bactrocera dorsalis*, biological control, *Ceratitis capitata*, Medfly, oriental fruit fly, Tephritids

## Introduction

The family Tephritidae (Diptera) commonly recognized as fruit flies, includes numerous damaging and invasive pests that pose a serious concern for agricultural production worldwide (Scolari et al. 2021). These fruit flies have the potential to establish into new areas, leading to ecological imbalances and environmental and economic consequences (Szyniszewska et al. 2014). People movements and import-export trade activities are the main factors that allow the transportation of arthropods from one continent to another (Lichtenberg and Olson 2018; Pace et al. 2022).

Among the fruit flies, *Ceratitis capitata* (Wiedemann, 1824) and *Bactrocera dorsalis* (Hendel, 1912) (Diptera: Tephritidae), along with other species belonging to the *B. dorsalis* complex, exhibit particularly high invasive potential. These entities share common biological traits such as high polyphagy, short life cycles, and excellent adaptive capacities (Pieterse et al. 2020).

*Ceratitis capitata*, commonly known as the Mediterranean fruit fly (Medfly), is native to sub-Saharan Africa. Since its initial discovery in some Southern European countries in the 19<sup>th</sup> century, this species has rapidly spread to several countries worldwide, often using European countries as bridgeheads. Additionally, this species is increasingly being detected in areas formerly free of infestation, such as Florida and California, where control and eradication strategies are being implemented (Calla et al. 2014; Szyniszewska et al. 2014).

On the other hand, *B. dorsalis*, native to Asia, has invaded a significant portion of the African continent (Goergen et al. 2011). It was first intercepted in Italy (Campania Region) in 2018 (Nugnes et al. 2018) and subsequently in 2019 (Gargiulo et al. 2021). However, active infestations of this species on different fruits were recorded in Italy for the first time in 2022, indicating a considerable shift in the scenario, and posing a significant challenge to the agriculture of several European countries (Mertens et al. 2022; Bernardo et al. 2023a; Nugnes et al. 2023). Both entities, *B. dorsalis* complex and *C. capitata* exhibit a wide host range, causing damage to 300 plant species including various fruits and vegetables (Gilioli et al. 2022). However, it is important to heed that the accurate host range of the *B. dorsalis* complex remains a challenge due to possible confusion with related species (Mertens et al. 2022).

The damage caused by these species is mainly due to larval trophic activity, which ultimately leads to fruit collapse and can result in the loss of fruits of high commercial value such as *Citrus* spp., *Malus* spp., *Diospyros* spp., and *Prunus* spp. (Shelly 2014).

Implementing phytosanitary measures is crucial for controlling these harmful species. Furthermore, as the number of active ingredients and the amounts allowed in cultivation continually decrease, an integrated approach that includes parasitoids has become essential (Jacquet et al. 2022).

Classical biological control, accomplished through the introduction of one or more parasitoids from the native area of the phytophagous pest, often represents the most effective and cost-efficient approach (Moore 2021). However, the effectiveness

of natural enemies can vary and be influenced by abiotic conditions, with the species' thermal requirements determining their success or failure (Adly 2016).

Biological control offers significant advantages, including enhanced security and cost-effectiveness. Numerous successful cases of tephritids management using parasitoids have already been documented, particularly in subtropical and tropical regions (Wharton 1997; Purcell 1998; Ovruski and Aluja 2000).

However, the introduction of exotic parasitoids for biological control purposes is a complex process that involves strict regulations and extensive preliminary studies. Conducting risk assessments can be time-consuming and expensive, resulting in bureaucratic challenges and delays in obtaining authorization for the release of biocontrol agents (Gay 2012; Barratt et al. 2018; Bernardo et al. 2023b).

However, the initial step of this lengthy process involves studying the indigenous parasitoid complex that has rapidly adapted to invasive pests. In addition, the parasitoid complex, both on endemic and invasive species, is constantly evolving, both qualitatively (different species) and quantitatively (varying percentages of relative parasitisation over time and space) (Mirchev et al. 2004). The parasitoid complex associated with tephritids present in Europe (Clausen et al. 1965; Papadopoulos and Katsoyannos 2003; Viggiani et al. 2006; Sasso et al. 2019) has been strongly influenced by the release of numerous parasitoids by Professor Silvestri (Silvestri 1916; Silvestri 1938) and other scientists (Marucci et al. 1952; Bokonon-Ganta et al. 2019; Wang et al. 2021; Coelho et al. 2023).

The aims of this study were manifold. First, we aimed to report the results of a survey conducted on parasitoids developing on *C. capitata* and/or *B. dorsalis* in Campanian fields. Second, we aimed to characterize the recorded parasitoid using a comprehensive integrative approach. This included a comparison with specimens obtained from a laboratory rearing in Spain, which, in turn, allowed us to infer the possible origins of the Italian population. Therefore, we assessed the establishment and distribution of this parasitoid in Campania (Italy), with a particular focus on territories affected by the recent invasive process of *B. dorsalis*. Lastly, the potential implications of the presence of the parasitoid in Italy for future tephritids control strategies are discussed.

## Materials and methods

### Monitoring sites and fruit samplings

As part of the compulsory monitoring of *B. dorsalis* and other non-European fruit flies from 2022 to the May of 2023, a fruit sampling with damages ascribable to fruit flies was conducted in various locations within the Campania region. Most samples were collected in mixed fruit-trees fields where the sampled fruit species varied with the progression of the seasons (Table 1). In each monitored area twenty fruits were collected from the ground and twenty directly from trees. The date and place of collection, along with the host plant species, were recorded for each sample.

**Table 1.** Locality, date of collection, sex, mt-haplotype, and Genbank accession number of *A. daci* specimens studied in the present work (codes in italics: molecularly characterised samples).

Locality	Coordinates	Date of collection	Specimen code	Number of specimens and sex	Mt-haplotype	Genbank accession number COI	ITS2-28S
Palma Campania (Na, Italy)	40°50'15"N, 14°32'54"E	13-Jan-23	Ad_1-Ad_7	5♀, 2♂	-	-	-
	40°51'36"N, 14°33'20"E	25-Oct-22	Ad_8, Ad_9, Ad_11-Ad_21 <i>Ad_10</i>	6♀, 7♂ 1♀	- Ha	- OR157906	- OR166972
	40°51'52"N, 14°33'7"E	24-Oct-22	Ad_22, Ad_24-Ad_26 <i>Ad_23</i>	2♀, 2♂ 1♀	- Hb	- OR157907	- OR166973
	40°52'46"N, 14°32'59"E	14-Sep-22	Ad_27, Ad_28	2♂	-	-	-
	40°51'41"N, 14°33'19"E	19-Oct-22	Ad_29	1♀	-	-	-
	40°52'5"N, 14°33'8"E	21-Sep-22 19-May-23	Ad_30 Ad_31	1♀ 1♀	- -	- -	- -
Portici (Na, Italy)	40°48'50"N, 14°20'47"E	4-Jul-23	Ad_60-Ad_62 <i>Ad_59</i>	1♀, 2♂ 1♂	- Hc	- OR536574	- OR539752
Quindici (Av, Italy)	40°52'12"N, 14°38'32"E	9-Nov-22	Ad_40-Ad_42, Ad_44 <i>Ad_43</i> <i>Ad_45</i>	2♀, 2♂ 1♀ 1♂	- Hb Hb	- OR157909 OR157910	- OR166975 OR166976
Sant'Agnello (Na, Italy)	40°37'4"N, 14°24'19"E	13-Jan-23 25-Oct-22	Ad_32-Ad_33 Ad_34-Ad_37, Ad_39 <i>Ad_38</i>	2♀ 3♀, 2♂ 1♀	- - Ha	- - OR157908	- - OR166974
Sant'Egidio del Monte Albino (Sa, Italy)	40°43'45"N, 14°35'15"E	03-Nov-22	Ad_46, Ad_47 <i>Ad_48</i>	1♀, 1♂ 1♂	- Ha	- OR157911	- OR166977
Moncada (Spain) rearing	39°35'21"N, 0°23'43"E	06-Mar-23	<i>Ad_49</i> <i>Ad_50</i> <i>Ad_51</i> <i>Ad_52</i> <i>Ad_53</i> Ad_54-Ad_58	1♀ 1♀ 1♀ 1♀ 1♀ 5♂	Hc Hc Hc Hc Hc -	OR157912 OR157913 OR157914 OR157915 OR157916 -	OR166978 OR166979 OR166134 OR166980 OR166981 -

The samples were transported in double-sealed bags to the CNR-IPSP laboratory. To allow mature larvae to pupate, infested fruits were isolated in plastic bugdorms (45×45×45 cm) and placed in a climatic chamber with the following conditions: 25±2 °C, 65±10% relative humidity and a 16:8 (L:D) photoperiod. Puparia were individually isolated in glass vials under the previously mentioned environmental conditions, and species and sex of the emerged tephritids and/or natural parasitoids were recorded. When a parasitoid emerged from an isolated puparium, the host species was identified by examining the mandible shape of the mature larva inside (Sabatino 1974; Balmès and Mouttet 2017). Sex ratio of parasitoids was calculated as in de Pedro et al. (2017a).

### Morphological identification

Both tephritids and emerged parasitoid specimens (Table 1) were examined under a Leica M165C auto montage microscope (Leica Microsystems, Mannheim, Germany)

equipped with a Leica DFC450 digital photo camera. The multifocal images were assembled using the Leica Application Suite software version 3.8.0 (Leica 2011). Some adult parasitoids were slide-mounted in Canada balsam phenol and observed under a Zeiss Axiophot 2 microscope (Carl Zeiss, Oberkochen, Germany).

To determine the genus of the emerged parasitoids, the key by Forshage and Nordlander (2008) was used. For species level, the keys Lin (1987) and Diaz and Gallardo (2001) were used. After the initial identification, a morphological comparison was conducted between the Italian specimens and twelve (6 males and 6 females) Spanish adults of *Aganaspis daci* (Weld 1951) (Hymenoptera: Figitidae: Eucoilinae).

The Spanish samples were provided in absolute alcohol, each contained within its own individual vial, and were obtained from the laboratory colony of the Valencian Institute of Agrarian Research (IVIA, Valencia, Spain). This colony was established in 2010 using several specimens obtained from medfly larvae collected from figs in a village near Valencia (Bétera, Spain) (de Pedro et al. 2016).

### DNA extraction, amplification and sequencing

Twelve adults (Table 1) were selected for molecular analysis and were singularly placed in Eppendorf containing 95% ethanol and preserved at -20 °C until use.

The DNA extraction from metasoma and legs (which do not present taxonomic characters at species level) was performed using a destructive method based on Chelex–proteinase K- protocol described in Gebiola et al. (2009). Part of the mitochondrial cytochrome oxidase C subunit I (COI) gene was targeted as it is a widely used marker for species-level systematics. PCR amplifications were performed in a 10 µl reaction volume on an Eppendorf Nexus GX2 thermocycler using primers as in Schulmeister et al. (2002) and the primer pair LepF1/LepR1 as described in Hajibabaei et al. (2006). The thermocycler conditions were set according to Nugnes et al. (2017). Furthermore, the inclusion of additional markers (ITS2-28S\_D1\_D2) for specimen characterization is motivated by situations where COI is shared among different species, thus necessitating the use of extra markers to distinguish between these entities (Nugnes et al. 2017; Bernardo et al. 2021; Wacławik et al. 2021).

Hence, the ribosomal gene ITS2, along with the expansion segments D1-D2 of the 28S ribosomal subunit (ITS2-28S\_D1\_D2) (~ 1200 bp), was amplified with primers ITS2F and D2R (Campbell et al. 1993) using the PCR cycling program reported in de Benedetta et al. (2022).

PCR products were checked on a 1.2% agarose gel stained with SYBR Safe (Invitrogen) and directly sequenced.

Chromatograms were assembled using BioEdit 7.0 (Hall 1999) and edited manually. The obtained sequences were compared with each other and with sequences in the genetic databases GenBank and BOLD ([www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/); [www.boldsystems.org](http://www.boldsystems.org); last accessed on 28 May 2023) and, subsequently, were submitted to GenBank.

## Results

### Monitoring activities

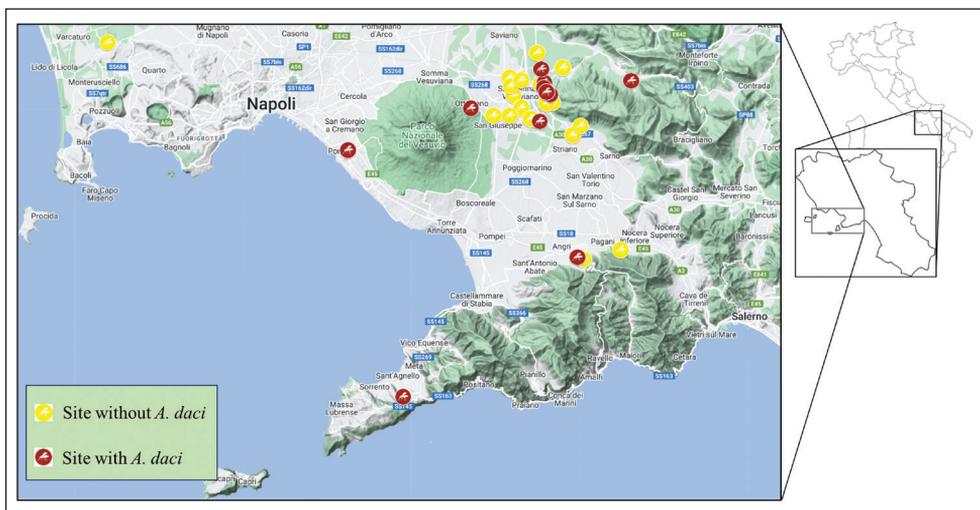
Parasitoids emerged exclusively from oranges infested by *C. capitata*, collected from 9 orchards located in three Campanian provinces. A total of 52 adult parasitoids (29 females and 23 males) were obtained from the sampled fruits, resulting in a female-biased sex ratio (0.56). The fruits were harvested between September 14, 2022 and December 15, 2022 and subsequently in May and July 2023 from sites where adults of *B. dorsalis* were trapped, except for Sant’Agnello and Portici (Naples), where the pest has not been detected previously (Bernardo et al. 2023a CNIE). Details of the emerged parasitoids are summarized in Table 1.

The maximum distance between the most extreme recorded localities was approximately 35 Km. The geographical distribution is depicted in Fig. 1.

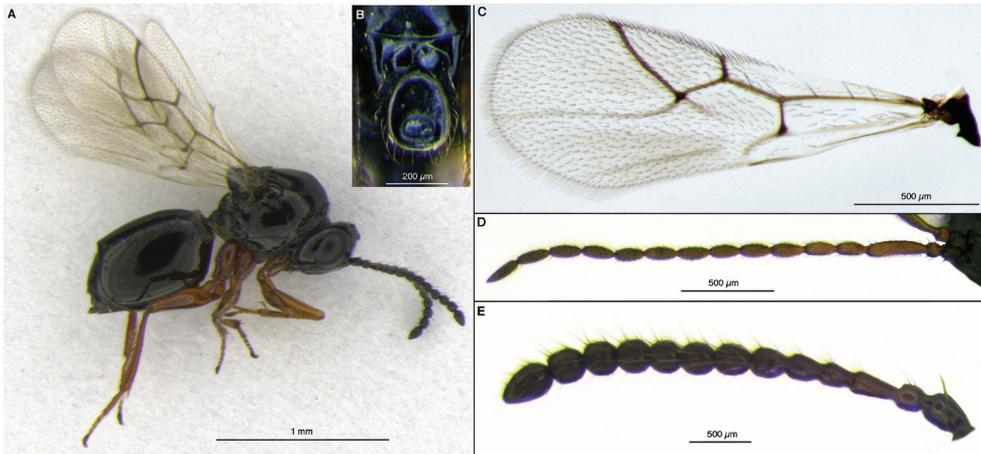
### Morphological identification

All collected specimens exhibited morphological characteristics consistent with *A. daci* (Fig. 2A–E) as diagnosed in Diaz and Gallardo (2001).

In the genus *Aganaspis* Lin, 1987, two species groups are recognised: *A. pelleranoi* group, [comprising *A. pelleranoi* (Brèthes, 1924) and *A. nordlanderi* Wharton (1998)], and *A. contracta* group, [comprising *A. contracta* Lin, 1987, *A. ocellata* (Lin, 1987), *A. major* (Lin, 1987), and *A. daci*]. The second group is characterized by eyes with setae and a conspicuous antennal club.



**Figure 1.** Sampling sites for *A. daci*: sites where *A. daci* emerged (red dots) or not (yellow dots) from puparia of *C. capitata*.



**Figure 2.** *A. daci* **A** female **B** scutellar plate **C** female forewing **D** male antenna **E** female antenna.

*Aganaspis daci* can be identified by the 9-segmented antennal club in females, featuring spherical segments (Fig. 2E). In males, antenna segment 3 is distinctly longer than segment 4 (Fig. 2D). The scutellar plate is oblong-ovate, very large, and extends beyond the disc (Fig. 2B). The black body exhibits weak bilateral compression in both sexes (Fig. 2A). When viewed from the dorsal perspective, the head is distinctly wider than long in both sexes, and the ocelli are always arranged in a broad triangle in both sexes (Diaz and Gallardo 2001). The comparison of the specimens obtained from field sampling with those obtained from the IVIA rearing confirmed that all specimens share the same morphological characteristics.

## Molecular characterization

The sequences, corresponding to the COI barcoding region, were obtained solely using the LepF1/LepR1 primer pair, resulting in a length of 632 bps, after editing and trimming. Sequencing analyses of *A. daci* specimens revealed the presence of at least three mitochondrial haplotypes (Ha, Hb, and Hc) where Ha and Hb differ each other by 1 bp and Hc showed 0.32% and 0.47% difference with Ha and Hb respectively. In Italian samples all the three mt-haplotypes were recorded (Table 1) while in the whole Spanish samples a single mt-haplotype (Hc) was found. However, these differences (2 or 3 out of 632 bp) do not infer any variations at the amino-acid level.

A BLAST search in the GenBank database showed an 88% similarity with samples identified as *Eucoilinae* sp. (without specific species information). The BOLD system did not find any similarities with sequences in the database since the only available sequences, belonging to *Aganaspis* sp., was short and referred to another COI region.

All specimens collected both in Italy and Spain shared the same nuclear gene sequence (ITS2-28S\_D1\_D2) with a single exception of the Spanish specimen *Ad\_52* that shows a three bases indel.

## Discussion and conclusion

*Aganaspis daci* is a solitary larval-pupal endoparasitoid of tephritids, recognized as an effective biocontrol agent (de Pedro et al. 2021). It was recorded for the first time in Malaysia and Taiwan as a parasitoid of *B. dorsalis* and subsequently released in Hawaii for the biocontrol of the same pest (Weld 1951; Clausen et al. 1965).

Additional releases of *A. daci* were carried out in various countries for the biological control of fruit flies such as *B. dorsalis*, and species belonging to the genus *Anastrepha* Shiner (Diptera: Tephritidae) (Clausen et al. 1965; Andleeb et al. 2010; Adly 2016; El-Heneidy 2019). Due to successful laboratory assay, the parasitoid has also been released in Egypt for the biocontrol of *Bactrocera zonata* (Saunders) (Diptera: Tephritidae), the peach fruit fly (Adly 2016; El-Heneidy 2019).

In Europe, *A. daci* was introduced in France in the 1970s to control *C. capitata*, but the results were inconclusive (de Pedro et al. 2021). Subsequently, it was recorded in the Greek Island of Chios and Spain, as a parasitoid of medfly, and its presence has not been reported in other European countries (Papadopoulos and Katsoyannos 2003; Verdú et al. 2011).

During the monitoring of *B. dorsalis* and other fruit flies, a few individuals of *A. daci* were found in the medfly-infested fruits in Italy. This discovery of *A. daci* represents the first documented finding of this Figitidae species in the country and highlights the expansion of its distribution range in Europe. The intricate history of its releases and findings, both in Europe and other countries within the Mediterranean region, complicates the reconstruction of its diffusion process, leaving its arrival and establishment unclear. Nevertheless, certain evidence permits to formulate hypotheses. Morphological and molecular analyses confirmed that the Italian and Spanish populations belong to the same species. However, mitochondrial studies suggest that only a single haplotype is shared (Hc) between Italian and Spanish individuals. This indicates a potential difference in the origin of at least a part of the Italian population. However, it is essential to consider that this difference might also be linked to the bottleneck phenomenon, particularly in terms of rearing practices, leading to a decrease in the variety of haplotypes (Prentis et al. 2008). Nevertheless, due to the limited number of Spanish specimens analysed, this result may not be conclusive.

Moreover, consistently with other instances of invasive Hymenoptera, where genetic analysis has indicated that invasive events are frequently attributable to populations comprising a solitary or a few haplotypes, the conducted analyses have revealed the presence of at least three haplotypes in Italy (Nugnes et al. 2015; Nugnes et al. 2016; Sabbatini et al. 2019; Sthal et al. 2019). Nonetheless, it is important to note that these analyses have been conducted on a relatively small number of specimens. Thus, the presence of other haplotypes could not be excluded a priori. Furthermore, the absence of similar sequences in the genetic databases prevented a comparison with other samples from different regions. For this reason, it is not possible to estimate the possible origin of the adult wasp here studied. The absolute absence of genetic sequences belonging to a species found and studied in multiple countries is highly unusual. Therefore, the molecular characterization of *A. daci* presented here, using a

mitochondrial region (COI) and two nuclear regions (ITS2-28S\_D1\_D2), not only represents the characterization of the entities found in Italian areas but also serves as a starting point for potential identifications and genetic studies by other researchers engaged in studies on this species and its congeneric entities.

The hypotheses regarding the arrival of *A. daci* in Italy include independent migration from neighbouring countries where the species is already present (Papadopoulos and Katsoyannos 2003; Verdú et al. 2011; Viggiani 2001; Beltrá and Soto 2011; Nugnes et al. 2016), host-tracking, following the invasion of its host fruit fly species (Radeghieri et al. 2002; Gebiola et al. 2014), or a combination of both. It is indeed plausible that *A. daci* may have arrived in Italy even together with its major host (*B. dorsalis*) which has been recently and repeatedly intercepted in recent years in Europe (Nugnes et al. 2018; Egartner et al. 2019; Vitiello et al. 2020).

The polyphagy of *A. daci* suggests that it could have established in Italy by reproducing on *C. capitata*. However, taking into account the number of Italian tephritids, which were probably not studied in depth because they are not related to agriculture, it cannot be excluded that *A. daci* may have adapted and reproduced on other hosts. Further investigations are required to determine the exact pathway of introduction and establishment.

The recorded sex ratio (0.56) is consistent with the previously calculated range (0.54–0.61) when the parasitoid was reared on *C. capitata*, as reported by de Pedro et al. (2017a).

The performance of *A. daci* as a biocontrol agent has been extensively studied (de Pedro et al. 2016, 2017a; El-Heneidy et al. 2019). Despite its female low fertility and longevity, *A. daci* can induce pupal mortality, resulting in significant reductions of pest populations. However, its effectiveness depends on environmental conditions, with high temperatures (30–35 °C) and low temperatures (about 15 °C) adversely affecting its development and survival (de Pedro et al. 2016). Conflicting reports exist regarding its field activity, with higher parasitism rates observed in some areas compared to others, and several reports indicating very low parasitisation percentages (Papadopoulos and Katsoyannos 2003; Ali et al. 2016; De Pedro et al. 2017b; El-Heneidy et al. 2019; Moraiti et al. 2020; de Pedro et al. 2021).

The presence of *A. daci* in Italy from September to December and again in May and July suggests that the parasitoid may be capable of completing more than one generation per year, particularly during seasons with favourable environmental conditions for its development. The observations made in May and July, following the winter period, further confirm its establishment in Italy.

The expansion of *C. capitata* northward in Italy, driven by climate change, may potentially enable *A. daci* to shift its distribution to other suitable environments.

The Mediterranean region has proven to be conducive to the establishment of *A. daci*, as demonstrated by its presence in Spain, Greece, and now Italy (Papadopoulos and Katsoyannos 2003; de Pedro et al. 2016; this work). The discovery of *A. daci* in Italy not only highlights its natural expansion in Europe, as we have previously discussed, but also its association with *C. capitata*, which presents a potential additional control strategy not only for this pest but also due to its polyphagy for other fruit fly species.

In the coming years, it will be intriguing to assess the stability of the parasitoid population, its spatial and temporal distribution, and the host range of *A. daci*, especially in certain Italian locations where another of its host, *B. dorsalis*, is also found.

It is important to note that European legislation currently imposes restrictions on the introduction of parasitoids for pest control into non-native areas. Despite such regulations, the discovery of *A. daci* in Italy underscores the ability of natural enemies to traverse the globe and establish in distant territories.

Comparable cases are becoming increasingly frequent (Radeghieri et al. 2002; Beltrá et al. 2011; Gebiola et al. 2014; Gebiola et al. 2015a, 2015b; Nugnes et al. 2016; Stahl et al. 2019).

Therefore, it is essential to reconsider some of these regulations in light of the fact that the arrival of any parasitoid, capable of controlling a phytophagous insect and released in a nearby country, can be prevented. Indeed, the delays caused by the mandatory studies only result in a period in which the alien species can develop undisturbed, leading to the massive use of chemicals that are probably more dangerous for the environment and humans than any parasitoid.

Considering the challenges associated with the importation of *B. dorsalis* parasitoids, the significance of this discovery cannot be understated. The presence and widespread distribution of its parasitoid in Italy greatly facilitate the implementation of a viable biological control strategy.

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