

# Drosophilidae monitoring in Apulia (Italy) reveals *Drosophila suzukii* as one of the four most abundant species

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## Abstract

The knowledge of the endemic drosophilid assemblage is a useful reference to study population dynamics when new species are introduced in a geographical area. The introduction of invasive species can change the structure of the drosophilid community; hence, the distribution data for endemic species are also essential to support efficient pest management. We provide the first description of the natural drosophilid populations (Diptera Drosophilidae) recorded in Apulia, in Southern Italy. The flies, which were collected in a field survey throughout a year, were classified by morphological and molecular analyses by sequencing the barcode fragment of the COI mtDNA gene. The identified species show a distribution of frequencies that varies throughout the year, reflecting a seasonal life cycle peculiar to each species. Among the recorded drosophilids, the potential pest species *Drosophila suzukii* represents one of the four most abundant species.

**Key words:** Drosophilidae, natural populations, seasonal variation, invasive species, spotted-wing *Drosophila*.

## Introduction

Among all Diptera, *Drosophila melanogaster* Meigen, commonly known as the fruit fly, is the most studied model organism in genetics and evolutionary biology since the first genetic discoveries of the early 1900s. *Drosophila* is one of over 65 genera belonging to the family of Drosophilidae, which includes more than 3,000 species distributed throughout the world (Wheeler, 1986). Knowledge of the composition of endemic drosophilid assemblages, which is currently lacking for Italy, is baseline information both for studying the adaptive process when new species are introduced in an area and for performing rapid and efficient pest management.

Many scientific reports in the last few years highlight the devastating effect on soft-fruit production caused by the infestation of invasive species of Drosophilidae, such as *Drosophila suzukii* (Matsumura) and *Zaprionus indianus* Gupta (van der Linde *et al.*, 2006; Hauser *et al.*, 2009; Bolda *et al.*, 2010; Calabria *et al.*, 2012; Walsh *et al.*, 2011; Cini *et al.*, 2012; Asplen *et al.*, 2015). Unlike the majority of Drosophilidae, which lay eggs in overripe or damaged hosts and are therefore harmless for fruit crops, *D. suzukii* lays eggs in healthy ripening fruits, promoting crop damage; rate of infestation is hastened by high female fecundity. Furthermore, this fly species has a wide range of hosts (Kanzawa, 1939; Walsh *et al.*, 2011; Lee *et al.*, 2015) and has been found over a wide range of latitude, altitude (up to 1,500 m above sea level), and temperature (Kanzawa, 1939; Dalton *et al.*, 2011; Tochen *et al.*, 2014). In an attempt to aid both basic and applied research due to its economic impact, the *D. suzukii* genome has been recently sequenced, and a high quality reference sequence has been obtained (Chiu *et al.*, 2013; Ometto *et al.*, 2013).

In the present study, we report data describing the natural drosophilid populations present in Apulia, in

Southern Italy for the first time. We performed a one-year field survey and identified the collected flies by morphological and molecular analyses. We recorded the potential pest species, *D. suzukii*, as one of the four most abundant species among the drosophilids; to monitor its trend, we compared the frequency of *D. suzukii* detection during autumn 2013 with that of the same season in the following three years.

## Materials and methods

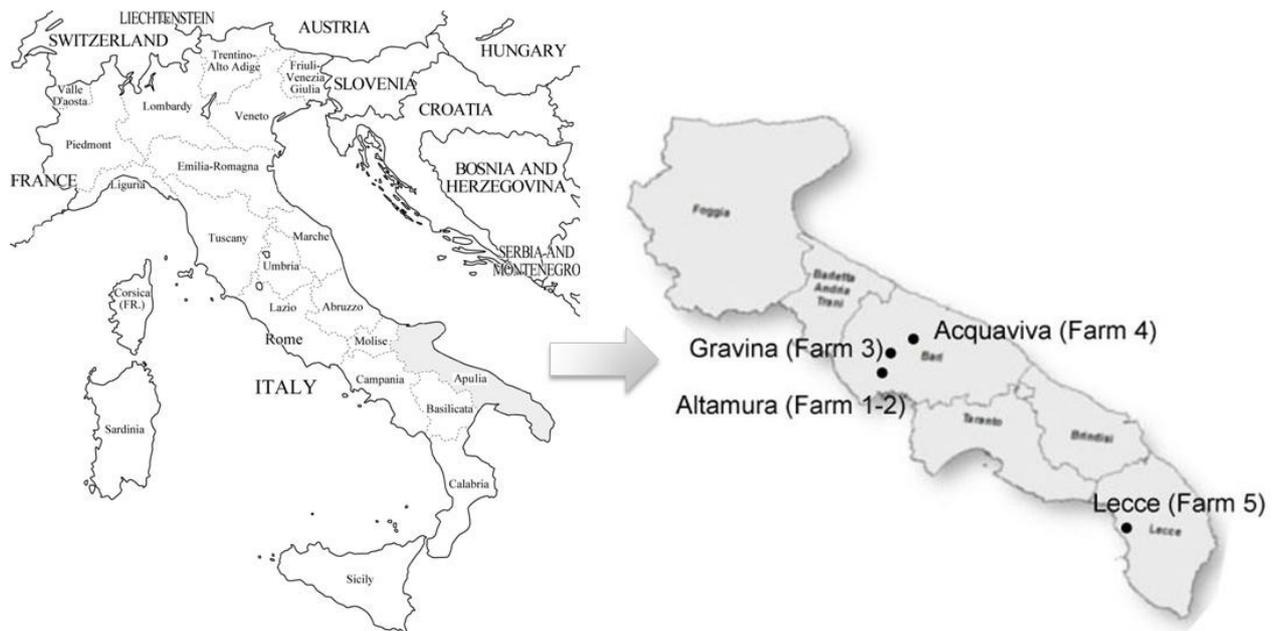
### Field survey

The field survey was performed in five localities of Apulia (Italy) (figure 1) throughout the year 2013. Locations and vegetation composition of the surveyed sites are reported in table 1. The altitude of Farms 1-3 is approximately 450 m above sea level, Farm 4 is approximately 300 m above sea level. Farm 5 is located approximately 150 km to the south of Farm 1, close to Lecce town at the altitude of 8 m above sea level.

Farm 1, in the locality of Pescariello, was monitored again during the month of November 2014, 2015 and 2016 (data of 2016 were obtained during the review period of the manuscript).

The staggered fruiting periods in the surveyed areas ensure that the drosophilids have a constant food supply for almost the entire year.

The flies were caught using transparent plastic bottles with holes (diameter of approximately 5 mm) and baited with 300 ml of apple cider vinegar with small apple slices. It is possible that some drosophilids were missed due to the trap bait method; it has been reported that both the bait, as well as the physical features of traps, have different sensitivity and selectivity for *Drosophila* species (Lee *et al.*, 2012; 2013). For each farm, four to five traps were placed on the plants in shady areas and removed weekly. Trap captures were not carried out in August.



**Figure 1.** Map of Italy depicting the localities in Apulia where the field survey was carried out (dots).

**Table 1.** Locations and vegetation composition of the surveyed sites

Site	Crop	Location, vicinity-town (site coordinates)
Farm 1	Cherry, plum, apricot, peach, apple, pear, persimmon, fig, olive, prickly pear, grape, strawberry; ornamental plants, such as myrtle, oleander, evergreen	Altamura (40°52'42"N 16°37'38"E)
Farm 2	Cherry, plum, apricot, apple, pear, persimmon, fig, olive, prickly pear, grape, evergreen	Altamura (40°47'34"N 16°32'51"E)
Farm 3	Vineyards and scattered fruit trees	Gravina in Puglia (40°46'23"N 16°22'05"E)
Farm 4	Cherry trees	Acquaviva delle Fonti (40°47'53"N 16°49'35"E)
Farm 5	Mediterranean scrub with scattered fruit trees	Lecce (40°08'38"N 18°02'24"E)

In the monitored farms fallen fruit was left on the ground; in the Farms 1-3 and 5, pesticides were not used (organic management).

The mean monthly values of temperature and humidity reported, have been retrieved from Centro Epon Meteo (CEM; <http://www.meteo.expert/>), the retrieved values were: 12 °C and 85% RH (November 2013), 13 °C and 88% RH (November 2014), 12 °C and 84% RH (November 2015) and 12 °C and 85% RH (November 2016).

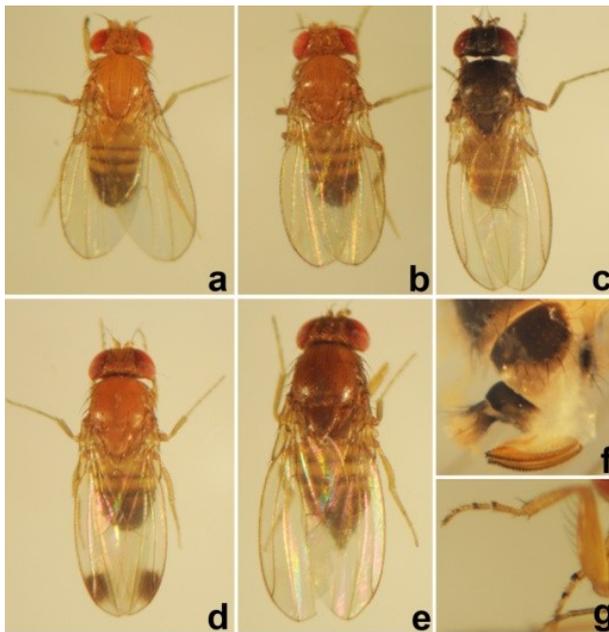
### Morphological analysis

The collected flies were placed in 70% ethanol; all specimens were analysed under a stereo-microscope, recorded and classified. The adult flies have a variety of structures that have been defined as useful morphological characters for species identification. Useful characteristics for the morphological identification of Drosophilidae are extensively reviewed in Fonseca (1965), Wheeler (1986), Grimaldi (1987; 1990), Markow and O'Grady (2006) and in the up-to-date database TaxoDros (<http://www.taxodros.uzh.ch>). Some of the morphological differences among the four most abun-

dant collected species are shown in figure 2.

The pigmentation pattern of adult tergites is one of the taxonomically characteristics used to identify the species. *D. melanogaster* (figure 2a) and *Drosophila simulans* Sturtevant (figure 2b) are yellowish species with an unbroken mid-dorsal line characteristic of the subgenus *Sophophora*, while *Drosophila immigrans* Sturtevant shows the interruption of the mid-dorsal line peculiar to most subgenus *Drosophila* species. *Drosophila subobscura* Collin (figure 2c) is dark in colour and the variation in the extent of the black pigment on the abdominal tergites (in females) is a taxonomic feature (Bächli and Burla, 1985). *Drosophila busckii* Coquillett shows a longitudinally striped pleurae. *Drosophila hydei* Sturtevant has a mesonotum with fused dark spots forming stripes and a dark line on the mesopleura. *Zaprionus tuberculatus* Malloch has multi-coloured vittae on the head and notum, frons with a fine white medial stripe anterior to ocelli and a yellow flagellomere.

Structures present on the forelegs, such as sex combs, spines and tubercles, were used to identify *D. immigrans*, *Z. tuberculatus* and *D. subobscura* males.



**Figure 2.** General view of the external morphology of the four most abundant species: *D. melanogaster* (a), *D. simulans* (b), *D. subobscura* (c), and *D. sukii* (d) males. Sexual dimorphism is evident in *D. sukii* males (d) and females (e): the black spots on the wings (d), the two sets of black sex combs on the foretarsi (g) in males and the serrated ovipositor in females (f).

*D. sukii* males (figure 2d, 2g) have two sets of black tarsal combs (one on the first and one on the second tarsal segment); the presence of a dark spot near the edge of each wing tip allows for easy identification of *D. sukii* males (Bock and Wheeler, 1972; Hauser, 2011).

Male and female genitalia are also essential for species-level identification. *D. sukii* females (figure 2e-f) have a long and narrowed ovipositor with dark teeth.

### Molecular analysis

Molecular identification was based on the sequences of the mitochondrial gene cytochrome oxidase subunit I (COI) and/or subunit II (COII). Total DNA was purified from a single fly using the DNeasy Blood & Tissue kit (Qiagen Inc.) according to the manufacturer's protocol. Mitochondrial DNA (mtDNA) amplification was performed using the universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) for COI and the TL2-J-3037 and TK-N-3785 primers (Simon *et al.*, 1994) for COII, which generate amplicons of 704 bp and 786 bp, respectively. Polymerase chain reaction (PCR) amplifications were set up in the thermal cycler (MJ Research PTC 100); the temperature profile for the amplification of COI was 94 °C for 5 min; followed by 30 cycles of 5 s at 94 °C, 40 s at 55 °C, and 1 min at 68 °C; and a final extension for 7 min at 68 °C. The temperature profile for COII gene amplification was the same as for COI, except that the annealing temperature was 52 °C. The purified PCR products were sequenced bi-directionally by a commercial service (BMR Genomics) using the amplification primers.

The mtDNA sequences were registered in the Euro-

pean Nucleotide Archive (ENA) for GenBank/ENA/DDBJ/IMG/IMG-DB (GEDI) public availability under the accession numbers from LN867072 to LN867084.

DNA sequence similarity searches were performed using the Barcode of Life Data Systems (BOLD) platform that contains a set of integrated databases for facilitating identification of unknown sequences (Ratnasingham and Hebert, 2007). We searched all published COI gene records from the BOLD and/or GenBank databases with a minimum sequence length of 500 bp.

The molecular analysis has been reiterated once a month during the field survey: one individual per species was randomly chosen from the recovered traps, and the COI and/or COII gene sequencing was repeated. Nucleotide multiple alignments were produced by Clustal W2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

### Statistical analysis

The statistical significance of the frequencies of drosophilid species, collected in Farm 1 during the month of November from 2013 to 2016, was determined by analysing a contingency table using chi-square test. The P value for each *Drosophila* species compared to the other collected species was adjusted by applying the Bonferroni correction for multiple tests.

Chi-square test was used also to compare the frequency of *D. sukii* occurrence in the five surveyed farms. Statistical tests were performed using Prism (GraphPad Software, Inc.).

## Results

### Drosophilid distribution in Apulia

The drosophilid species and the relative monthly frequencies of the adult flies captured in five Apulian localities are reported in table 2. To prevent errors due to the misidentification of the females in the sibling species *D. melanogaster* and *D. simulans*, the frequencies of the two species were combined. After morphological analysis of the collected flies, species identification was established molecularly by sequencing the barcode fragment of the COI mtDNA genes for all of the collected species and registered in ENA.

DNA sequence similarity searches using the BOLD system, in the majority of cases, allowed a solid identification of the species, with a percentage of nucleotide identity greater than 98% to 100%. In the case of a low percentage of nucleotide identity (<98%) with the publicly available sequences, the COII fragment was also sequenced. Table 3 summarizes the percentages of sequence identity of the COI barcode fragment of the collected species, with the publicly available sequences. No match in BOLD and hits markedly below the threshold of 98% in GenBank resulted for the *Hirtodrosophila cameraria* COI sequence. In this case, the COII gene sequence was determined (Acc. number LN867084), and the BLAST search of the GenBank database found a *H. cameraria* COII gene sequence with a percentage of nucleotide identity of 99.7%, confirming our previous morphological data.

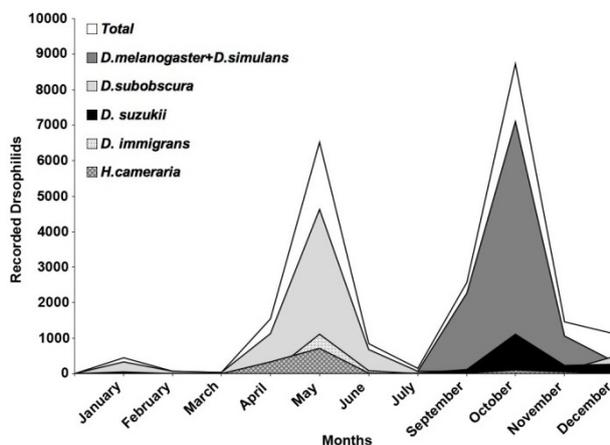
**Table 2.** Frequency of drosophilid species distribution per months during 2013 in Apulia.

Collection date	Jan	Feb	Mar	Apr	May	Jun	Jul	Sep	Oct	Nov	Dec
<i>Drosophila melanogaster</i> Meigen 1830	0.11	0.03	0.05	0.02	0.008	0.04	0.40	0.90	0.81	0.72	0.31
<i>Drosophila simulans</i> Sturtevant 1919											
<i>Drosophila subobscura</i> Collin 1936	0.72	0.95	0.95	0.74	0.70	0.82	0.47	0.02	0.02	0.05	0.43
<i>Drosophila suzukii</i> (Matsumura 1931)	0.09			0.001	0.002	0.009	0.08	0.05	0.12	0.16	0.23
<i>Hirtodrosophila cameraria</i> (Haliday 1833)				0.22	0.11	0.02	0.03		0.01	0.03	
<i>Drosophila immigrans</i> Sturtevant 1921				0.01	0.17	0.09	0.007	0.005		0.004	0.001
<i>Drosophila busckii</i> Coquillett 1901	0.005	0.02		0.001	0.002	0.006		0.01	0.004	0.006	0.006
<i>Drosophila hydei</i> Sturtevant 1921	0.05				0.004	0.01	0.007		0.03	0.05	0.015
<i>Drosophila funebris</i> (F. 1787)	0.01			0.007						0.004	0.004
<i>Scaptodrosophila</i> sp.						0.003		0.007	0.004		0.004
<i>Zaprionus tuberculatus</i> Malloch 1932										0.006	
<i>Drosophila phalerata</i> Meigen 1830										0.002	
Total number of drosophilids collected	666	62	40	1524	6556	834	147	2578	8729	1462	939

**Table 3.** COI mtDNA gene sequences.

Drosophilid species	Accession Number	Nucleotide identity with Publicly DNA sequences
<i>D. melanogaster</i>	LN867079	100% (JQ350715)
<i>D. subobscura</i>	LN867074	100% (KR682371)
<i>D. suzukii</i>	LN867072	100% (KJ671597; KJ671588; HM636439)
<i>D. phalerata</i>	LN867082	100% (FIDIP858-12)
<i>D. busckii</i>	LN867078	100% (KR772761)
<i>D. immigrans</i>	LN867076	100% (KJ 671616)
<i>D. hydei</i>	LN867077	99.86% (KJ463782)
<i>D. funebris</i>	LN867080	99.06% (EU390731)
<i>D. simulans</i>	LN867081	98.33% (KJ767234)
<i>H. cameraria</i>	LN867075	< 98%

The COII gene was also sequenced for *D. suzukii* species (Acc. number LN867083); the sequence analysis has revealed 99.84% nucleotide identity, with a corresponding sequence present in the GenBank database. A significant species level match was not found for either the COI or COII sequences for a specimen whose morphology indicates it belongs to the genus *Scaptodrosophila*.



**Figure 3.** Monthly records (absolute numbers) of the most abundant drosophilid species in Apulia during the year 2013. Monitoring was not carried out in August. The graph shows the species recorded with a frequency greater than 0.1 in at least one month.

A species level match could not be made for the *Z. tuberculatus* COI sequence. The high COI nucleotide identity did not allow us to distinguish among *Z. tuberculatus*, *Zaprionus verruca* Burla and *Zaprionus badyi* Chassagnard et McEvey, and the COII gene sequence cannot be used to solve the triad (Yassin *et al.*, 2008). Hence, the species *Z. tuberculatus* was identified by morphological analysis only.

The sequence analysis revealed 100% identity between mtDNA samples derived monthly from the same species confirming the number of thirteen haplotypes found.

Among the 23,537 collected flies, we systematically identified 12 Drosophilidae species belonging to *Drosophila* (nine species), *Hirtodrosophila* (one), *Zaprionus* (one) and *Scaptodrosophila* (one) genera. The abundance of the different collected species varied throughout the year, with a prevalence in spring and autumn, as depicted in figure 3, reflecting a seasonal life cycle peculiar to each species. *D. melanogaster* plus *D. simulans* and *D. subobscura* are the most abundant species; the first two represent the majority of the species recorded in autumn, and *D. subobscura* is the prevalent species throughout the winter and the spring. *D. suzukii* appears in spring, while the most abundant presence is recorded between September and December; note that *D. suzukii* is one of the four most abundant species. *H. cameraria* and *D. immigrans* are present mainly in spring. *D. busckii*, *D. hydei* and *D. funebris* are detected at a

low percentage during almost the entire year. Lastly, very few specimens of *Drosophila phalerata* Meigen, *Z. tuberculatus* and specimens belonging to the *Scaptodrosophila* genus were sporadically recorded.

### *Drosophila suzukii*

*D. suzukii* was found in all of the monitored farms. In Farm 2, during a preliminary field survey conducted during September 2012, we collected two *D. suzukii* females, recording the occurrence of this potential pest in Apulia in accordance with a recent report (Baser *et al.*, 2015). The analysis of the *D. suzukii* trend during a year (table 2 and figure 3) shows that the first occurrence of this species is in spring, when cherries and strawberries ripen in Apulia, and its presence increases again in autumn, when grapes ripen.

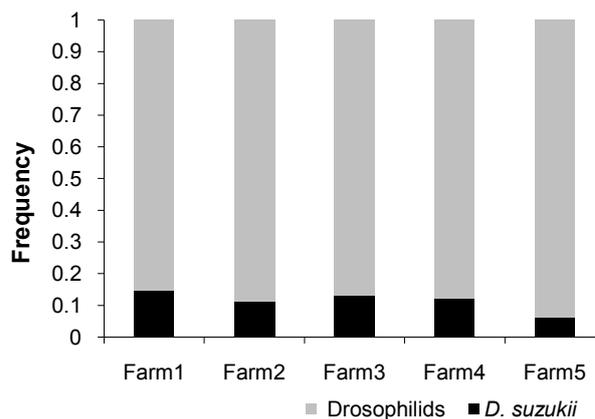
Figure 4 shows the frequency of *D. suzukii* recorded during October 2013; a slight but significant difference in the frequency occurrence among the locations was observed.

To study the presence of *D. suzukii* over time, we repeated the drosophilid survey in autumn, during November 2014, 2015 and 2016 in Farm 1, where it was present with the highest frequency (figure 4). We recorded all of the fly species to be able to monitor variations in the *D. suzukii* presence relative to the drosophilid assemblage. The frequencies comparison indicates slight differences, statistically tested for their significance, of *D. suzukii* presence (figure 5).

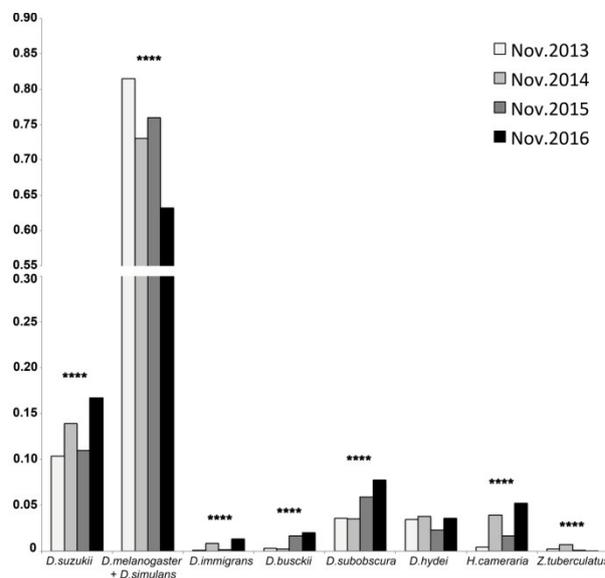
To examine the source of the *D. suzukii* introduction in Apulia, the sequence of the COI mtDNA fragment of our collected specimen was compared with the corresponding gene sequences available in public databases taken from specimens collected in other Italian regions. This analysis revealed 100% sequence identity among the Apulian *D. suzukii* sample and the specimens collected in Emilia-Romagna and Trentino (Acc. number KJ671597 and KJ671591, respectively; Dhimi and Kumarasinghe, 2014).

## Discussion

This study outlines the composition of the drosophilid communities present in cultivated areas in a region of Southern Italy; no data concerning this topic have been previously published. The only data available on drosophilid distribution in Italy refer to the North of the country (Zangheri, 1969; Nigro, 1979). Our detailed data on the occurrence and abundance of endemic drosophilid species in Apulia could be the basis for future studies of population dynamics. It is known that diversity and population density of a species in a locality is influenced by the species that are ecologically related (Begon, 1996). A framework of reference is useful to address how the endemic drosophilids respond to the introduction of new species as the case of *D. suzukii* and *Z. tuberculatus* recently detected in Italy for the first time. With regard to the Afrotropical Drosophilidae *Z. tuberculatus*, Raspi *et al.* (2014) sampled this species for the first time on the European mainland, in Northern Italy, in 2013. This species, which is a native of Africa,



**Figure 4.** Frequency of *D. suzukii* occurrence in the five surveyed areas, recorded during October 2013. Statistical analysis was performed by the  $\chi^2$  test, P value < 0.0001.



**Figure 5.** Frequencies of the most abundant drosophilid species, collected in Farm 1 in November 2013, 2014, 2015 and 2016. Statistical significance refers to each species during the years. Statistical significance was determined by analysing a contingency table using  $\chi^2$  test. P-values smaller than 0.05 were considered statistically significant, with the following ranking:  $p \leq 0.05$ (\*);  $p \leq 0.01$ (\*\*);  $p \leq 0.001$ (\*\*\*);  $p \leq 0.0001$ (\*\*\*\*). Statistical tests were performed using Prism (GraphPad Software, Inc.).

has spread to the other continents. Although *Z. tuberculatus* is not harmful to agricultural products, a closely related invasive species, *Z. indianus*, is considered a pest for fig production in some areas of America (van der Linde *et al.*, 2006). Therefore, in an age when one of the most likely routes of fast introduction of pests is the trade of fresh fruits (Rota-Stabelli *et al.*, 2013), attention should be paid to the presence of *Zaprionus* species during future monitoring.

*D. suzukii*, first described by Matsumura in 1931, was recorded in Japan at the beginning of the 20<sup>th</sup> century

(Hauser *et al.*, 2009). The first report of *D. suzukii* recorded outside Asia came from the Hawaiian Islands in 1980 (Kaneshiro, 1983), while in the mainland United States, the first record dates back to 2008 in California (Hauser *et al.*, 2009). That same year *D. suzukii* adults were collected in Europe, in Spain (Rasquera Province) (Calabria *et al.*, 2012) and Italy (Tuscany) (Cini *et al.*, 2012). During the following years *D. suzukii* was recorded in several other European countries (Calabria *et al.*, 2012); in Italy, it was reported in other regions along the country (Grassi *et al.*, 2009; Süß and Costanzi, 2011; Gargani *et al.*, 2013; Baser *et al.*, 2015). Currently the species appears to be spreading rapidly in most of the American and European countries (CABI, 2015; Asplen *et al.*, 2015).

A recent population analysis of this pest species has revealed that the colonisations of the continental United States and Europe were separate demographic events (Adrion *et al.*, 2014).

The sequence analysis of the COI mtDNA fragment of our collected specimens revealed a perfect sequence identity among the Apulian *D. suzukii* sample and the specimens collected in Emilia-Romagna and Trentino (Acc. number KJ671597 and KJ671591) regions that are not close to each other. The unique COI haplotype would speculate a single introduction route of the species in Italy. Furthermore, the same haplotype has also been found in Spain (Acc. number HM636439) (Calabria *et al.*, 2012). However, a population genetic analysis based on polymorphic molecular markers on more *D. suzukii* populations collected throughout the Italian Peninsula is necessary to reconstruct the origin of this invasion.

A slight difference in the frequency of *D. suzukii* occurrence among the five surveyed localities was observed; this variation may be due to the different crops cultivated in those areas, as already reported (Baser *et al.*, 2015), as well as to differences in altitude.

Throughout the *D. suzukii* monitoring in Apulia, we confirmed its greatest presence during the autumn with slight differences in its occurrence among the years. These differences may be related to the variations in environmental conditions such as temperature and humidity that, as already demonstrated, influence *D. suzukii* activity and development (Kanzawa, 1939; Tochen *et al.*, 2014; 2016; Tonina *et al.*, 2016). The highest *D. suzukii* presence is recorded during November 2016, when the mean temperature and humidity percentage monthly reported were 12 °C and 85%, respectively. It is noteworthy that the average conditions of temperature and humidity were the same in the year 2013 and 2016 (see material and methods) so that we might speculate a tendency to an increase in the presence of *D. suzukii*. However, data from the monitoring during the following years should be helpful to understand the trend of this invasive species in Apulia. The presence or disappearance of a suite of drosophilid species may be an indicator of changes in ecosystem integrity (Leblanc *et al.*, 2013). The nontarget effect of practices to control *D. suzukii* may impact endemic drosophilids assemblage (Leblanc *et al.*, 2013); therefore the *Drosophila* community structure should be steadily monitored.

In many countries around the world, the estimation of fruit-yield losses due to *D. suzukii* infestation predicts economic damages (Bolda *et al.*, 2010; Hauser, 2011; Cini *et al.*, 2012). In Italy, damages have been reported to small-fruit production (De Ros *et al.*, 2012; Ioriatti *et al.*, 2015). Multiple strategies are required to set up an effective pest management (Dreves, 2011). A proper monitoring of the *D. suzukii* presence is the first step for a successful integrated pest management (Cini *et al.*, 2012). Even the extent of the pest population seasonal variation is useful to define key period to manage the choice in sanitation, chemical treatments, cultural control and biological control.

*D. suzukii* in Apulia could attack a range of commercial crops, such as cherries and grapes, which are essential for the economy of this region. A constant monitoring of the invasive species is essential to support an appropriate pest risk management to avoid yield losses not only for Apulia but also for all Mediterranean countries with similar environments, climate conditions and fruit production.

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