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ALIEN FLORAS AND FAUNAS 14

Discovering the Pandora's box: the invasion of alien flatworms in Italy

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Abstract Alien land planarians have been scarcely recorded in Italy and the aim of this work was to update the distribution of alien planarians in Italy using a citizen science and, whenever possible, a molecular approach. We received 133 records of at least 15 species (*Anisorhynchodemus* cf. *signata*, *Australopacifica atrata*, *Australoplana* cf. *sanguinea alba, Bipalium kewense*, *B. vagum*, *Caenoplana* cf. *dendyi*, *C.* cf. *decolorata*, *C. coerulea*, *C. variegata*, *Diversibipalium multilineatum*, *Diversibipalium* "black", *Endeavouria septemlineata,* and *Obama nungara*) and some undescribed or unidentifiable-to-species taxa. Records came from all Italian regions except for those characterized by the lowest human population densities (Valle d'Aosta, Molise, and Basilicata) and 83% of records come from private gardens. Most records have been observed in spring and early autumn and seem to increase with increasing rainfall. Citizen-science data significantly expanded the distribution area of these species in Italy, and thus the citizen-science platforms represent an effective tool for the early detection of these alien pest species.

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Introduction

The Mediterranean basin is highly exposed to biological invasions because of its mild climate and high habitat diversity, potentially suitable for species of tropical/subtropical origin, especially in a scenario of rising temperatures (Di Castri et al. 2012).

Among successful invaders, land flatworms (Platyhelminthes: Tricladida: Continenticola: Geoplanidae) include more than 900 species which live throughout the world with exception of Antarctica, although most species are native to moist soils of tropical forests (Sluys 2016). Based on traditional taxonomy, it seems that the European fauna of terrestrial planarians includes few indigenous species compared to other continents (N = 9 species: Minelli 1977; Jones 1998), but recent works using molecular methods have shown that many more species, especially microplanids, are present (Mateos et al. 2017; Álvarez-Presas et al. 2018).

Currently, many places in the world have been invaded by land flatworm species, accidentally stowed away in the soil of imported ornamental plants (Sluys 2016; Vardinoyannis and Alexandrakis 2019), and a total of 36 species of terrestrial planarians are known to have been introduced in different countries around the globe (Álvarez-Presas et al. 2014). Invasion by land planarians has been long overlooked, as impacts of these species on native ecosystems are still mostly unknown. On the contrary, some species are being reported as voracious, potentially affecting populations of native invertebrate species (snails, slugs, earthworms, and even other planarians) by predation and in several cases strongly affecting local ecosystems and human health (Boag et al. 2010). Despite tetrodotoxin has been detected in a land planarian, there is no evidence yet that alien flatworms represent a direct threat to human health (Stokes et al. 2014). Invasive edaphic organisms can indeed have dramatic effects on the environment, due to the direct effects on native soil organisms, and through their interactions with the environment aboveground (Sluys 2016).

In Europe, at least 20 species of alien planarians have been recorded, and some of them are considered as invasive, e.g., *Platydemus manokwari* (Álvarez-Presas et al. 2014; Justine et al. 2014, 2018, 2020). Several species have been described in the invasive range before being discovered in the native one (e.g., *Bipalium kewense* in the UK, *Caenoplana decolorata* in Spain: Moseley 1878; Mateos et al. 2020). Determination of land planarians at the specific level generally demands genetic analyses apart from some species which can be identified by their morphology and color pattern (Álvarez-Presas et al. 2014).

According to the Fauna Europaea Database (de Jong et al. 2014), Italy is the European country with the largest number of confirmed animal and plant species. Also, Italy hosts the highest species richness in terms of animal species, including endemic ones (Blasi et al. 2007). Alien land planarians have been scarcely recorded in Italy since 2015, with only a few species recorded: *Bipalium kewense*, *Caenoplana variegata* (reported as *C. bicolor*), *Diversibipalium multilineatum,* and *Obama nungara* (Carbayo et al. 2016; Mazza et al. 2016; Dorigo et al. 2020; Justine et al. 2020; Novarini and Lebech Nässling Iversen 2020).

Since our first work (Mazza et al. 2016), a major media campaign was followed by a high number of photographic reports of alien terrestrial flatworms from non-scientists in several Italian localities. Alien flatworms are often brightly coloured and are able to capture the attention of the general public, and to be recorded through citizen-science platforms (Álvarez-Presas et al. 2014; Justine et al. 2020). Thus, this work is aimed to update the distribution of alien planarians in Italy using a citizen science approach and to collect samples from the greatest possible number of sites

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where they are reported to occur and to determine the number of terrestrial flatworm species introduced in Italy through visual identification of photos and molecular analyses.

Materials and methods

Since the first report of an alien land planarian population in Italy (Mazza et al. 2016), we started a media campaign in main newspapers and on social networks (Facebook, Twitter) to collect as much data as possible of the occurrence of alien land planarian species. Further data were searched on the platform iNaturalist (www.inaturalist.org). Every photograph sent by citizen have been examined (by EM, GM, MM and ES) and the identification has been validated by specialists (i.e., by JLJ, LW).

We made contact with the authors of each record asking for dates, coordinates, and if possible, the number of observed individuals and to send us specimens stored in absolute ethanol to preserve them for molecular identification analyses. We then filled a dataset including an unequivocal code for each record, some photos of each individual, the locality, the coordinates, the date, the habitat type where each photo was taken, and whether a sample for molecular analyses was collected. Concerning habitat type, we followed Álvarez-Presas et al. (2014) and we identified: (1) private gardens, (2) nurseries and plantations, (3) semi-natural areas, (4) human indoor (including houses and cellars), (5) urban areas (including urban parks) and (6) botanical gardens. Species for which reliable information on their habitat type was unavailable were classified as "not available: NA". We performed a Hermans-Rasson test to evaluate whether a random detection of records was exhibited over the 12 months (Landler et al. 2019).

At first, morphological identification was conducted through the analysis of received photos, which allowed us to determine flatworm species through an expert-based approach. In other cases, samples were collected and stored in Eppendorf tubes with absolute ethanol for molecular analyses (see below).

DNA extraction, PCR and sequencing

Total DNA was extracted from a 3 mm^3 fragment of body tissue taken from each individual using phenol–chloroform method described by Mereghetti et al. (2019), with a modification in tissues disruption step, that in this study was performed using sterile pestles. DNA was then used as template for the amplification of a segment of the gene cytochrome c oxidase subunit I (COI) by PCR using the primers pair BarS (Álvarez-Presas et al. 2011) and COIR (Lázaro et al. 2009). PCRs were performed in a volume of 25 µL reaction mix containing: 1X GoTaq reaction Buffer, 0.2 mM of each deoxynucleoside triphosphate, 0.5 pmol of each primer, 0.6 U of GoTaq G2 Hot Start Polymerase and 100 ng of template DNA. The following thermal protocol was adopted: 2 min at 95 °C followed by 30 cycles of 50 s denaturation at 94 °C, 45 s annealing at 43 °C and 1 min e 10 s extension at 72 °C, with a final single extra extension step of 10 min at 72 °C. Positive amplicons, determined via agarose gel electrophoresis, were directly sequenced on both strands using Sanger sequencing (Microsynth, Balgach, Switzerland). Consensus sequences were obtained after assembling and editing electropherograms through Geneious 10.0.9 (Biomatters Ltd., Auckland, New Zealand); the presence of open reading frame was verified using the on-line tool EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). All nucleotide sequences developed in this study were deposited in the European Nucleotide Archive (accession numbers: FR989842-FR989861; Supplementary Material Table S1).

Molecular identification and phylogenetic inference

The obtained COI sequences were compared with those available in GenBank database by performing Basic Local Alignment Search Tool analyses (BLAST: http://www.ncbi.nlm. nih.gov/BLAST; Altschul et al. 1990) with default parameters. Species-level identification was assigned only when a sequence identity $\geq$ 99% and a E-value $< 1 \times 10^{-20}$ were obtained in the comparison between query and reference sequences, as a standard procedure in molecular identification (e.g., Pajoro et al. 2018; Kubisz et al. 2020; Olivieri et al. 2021). To further support the molecular identifications obtained, for the genera *Caenoplana*, *Bipalium* and *Obama* a phylogenetic analysis was carried out. A COI tree for each genus was thus inferred on alignments including the sequences developed in this study plus a selection of orthologous sequences of the genus available in GenBank and one outgroup sequence for each genus. For the other two genera molecularly identified in this study, *Endeavouria* and *Diversibipalium*, phylogenetic analysis was not performed since in GenBank orthologous sequences were available only for one species. The COI sequences used for phylogenetic analyses were aligned using MUSCLE (Edgar 2004) with default parameters and then haplotypes reduced using R library *Haplotypes* (https://biolsystematics.wordpress.com/r/). Phylogenetic inference was conducted as in a previous study (Montagna et al. 2013). Briefly, the best nucleotide substitution models were estimated for each data set using jModelTest 2 (Darriba et al. 2012) and selected according to Akaike Information Criterion (AIC) (Akaike 1974). In the case of *Obama* sequence alignment, the best model of nucleotide substitution resulted the General Time Reversible (GTR; Lanave et al. 1984) + gamma distribution (Γ) and a proportion of invariant sites; for *Caenoplana* alignment the best model of nucleotide substitution resulted the GTR (Lanave et al. 1984) + Γ; while for *Bipalium* the best model of nucleotide substitution resulted the GTR + a proportion of invariant sites. Maximum likelihood phylogenetic inferences were performed using PhyML version 3.0 (Guindon et al. 2010) with the following options: evolutionary nucleotide substitution models as obtained by model selection procedure; the best of NNI and SPR tree searching operation; approximate likelihood ratio test as node support (aLRT; Anisimova et al. 2011).

Results

We collected 133 records of alien land planarians in Italy between 2010 and 2021, distributed in all Italian regions except for Valle d'Aosta (i.e., an Alpine mountainous region) and the two regions with the lowest human density in Italy (Molise and Basilicata: Fig. 1). The highest number of records came from Sicily (n = 27), followed by Latium (n = 23) and Tuscany (n = 21, Fig. 1a). Most recorded individuals were morphologically identified from the photos provided by citizen-scientists (individuals from 113 records); in the cases in which citizen-scientists were able to collect and appropriately store the recorded individuals both morphological and molecular identification were performed (individuals from 13 records). In some cases, when only partial individuals were collected, only molecular identification was performed (7 records). Molecular and morphological approaches led to the identification of eight genera of alien land planarians in Italy, in three subfamilies: Bipaliinae, Rhynchodeminae, and Geoplaninae (Figs. 1b-1f and 2d).

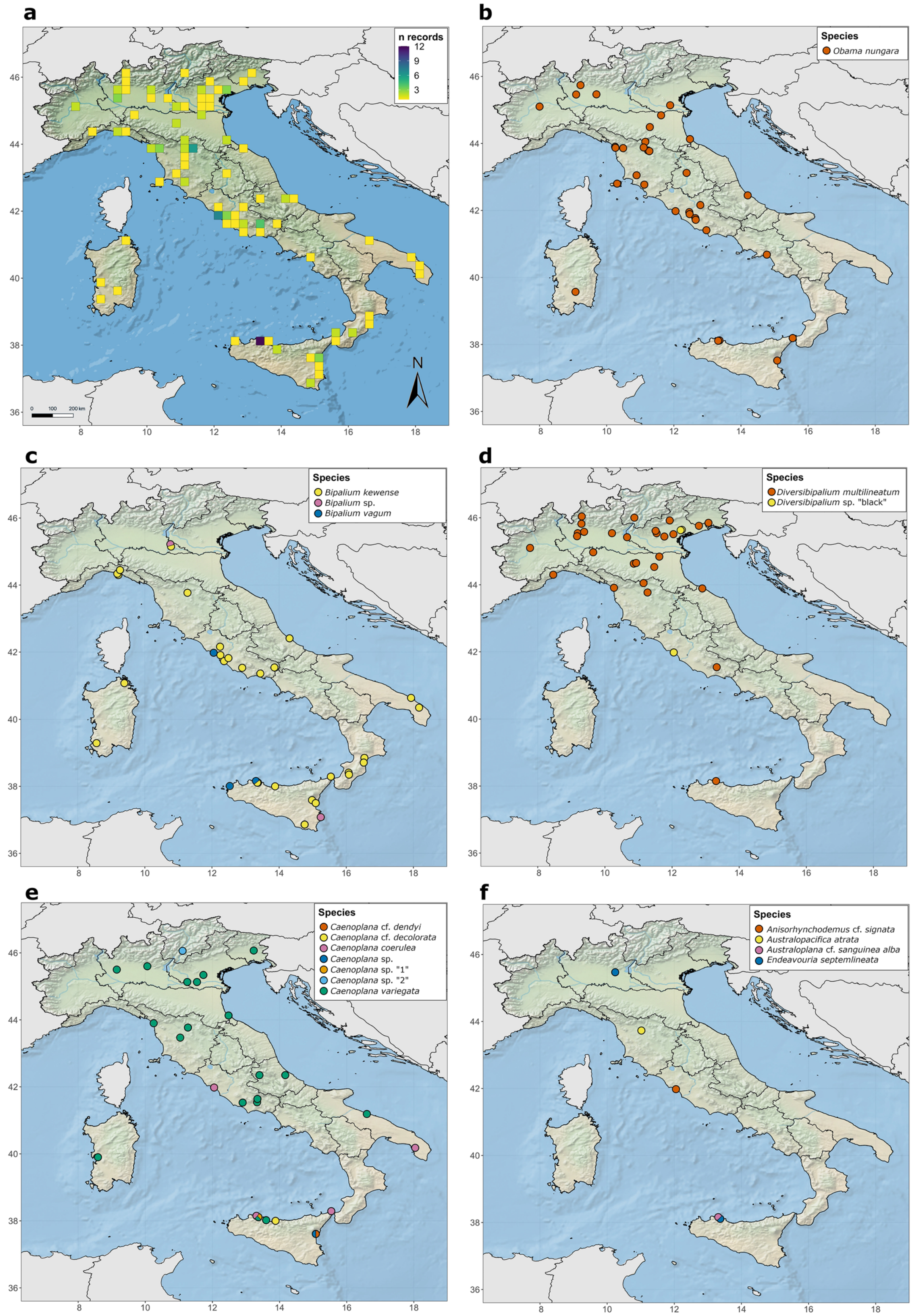
Fig. 1 Distribution of records of alien land planarians in Italy. ▶ **a** Distribution across regions; **b-f** each map corresponds to a genus, with records of genera represented by 1–2 observations clumped in only one map

The number of records increased between 2015 and 2020–2021. The low number of records in 2017 might be related to low rainfall that year (Fig. 2a).

Most records were observed in spring and early autumn (peaks in May and October, Hermans-Rasson test: $r = 67.27$, $p < 0.05$: Fig. 2b), i.e., at the peaks of rainfall in Italy. Of the 133 records, 111 were from domestic gardens (83.45%), six from botanical gardens, five from urban areas, four from plant nurseries, three from human indoor sites, three not known and only one from seminatural areas.

In our records, 94% of individuals were identifiable to species, the remainder to the level of genus. A total of 15 species of eight genera were identified (*Anisorhynchodemus* cf. *signata*, *Australopacifica atrata*, *Australoplana* cf. *sanguinea alba, Bipalium kewense*, *B. vagum*, *Caenoplana* cf. *dendyi*, *C.* cf. *decolorata*, *C. coerulea*, *C. variegata*, *Diversibipalium multilineatum*, *Diversibipalium* "black", *Endeavouria septemlineata,* and *Obama nungara*) with further unidentified taxa requiring investigation (Table 1; Supplementary Material Table S1).34

Among unidentified species, some individuals belonging to the genus *Diversibipalium* were recorded in three Italian localities (Latium and Veneto regions). They present morphological characters attributable to a taxon recorded for the first time in France (unnamed *Diversibipalium* 'black', Justine et al. 2018), belonging to a new species under description. Other two taxa not identified to the species level, for which only one individual each was recorded, were both morphologically assigned to the genus *Caenoplana*. For these individuals, molecular identifications were also performed. One of them (named *Caenoplana* sp. 1 FR989859) has the highest identity (> 99%) with a


a
n records
12
9
6
3
N
0 100 200 km
b
Species
Obama nungara
c
Species
Bipalium kewense
Bipalium sp.
Bipalium vagum
d
Species
Diversibipalium multilineatum
Diversibipalium sp. "black"
e
Species
Caenoplana cf. dendyi
Caenoplana cf. decolorata
Caenoplana coerulea
Caenoplana sp.
Caenoplana sp. "1"
Caenoplana sp. "2"
Caenoplana variegata
f
Species
Anisorhynchodemus cf. signata
Australopacifica atrata
Australoplana cf. sanguinea alba
Endeavouria septemlineata

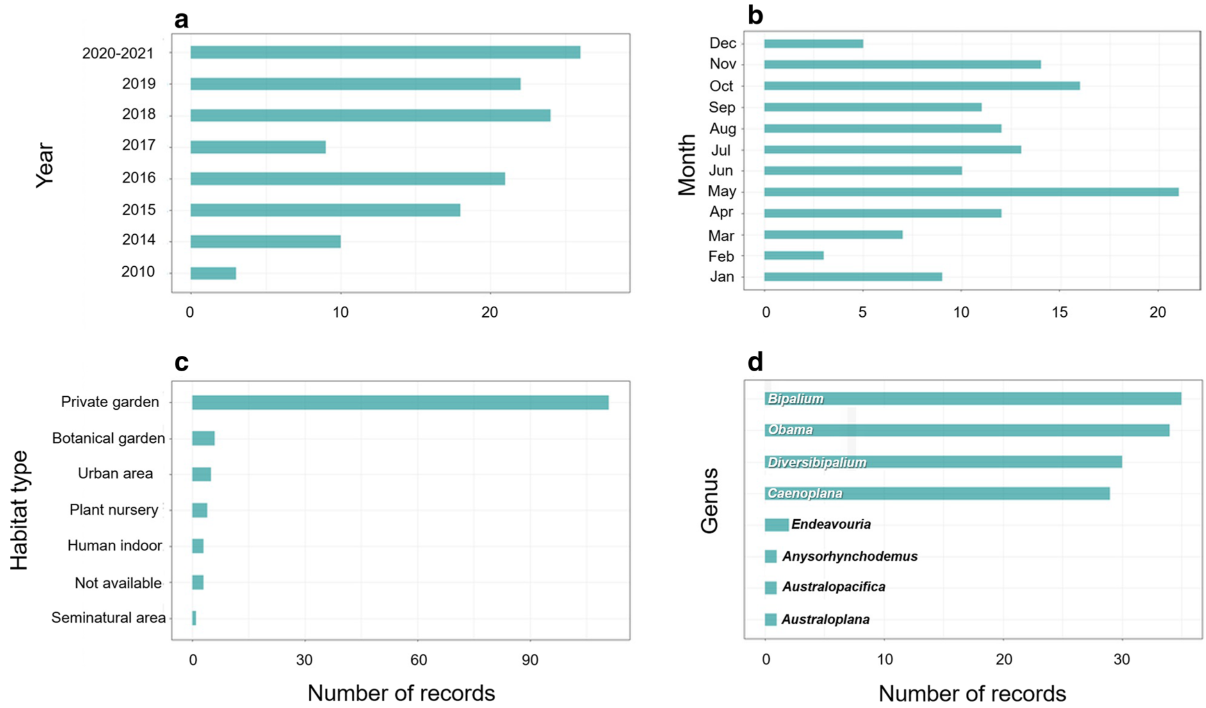


Fig. 2 Number of records per **a** year, **b** month, **c** habitat type and **d** genera

sequence labelled as *Caenoplana* sp. present in GenBank (accession number: KJ659650), the other (named as *Caenoplana* sp. 2 FR989860) has an identity < 89% with different sequences belonging to Caenoplana present within the same reference database. The maximum likelihood tree inferred from the alignment of Caenoplana COI sequences confirms the placement of the two Caenoplana variegata individuals collected in Italy (FR989857-FR989858) in a well-supported (aLRT = 1) monophyletic group together with other sequences of *Caenoplana variegata* mined from GenBank (Fig. 3). Whereas, the other two individuals of the genus included in this study, *Caenoplana* sp. 1 FR989859 and *Caenoplana* sp. 2 FR989860, grouped into two separate groups (aLRT of 0.99 and of 0.97, respectively) (Fig. 3).

In the maximum likelihood tree inferred from the *Obama* COI sequences alignment, the sequences developed in this study resulted in a monophyletic group consisting of *Obama nungara* (aLTR = 0.99), thus confirming the molecular identification obtained through BLAST. Specifically, the *Obama nungara* individuals collected in Italy clustered into two separate and well supported clades, one including representative of the Argentina 1 clade (according to Justine et al., 2020) and the others grouping with representatives of Argentina 2 clade (Justine et al., 2020; Figure S1 in Supplementary Material 2). The phylogenetic placement of the *Bipalium* individual recorded in this study (accession number FR989861) was consistent with BLAST identification to *Bipalium vagum* (Figure S2 in Supplementary Material 2).

Discussion

Our work showed an extensive level of planarian invasion in Italy, with at least 15 species and some undescribed and unidentified at species-level taxa detected in five years of monitoring, making Italy one of the European countries, together with UK and France, with the highest diversity of alien planarian species. Among those, *Obama nungara,* native to South America, seems to be the most successful invader, with 34 records from 11 Italian regions and more than 100 specimens found in a private garden in Lombardy. This finding is supported by the fact that most of Italy is suitable for the establishment of this flatworm (Negrete et al. 2020; Fourcade 2021), as well as most of Southern Europe (Carbayo et al. 2016). We

Table 1 Genera and species of alien land planarians detected in Italy. N, number of records; Mor, morphological identification based on photo analysis; Mol, molecular analyses

Genus and species	N	Identification	BLASTN % of identity with best match	Accession numbers	Native range
Anisorhynchodemus	1				
cf. *signata*	1	Mor			Far East
Australopacifica	1				
atrata	1	Mor			New South Wales and Moluccan Islands
Australoplana	1				
cf. *sanguinea alba*	1	Mor			Australia
Bipalium	35				
kewense	29	Mor			South-East Asia
sp.	2	Mor			Unknown
vagum	4	Mor mol	100% *Bipalium vagum* MG655593	FR989861	Australasia
Caenoplana	29				
cf. *dendyi*	1	Mor			Australia
coerulea	4	Mor			Australia
sp.	1	Mor			Unknown
sp. 1	1	Mor and mol	99.8% *Caenoplana* sp. KJ659650	FR989859	Unknown
sp. 2	1	Mor and mol	88.1% *Caenoplana* sp. KJ659643	FR989860	Unknown
cf. *decolorata*	1	Mor			Unknown
variegata	20	Mor and mol	99.3% *Caenoplana variegata* MN990648	FR989857; FR989858	Australia
Diversibipalium	30				
multilineatum	27	Morl and mol	100% *Diversibipalium multilineatum* MG655612	FR989851-FR989854	Japan
sp. "black"	3	Mor			Unknown
Endeavouria	2				
septemlineata	2	Morl and mol	100% *Endeavouria septemlineata* MH572010	FR989855; FR989856	
Obama	34				
nungara	34	Mor and mol	> 99.2% *Obama nungara* MN529567/ MN529563/MN529572/KJ659654 /	FR989842-FR989850	South America

also provided the first molecular evidence of the occurrence of *Bipalium vagum* and *Endeavouria septemlineata* in Europe. All planarian species are hermaphrodites, which means that their reproductive system has both male and female genital structures. Furthermore, planarians show an amazing ability to regenerate missing body part, i.e. different pieces of a single planarian may regenerate missing body parts, forming a complete individual. Therefore, even where single individuals have been observed, it is not possible to assess the invasion status (sensu Blackburn et al. 2011), although we are confident that areas hosting over 10 individuals (cf. Supplementary Material 1) includes self-sustaining populations.

Records of alien planarians in Italy came from all Italian regions except for those characterised by the lowest human population densities (i.e., 38–67 human inhabitants/km^2), and the smallest area (3,260–10,070 km^2: www.istat.it, accessed on 15.03.2021). In these regions, fewer introductions may have occurred, therefore resulting in a lower density of populations and variety of introduced planarian species. This may represent a bias of citizen-science sampling, thus not reflecting an actual absence of alien planarians, but

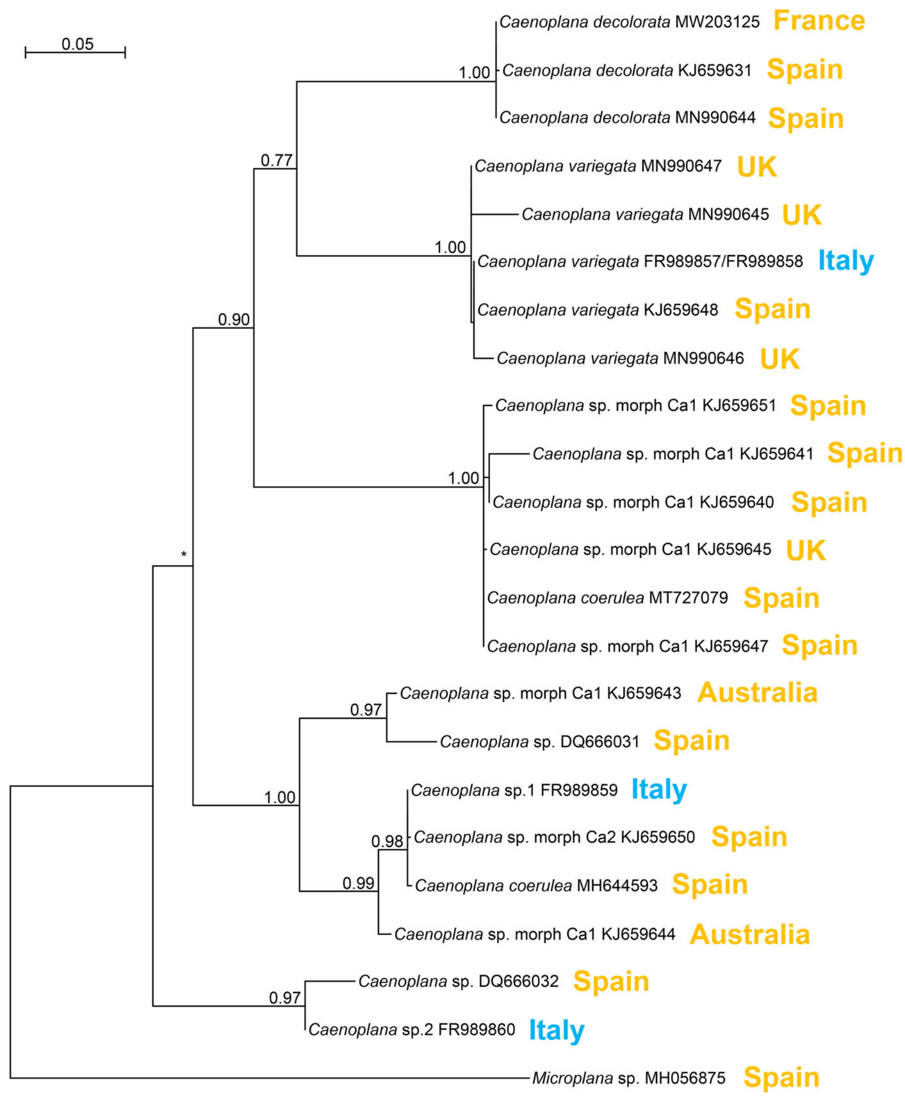


Fig. 3 *Caenoplana* maximum likelihood COI tree. GenBank accession numbers are reported on the tips; outgroup *Microplana* sp. MH056875. The aLRT values are reported on nodes; * represents aLRT values < 0.70. The scale bar indicates the distance in substitutions per site

possibly due to cultural differences between urban populations accustomed to using smartphones and social media, and rural populations, often characterised by people less familiar with modern technology (Justine et al. 2020). Furthermore, the lack of records in winter may be due to a combination of a lower number of worms in this season and of the fact that people usually do not work in their gardens in cold months. Accordingly, Justine et al. (2020) reported that, although it is probably true that *O. nungara* is less abundant in winter (December–February), it is not possible to exclude with certainty if this is an actual decrease of abundance, or if the number of records is low simply because gardeners spend less time in their gardens in cold months. Moreover, during the winter the terrestrial planarians limit their activity in a sort of hibernation period, staying in the interstitial zones of the soil and emerging later on, in spring, i.e. when the conditions are more suitable for their vital activity (Cannon et al. 1999).

Anecdotal observations showed that successful invasion of alien flatworms in Italy is influenced by soil moisture, in line with previously published works (Fraser and Boag 1998). Alien planarians were mostly recorded in private gardens or at least in human-modified habitats (possibly because these areas are more sampled by citizen-scientists with respect to natural ones). Where detected, alien planarians were always associated with ornamental plants (Boag et al. 1994; Alford et al. 1996; Moore et al. 1998) and found in microhabitats with moist conditions. This may explain why the highest number of records came from the largest regions, where human population density is high (e.g. Tuscany, Latium, Sicily, and Lombardy). Citizen-science data significantly expanded the distribution area colonized by these species in Italy, and thus social networks and citizen-science platforms represent an effective tool for the early detection of pest species as already highlighted in Mazza et al. (2020) and Farina et al. (2021).

Since many of these alien planarians are recent colonizers (Justine et al. 2014), it is not possible to predict what their status will be in the future, because alien species often exhibit their invasiveness only after an initial period of adaptation (lag phase), which may be rather long (Crooks 2005), as acclimatization to new environmental conditions plays an important role during establishment (Hänfling et al. 2011).

Moreover, for several species, taxonomy is debated or undefined. Thus, there is a remarkable doubt on their geographic origins. An exemplar case is those of individuals belonging to *Caenoplana* genus recorded within this study. The maximum likelihood tree inferred from COI sequences of this genus confirmed the results obtained from the molecular identification using BLASTn tool (Altschul et al. 1990), including the impossibility to define the species to which two *Caenoplana* individuals recorded in Italy (*Caenoplana* sp. 1 FR989859 and *Caenoplana* sp. 2 FR989860) belong. From the tree, it is possible to define that these individuals belong to two different entities, both possibly attributable to undescribed new species of the genus *Caenoplana*. In any case, the possibility that they belong to morphologically described species that are not represented in GenBank from conspecifics identified to species level has also to be considered. In general, the inferred tree is representative of the taxonomic uncertainly related to the genus *Caenoplana*. Individuals identified based on their morphotypes are split into few groups, forming well-supported clusters with individuals identified to genus-level, suggesting that a taxonomic redefinition of alien planarians in general may be advisable and possibly resulting in the description of species new to science.

Colonization by alien species is promoted by international trade and their establishment is favoured by human-driven climate change (Bertolino et al. 2020). Ecosystem resistance and resilience encountered by invaders are weakened by environmental alterations and by higher environmental temperatures in human settlements, factors that may facilitate the establishment of newcomers (Masters and Norgrove 2010; Pyšek et al. 2020).

The introduction of land planarians, recognized as top predators of the invertebrate soil fauna, poses a risk to the native invertebrates in those areas where they have been accidentally introduced (Murchie and Gordon 2013; Molfini et al. 2020). For instance, *Platydemus manokwari*, categorized as one of the 100 worst invasive species, has caused a rapid decline in terrestrial invertebrates in areas where it has been introduced (Gerlach et al. 2021). The ''New Zealand'' flatworm *Arthurdendyus triangulatus* was introduced in the UK, Ireland, and Faroe Islands, causing a serious risk to earthworms and, potentially, to native planarians (Boag et al. 2006; Murchie and Gordon 2013). As to Italy, a quantification of impacts by introduced planarians is still lacking but deserves further research. In addition, since the vast majority of the observations comes from anthropogenic areas, further research should focus the evaluation of the expansion from private areas to nearby natural ones.

When prevention fails, early warning and rapid response is the best option to avoid the spread of alien species (Genovesi et al. 2015). Threats posed by introduced alien species should be thus monitored, starting from the distribution of established ones, the assessment of their impacts, and the early detection of new invasions (Bertolino et al. 2020; Molfini et al. 2020).

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Data availability Data used in this study is available in Table S1 of the Electronic Supplementary Material.

Declarations

Conflict of interest The authors state no conflicts of interest.

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