

## ***Oscheius tipulae* in Italy: Evidence of an Alien Isolate in the Integral Natural Reserve of Montecristo Island (Tuscany)**

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**Abstract:** Montecristo Island is an integral natural reserve of the Tuscan Archipelago National Park (Central Italy), characterized by a peculiar assemblage of flora and fauna, with several endemic taxa, and also with a high number of alien species. During a soil survey, we found an alien *Oscheius tipulae* Lam & Webster, 1971 isolate, phylogenetically close to others from South America. In this article, we examined the possible pathways of introduction of this nematode. Because of the high number of alien plants in this protected area and the low desiccation survival ability of *O. tipulae*, we hypothesized that the presence of this alien nematode isolate may be related to the soil of introduced plants, although historical association with plant-associated invertebrates is also possible. Further studies with more populations and marker molecules are necessary to investigate the distribution of *O. tipulae* and the possible impact on this natural reserve.

**Key words:** alien nematode isolate, Central Italy, desiccation assay, genetics, nematode introduction.

Insular biodiversity is characterized by a peculiar fauna and flora, with high levels of endemism (Whittaker and Fernández-Palacios, 2007). The Tuscan Archipelago consists of seven major and several minor islands located between Corsica and Tuscan coast. Most of the islands of the Tuscan Archipelago are included, partly or entirely, in the Tuscan Archipelago National Park, established in 1999, while Montecristo Island has been a state natural reserve since 1971. Montecristo Island is one of the most peculiar ones, because it is a biogenetic reserve established in 1971 by a ministerial decree to protect its unique nature. Visitors face many restrictions and this management strategy helped the preservation of a peculiar combination of flora and fauna (<http://www.corpoforestale.it>). Notwithstanding the rigorous protection, several alien species, both animals and plants, are present in this island, mainly as historical-cultural legacies (<http://www.montecristo2010.it>, <http://www.restoconlife.eu/>).

Despite the importance of these islands and the presence of several protected areas, comprehensive projects are currently carried out only for some taxonomic groups (AA. VV., 1976; Cianferoni et al., 2013). Data on nematodes of the Tuscan Archipelago National Park are almost absent. The only available concern is endoparasites, e.g., stomach nematodes of goats, *Capra hircus* Linnaeus, 1758 (Lucchesi et al., 2011). For this reason, we began a survey of nematodes to fill the gap of the knowledge on these taxa.

During a soil survey in Montecristo Island, we found an alien *O. tipulae* strain, phylogenetically close to the South American ones. Alien species (Scalera and Zaghi, 2004) are present in this area and in the other islands of the Tuscan Archipelago National Park (Mazza et al., 2012; Inghilesi et al., 2013a; Lazzaro et al., 2014). For this reason, several projects were conducted for the eradication of invasive alien species in Europe, as they are recognized to cause heavy impacts on native biodiversity (Kettunen et al., 2009; Shine et al., 2009) and for the protection of native species and habitats in the Archipelago (<http://www.montecristo2010.it>, <http://www.restoconlife.eu/>). Nematodes with other organisms present in the soil can be accidentally introduced via a range of human-associated pathways (Meagher, 1977; Hulme et al., 2008; Hughes et al., 2010), in particular as contaminant of traded plant propagation material (Davidson et al., 2005; McNeill et al., 2006) and through clothing and footwear (Baker, 1966; Davidson et al., 2005; Webber and Rose, 2008).

Unfortunately, there is still a considerable lack of knowledge about the incidence and distribution of terrestrial nematodes (new species are continuously described, such as *Oscheius onirici* Torrini et al., 2015) and their status as alien or endemic. The only data concerning some heavy and famous pests of agriculture (e.g., *Globodera* spp.) and forests (e.g., *Bursaphelenchus xylophilus* (Steiner and Buhner 1934) Nikle), or parasites of alien animals introduced to Europe (reviewed in Roques et al., 2009).

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In this article, we report the finding of an alien *O. tipulae* isolate and we attempt to examine the possible pathways of its introduction, highlighting human-associated pathways, such as contamination of 1) alien plants and 2) soil under tourist footwear.

#### MATERIALS AND METHODS

*Isolation of nematodes from Montecristo soil:* Three samples of soil (about 2 dm<sup>3</sup>) were collected between 17 and 20 May 2014 on the three more accessible sites of Montecristo Island: Monte della Fortezza (42.342922 N, 10.307620 E), Cima dei Lecci (42.326680 N, 10.306847 E), and the vegetable garden near Cala Maestra (42.334675 N, 10.295432 E). For each location, six scores were randomly sampled and then mixed to form one composite sample.

We assessed the presence of entomopathogenic nematodes using the *Galleria* bait method (Bedding and Akhurst, 1975). The soil samples were placed in plastic containers and one steel mesh pocket containing three last larval instars of *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) was placed in each container and kept at room temperature (20 ± 3°C) in the Nematology Laboratory of Centro di Ricerca per l'Agrobiologia e la Pedologia (CREA), Florence, Italy. Cadavers with nematodes were placed on modified White traps (Kaya and Stock, 1997). Juveniles emerging from the *Galleria* larvae were collected and stored in distilled water in 50-ml tubes at 12°C. Part of them was used for identification and the remaining part for desiccation tolerance experiments.

*Identification and phylogenetic analysis:* Nematodes (juveniles and adults) were mounted on temporary slides and observed under Leitz Orthoplan light microscope at up to ×1,000 magnification.

For molecular analysis, five nematodes of Montecristo Island were placed individually in 50.0 µl of InstaGene Matrix (Bio-Rad, Milan, Italy) and the DNA was extracted according to the manufacturer's protocol. The amplification of 18S and ITS loci was performed according to Foucher and Wilson (2002) and Joyce et al. (1994). Amplicons were sequenced at the CREA-Centro di ricerca per la genomica vegetale, Italy, and the sequences were submitted to GenBank (accession number: KT728762). The other *O. tipulae* strains used in the phylogenetic tree reconstruction were the following: GT4, F1, CEW1, JU956, JU1061, JU1062, JU1063, JU1131, JU1173, JU1417, and SB283. The amplification of 18S and ITS loci was performed as described above and the sequences were submitted to GenBank (accession numbers: KT728763, KT728764, KT728756, KT728760, KT728759, KT728761, KT728757, KT728755, KT728753). Phylogenetic inferences using maximum likelihood (ML) method were performed with Molecular Evolutionary Genetics Analysis Version 6.0 (Tamura et al., 2013) on ITS locus adopting T92 as substitution matrix and performing 1,000 bootstrap replicates;

the reconstruction of phylogenetic tree was tested with a moderate branch-swapping algorithm. The choice of an appropriate substitution model was evaluated using jModelTest2. Bayesian inference (BI) of phylogenetic tree was performed with BEAST2 under the coalescent constant population model and a relaxed log-normal clock assumption. Analyses were run for 10 million generations, sampling every 1,000 generations (first million discarded as burn-in). Five independent Markov chain Monte Carlo analyses were performed starting from a randomly chosen tree. Maximum clade credibility tree was summarized.

*Data on alien plants and visitors:* An accurate screening of the more recent literature data sources on Montecristo was performed (Paoli and Romagnoli, 1976; Lazzaro et al., 2014). Field surveys, aiming at assessing the actual presence of alien plants, were also conducted in the years 2013–14. Nomenclature is after Celesti-Grapow et al. (2009) and Banfi and Galasso (2010); for the additional data on the *taxa*, native range, introduction mode, and the invasion *status* terminology, we followed Blackburn et al. (2011), Celesti-Grapow et al. (2009, 2010a, 2010b), and Pyšek et al. (2004), also checking online databases.

Information about the flux of tourists and travellers in the Island from 1984 to 2014 were gathered using the State Forestry Corps register of visitors. Information on visitors from 1971, year of the establishment of the reserve, to 1984 is not available.

*Surviving ability to desiccation of *Oscheius tipulae*:* As discussed in McNeill et al. (2011), since the soil of footwear (in particular hiking boots) contains non-indigenous species such as nematodes, we performed an experiment to assess the risk of a possible introduction of *O. tipulae* through soil adherence to footwear. Laboratory experiments were conducted to investigate desiccation stress tolerance of *O. tipulae* collected from Montecristo soil.

Nematodes were kept at 20°C for 24 hr prior to testing. Experimental units consisted of petri dishes (10 cm diam.), each one containing 20 ml of sterile (autoclaved) soil volume. The textures of the soil used were classified as clay and sand according to the U.S. Department of Agriculture Soil Taxonomy. In particular, clay soil was characterized by 14.8% ± 0.27% of sand, 34.1% ± 0.95% of silt, and 51.1% ± 0.88% of clay, and sandy soil by 100% of sand. A distilled water suspension of 1.0 ml containing about 250 dauer juveniles was inoculated into each petri dish. The petri dishes containing both sandy soil ( $n = 20$ ) and clay soil ( $n = 20$ ) were opened for 1 d at 20 ± 2°C for the desiccation. The same set of trials ( $n = 20$  for sandy soil and  $n = 20$  for clay soil) was dissected after 1 wk. Live nematodes were extracted after the desiccation periods through the Baermann funnel technique involving immersion of the soil samples in water for 48 hr. Nematode survival in soil was measured by direct counting.

**Data analyses:** Statistical comparisons of frequencies were made using Wilks' test after Williams' correction (statistic: G). The level of significance at which the null hypothesis was rejected is  $\alpha = 0.05$ . Nematode survival following desiccation trials was analyzed by one-way analysis of variance. The differences were compared using Duncan's test (CoStat statistical software package; <http://www.cohort.com/costat.html>).

## RESULTS AND DISCUSSION

Nematodes isolated using *Galleria* bait method from the soil collected in Monte della Fortezza corresponded with the description of *O. tipulae* in Sudhaus (1993), based on morphobiometrical observations. The sequences of the 18S locus of the Montecristo isolate (named MC1) confirm the species attribution to *O. tipulae*, a widespread hermaphroditic soil nematode, which displays a high genetic diversity and a complex large-scale geographical structure (Baïlle et al., 2008). No *O. tipulae* were obtained from the other two soils of Montecristo Island.

Sequence analysis of 18S locus of all strains did not show the presence of polymorphic sites, but the ITS locus instead showed 12 SNPs and 2 insertions that were used for phylogenetic inferences. The phylogenetic relationship between *O. tipulae* MC1 and other strains are shown in Fig. 1. The final tree shows two well-supported clades using the three reconstruction methods (ML and BI): Clade I could be subdivided in tree subgroups called the Sicilian, the hybrid Japanese-South American and South American group while Clade II includes European and North American strains.

The *O. tipulae* MC1 sequence was significantly different not only from the Tuscan ones (GT4 and F1) that are geographically closer to Montecristo Island, but also from the other Italian strains, the Sicilian ones that

grouped together (Fig. 2). The close phylogenetical relationship of the MC1 strain to the South American strains leads us to hypothesize its possible introduction from that area. Although not easily quantifiable, movement of plants and soil from Brazil to Portugal could have been the historic route of nematode introduction. However, as for *Pristionchus pacificus*, the ecological knowledge of geographic endemicity of *O. tipulae* is incomplete and far away to be solved (Baïlle et al., 2008).

In Montecristo Island, we found 69 alien plant species belonging to 40 families. Most of the species come from Asia (G corr. Williams = 36.79; df = 7;  $P < 0.001$ ; Fig. 3). As expected, deliberate arrival is the main pathway of introduction (81%; G corr. Williams = 66.24; df = 2;  $P < 0.001$ ) and plants are mostly cultivated (62%; G corr. Williams = 70.14; df = 4;  $P < 0.001$ ). No analyses of soil of transplanted plants (with the roots and intact soil) were performed, since no plants have been recently introduced. Although the richness of alien plants on islands is strictly related to human pressure (Kueffer et al., 2010), the census data of the alien flora in Montecristo, currently an uninhabited island, highlighted the presence of a high number of taxa and the high related probability of introduction of alien species, in particular invertebrates. In-depth information about the introduction pathways of the alien species are lacking. However, the direct introduction of some plants (with the associated fauna; Mazza et al., 2012; Inghilesi et al., 2013b) from their own home ranges (e.g., the members of the genus *Eucalyptus* from Australia) has been ascertained, whereas we found no information about possible indirect introductions of alien cultivated species from continental Italy.

Visitors come mainly from Italy, whereas other countries are less represented (Fig. 4). A secondary introduction from the nearby Italian soil is improbable, because MC1

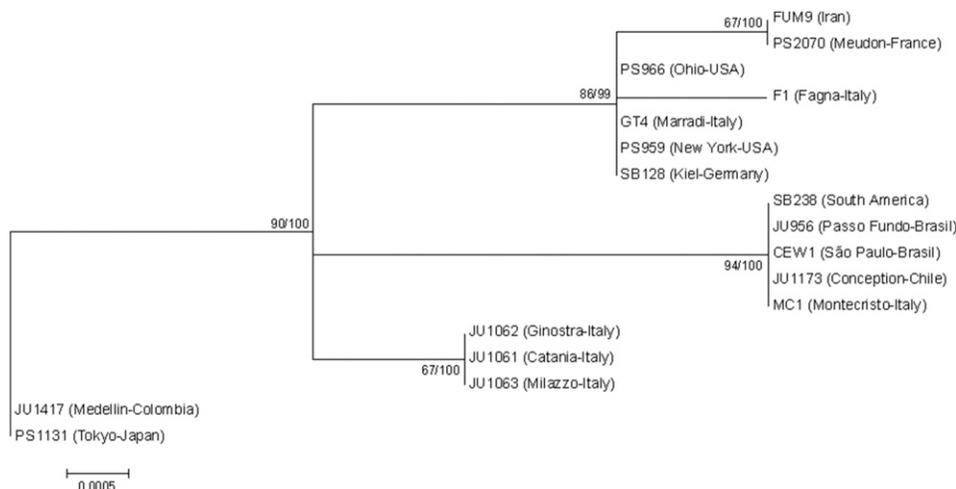


FIG. 1. Phylogenetic identification of *Oscheius tipulae* MC1 based on ITS locus. The tree is displayed in a condensed form with a 50% cut-off value and a 0.9 posterior probability limit; the identifying marks of strains are followed by their collection sites in brackets. Bootstrap support values maximum likelihood and posterior probability Bayesian inference were reported for each node.

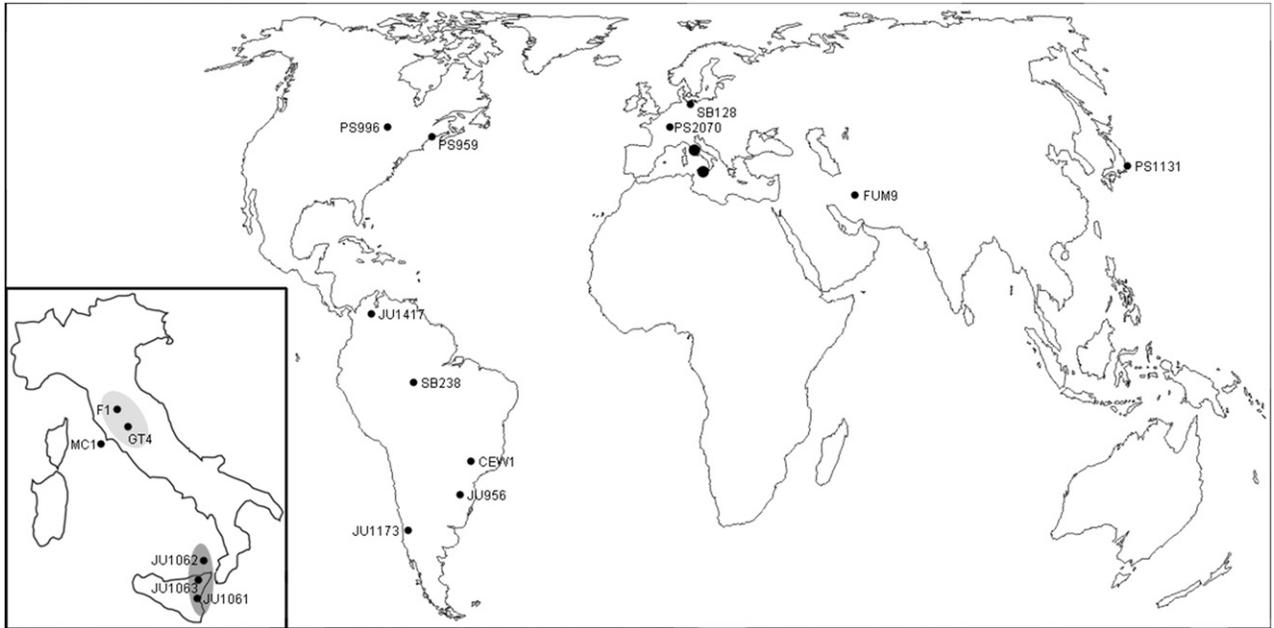


FIG. 2. Distribution of *Oscheius tipulae* isolates used for the phylogenetic tree reconstruction. The inset shows the geographical position of the sampling locations in Italy and the phylogenetic groupings.

strain was not found in other parts of Italy. Anyway, further surveys are needed to exclude this possibility. Moreover, desiccation stress tolerance of *O. tipulae* revealed a low probability of nematode introduction through soil adherence to footwear. After 1 d, only  $17.54\% \pm 0.96\%$  and  $6.24\% \pm 0.44\%$  of nematodes were live in clay and sandy soils, respectively. The percentage reached almost zero value after 1 wk in both soils (clay:  $0.78\% \pm 0.19\%$ ; sandy:  $0.06\% \pm 0.03\%$ ). Significant differences were found only between clay and sandy soils after 1 d, and between different days ( $F_{3, 76} = 226.89$ ;  $P = 0.00001$ ; Fig. 5). However, we cannot discard the possibility of introduction of *O. tipulae* by soil of footwear and the consequent establishment, since this nematode reproduces through self-fertilizing hermaphrodites and facultative males (Baillie et al., 2008) and thus few specimens are sufficient to create established populations.

From our results, import of plants and associated insects, isopods, or snails before phytosanitary restrictions has been historically reported and could have been the source of the nematode more than the recent soil from tourist foot traffic. Baillie et al. (2008) suggested the possibility of nematode dispersion through wind and birds, but in this case, we consider it highly improbable. The isolate we found in this protected area is very similar to the CEW1, isolated in São Paulo, Brazil, which is currently used as the reference strain for molecular and genetic studies and thus largely diffused in

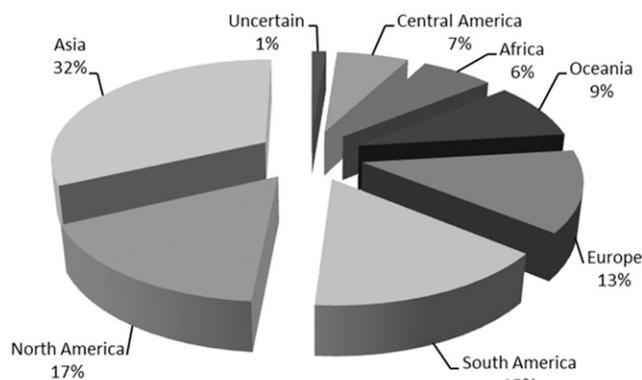


FIG. 3. Donor countries of alien plants found in Montecristo Island.

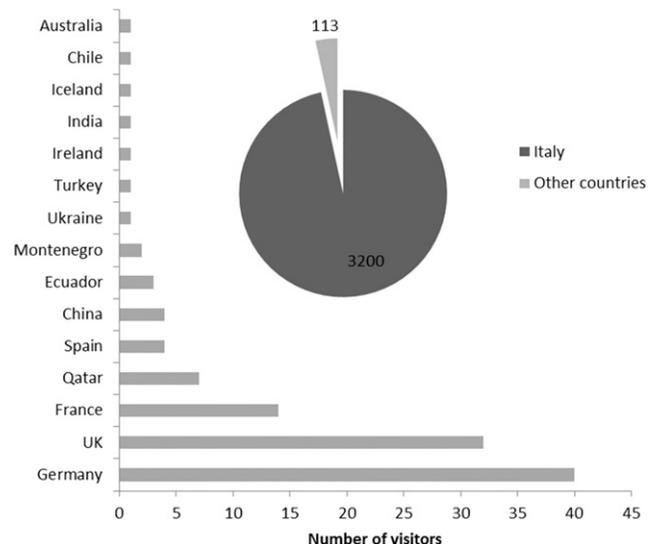


FIG. 4. Number of visitors in Montecristo Island from 1984 to 2014. Histogram describes the origin of people from “other countries.” Tourists from Russia, United States, Norway, Brazil, Canada, and Egypt are present in the register, but their number is unknown.

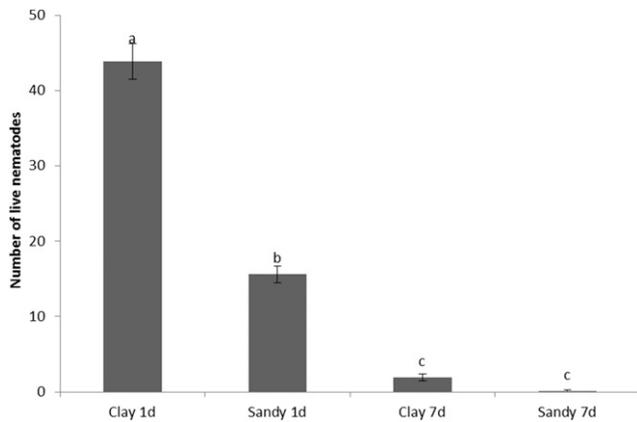


FIG. 5. Number of live *Oscheius tipulae* nematodes (mean  $\pm$  SE) in desiccation stress tolerance trials. Different letters up the histograms indicate significant differences at  $P < 0.05$ .

laboratories (Evans et al., 1997; Dichtel et al., 2001; Louvet-Vallée et al., 2003; Dichtel-Danjoy and Félix, 2004; Ahn and Winter, 2006). Indeed, the introduction of species for research purposes, i.e., the use of animals in research laboratories, is a serious concern as escaped or released animals can represent a threat for the local communities (Springborn et al., 2011).

It is noteworthy that one of the first nematodes found in this protected area is an alien isolate. An accurate monitoring program should be carried out in the countryside nearby the site of the first report to assess the real distribution of this nematode and the presence of other nematode species in Montecristo and in the other islands of the Tuscan Archipelago. Finally, since this species was originally isolated from *Tipula* larvae, and is also found on isopods, snails, and adult insects, the analysis of possible impacts on the invertebrate fauna would be necessary, in the light of the high levels of endemic taxa of Montecristo Island (Vignoli et al., 2007).

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