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Research Notes

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A singular case of early-stage long-distance dispersal of the smooth newt, *Lissotriton vulgaris* (Linnaeus, 1758) (Amphibia, Caudata)

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Abstract: To date, the global introduction of alien amphibians and reptiles has likely been facilitated by the expanding plant nursery trade. Here, we report in detail a singular case of unintentional introduction of *Lissotriton vulgaris* in Sicily. A newt in the final stages of its larval development was found in a private garden pond in north-western Sicily, where aquatic plants from a nursery in Veneto (northeastern Italy) had been introduced two months earlier. The analysis of the mithochondrial markers ND2 and ND4 revealed a shared haplotype between the Sicilian newt and a sample from Treviso, the province of the putative source. The timing of the plant introduction to the garden pond suggests that the translocation occurred during the newt's egg or early larval stage. Although based on a single case study, our results provide clear evidence of amphibian egg/early stage translocation and their ability to survive as contaminants in aquatic plants. This underscores the need to strengthen prevention and early detection programs in the plant trade and nursery management supply chain, involving institutions, regulatory bodies, traders, as well as buyers and hobbyists.

Key words: Nonnative species, translocation, plant nurseries, Sicily

1. Introduction

Among the most impactful nonnative invasive species, amphibians and reptiles are a well-represented group (Wiles et al., 2003; Shine, 2010). Despite the extent of their impact on ecosystems, many species possess a strong capacity for passive dispersal, which has sometimes led to an almost cosmopolitan spread (Rödder et al., 2008, 2017). The current global introduction of allochthonous amphibians and reptiles has likely been facilitated by the sharp increase in international trade (Westphal et al., 2008) and climate change (Sutherst, 2000). Regarding the first factor, herpetofauna is continuously translocated both voluntarily, particularly through the pet trade (Krysko et al., 2011) and as food resource (Bellati et al., 2019), and involuntarily, through the transport of goods (Faraone et al., 2019; Mori et al., 2022). One of the most effective dispersal factors is the plant nursery trade, driven by the growing volume of translocated goods and shorter travel times (Kraus, 2007).

The interactions between introduced herpetofauna and ecosystems are well known and, for amphibians, they often involve multiple impacts, such as predation of native species (Oda et al., 2019), introduction of pathogens (Fisher and Garner, 2007), and negative effects on native predators (Letnic et al., 2008). The cumulative effects of certain nonnative reptile and amphibian species on ecosystems, as well as their management, can also require significant efforts and incur substantial costs from an economic perspective (Soto et al., 2022 and references therein). This underscores the need for heightened attention to the prevention of potential future invasions.

Here, we report in detail a singular case of unintentional amphibian introduction, in which we precisely traced the origin, timing, and method of translocation. Furthermore, we discuss the risks associated with the plant trade and the need for preventive measures.

2. Materials and methods

On 26 June 2021, while maintaining a garden pond on a private property near the town of Altavilla Milicia (Province of Palermo, NW Sicily; 38°02’E, 13°33’N), the author SC found a newt larva, along with numerous tadpoles of the painted frog, *Discoglossus pictus* Otth, 1837, at various stages of development (Figures 1A and 1B). Since Caudata species are not native to Sicily (Lo Valvo et al., 2017), the newt was collected for ex situ raising in a dedicated aquarium and subsequently identified following Razzetti et al. (2016). The newt was photographed three days after capture (Figure 1C) using a digital camera (Nikon Coolpix P600, Nikon Corporation, Tokyo, Japan)

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and then measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

The garden pond was established in early spring 2021, and on 28 April 2021, three aquatic plant species were introduced: *Typha minima* Funck, *Persicaria amphibia* L., and *Utricularia australis* R.Br., 1810. The plants were purchased from a nursery in the Veneto region of northeastern Italy (Province of Treviso) and arrived in Sicily three days after shipment. All plants were transported in pots containing soil, except *U. australis*, which was wrapped in damp paper and packed in a plastic bag (Figure 1D). This species was briefly rinsed in warm water before being placed in the pond.

In order to confirm the species identity of the specimen and determine its geographic origin, a biomolecular analysis was performed. Genomic DNA was extracted from a 2-mm tail tip tissue sample preserved in 96% ethanol, using MyTaq Extract-PCR Kit (BIO-21127; Meridian Bioscience, Cincinnati, Ohio, USA). PCR amplification was performed using MyTaq HS Red mix (Bioline) 2×, following the manufacturer's protocol. Two mitochondrial DNA (mtDNA) fragments were subsequently amplified and sequenced: a portion of the NADH dehydrogenase subunit 4 gene (hereafter ND4) and the NADH dehydrogenase subunit 2 gene (hereafter ND2). Amplifications and sequencing were performed using the primers ND4 (CACCTATGACTACCAAAAGCTCATGTAGAAGC) and Leu (CATTACTTTTACTTGGATTTGCACCA) for the ND4 fragment, L3780 (CCCCAAATATGTTGGTGGAA) and H5018 (TCTGGGTTGCATTCAGAAGA) for the ND2 fragment. Primers were selected following Maura et al. (2014) to adequately position our sample within the context of the Italian haplotypic diversity of *L. vulgaris*. The PCR was performed as follows: 94 °C for 2 min, 56 °C for 45 s, 72 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 56 °C for 45 s, 72 °C for 90 s, with a final extension step at 72 °C for 3 min. PCR products, verified by electrophoresis in a 1% agarose gel, were sent to BMR Genomics for purification using Expo-SAP (BMR Genomics, Padua, Italy) and sequencing via the Sanger method (Sanger et al., 1977).

The obtained electropherograms were visually inspected using Chromas v. 2.6.2 software (Technelysium Pty. Ltd., 1998, Queensland, Australia). The ND2 and ND4 sequences were analysed using the BLAST algorithm[1] to confirm the taxonomic identity and to select appropriate comparative datasets. The novel sequences were deposited in the GenBank public database (accession numbers: PV527312 for ND2; PV527313 for ND4). A total of 94 *Lissotriton vulgaris meridionalis* (Boulenger, 1882) sequences (47 for ND2 and 47 for ND4) were downloaded from GenBank (accession numbers: KM262086–KM262179; see Maura

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[1] National Library of Medicine (2024). Basic Local Alignment Search Tool [online]. Website https://blast.ncbi.nlm.nih.gov/Blast.cgi [accessed 22 October 2024].

Figure 1. A: garden pond where the smooth newt, *Lissotriton vulgaris* (Linnaeus, 1758), was found; B: the smooth newt at the time of capture, alongside the painted frog, *Discoglossus pictus*, Otth, 1837 tadpoles; C: the smooth newt three days after discovery (background square width: 10 mm); D: *Utricularia australis* R.Br., 1810 at the time of delivery, prior to being introduced into the garden pond.

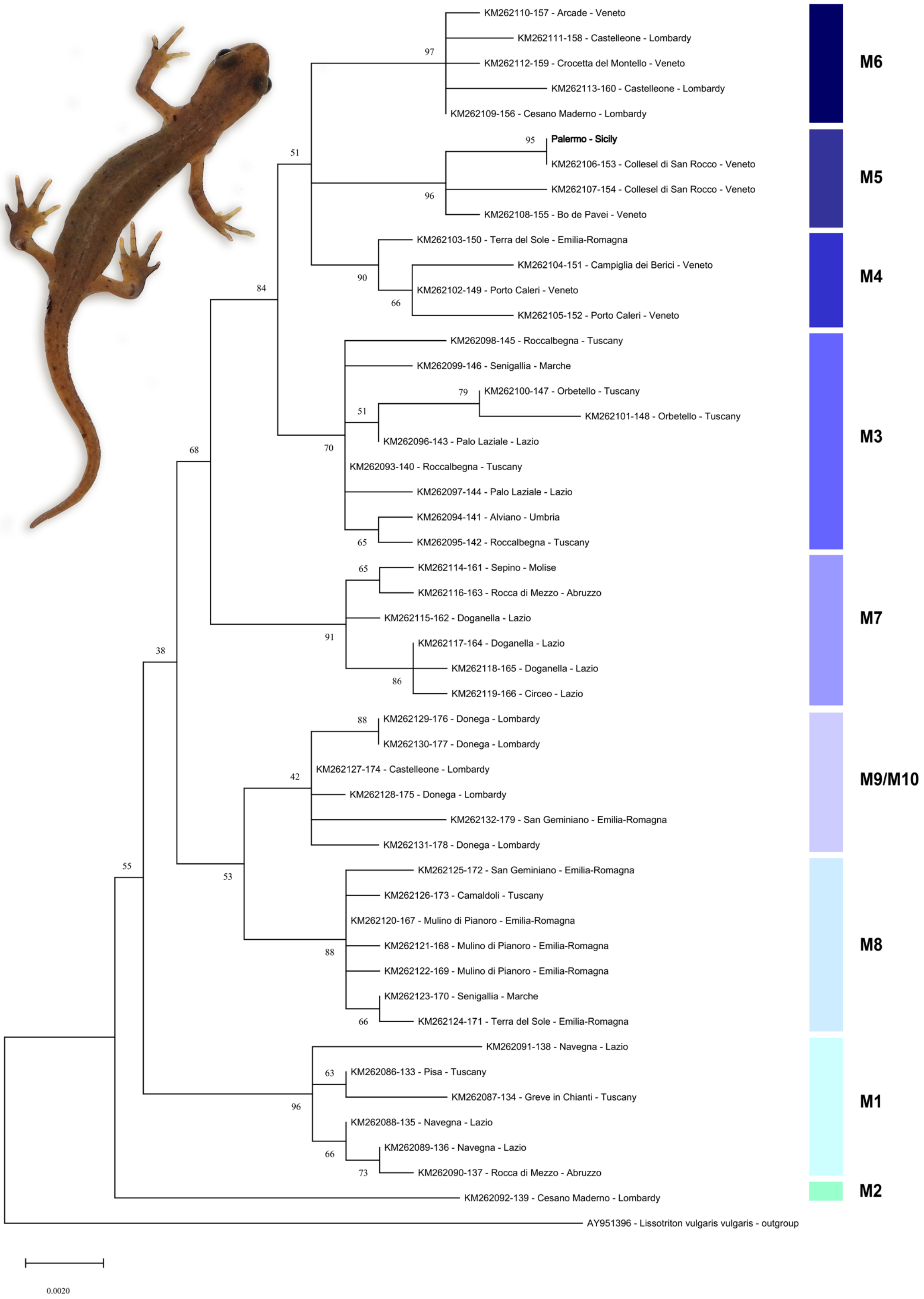

Figure 2. Tree with the highest log likelihood (–2637.73) is shown. The percentage of trees in which the associated taxa clustered together is indicated next to the branches. Initial tree(s) for the heuristic search were automatically generated by applying Neighbour-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) method, followed by selection of the topology with the highest log likelihood value. The tree is drawn to scale, with branch lengths representing the number of substitutions per site. Coloured bars labelled from M1 to M10 indicate the main haplogroups identified in Italy by Maura et al. (2014).

et al., 2014 for details). The best evolutionary model for the ND2 and ND4 datasets was selected separately using the IQ-TREE platform[2] (Nguyen et al., 2015), applying the ModelFinder algorithm in combination with tree reconstruction analysis (Kalyaanamoorthy et al., 2017). The TN + F + G4 model was identified as the best fit for both datasets.

ND2 and ND4 sequences were concatenated (1155 bp), and a total of 49 sequences— including *Lissotriton vulgaris vulgaris* (Linnaeus, 1758) as outgroup (GenBank accession numbers: AY951562 and AY951396)—were aligned using MEGA11 (Tamura et al., 2021) with the CLUSTAL W algorithm (Thompson et al., 1994). The novel sequences were translated to amino acids to check for frameshifts or stop codons. Phylogenetic analyses were performed in MEGA11 (Tamura et al., 2021) using the maximum likelihood (ML) method with Kimura 2-parameter model (Kimura, 1980). Node support was assessed through 1000 bootstrap replicates. Initial tree(s) for the heuristic search were automatically generated by applying Neighbour-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) method, followed by selection of topology with the highest log-likelihood value.

3. Results

A single newt, measuring 40 mm in total length at the time of discovery, was identified as *Lissotriton vulgaris* (Razzetti et al., 2016), as confirmed by the BLAST analysis. The specimen was at the end of the premetamorphic stage. No additional newts were found during the cleaning and draining of the garden pond or in the subsequent years.

The ML tree (Figure 2) exhibited a topology similar to that previously reported by Maura et al. (2014) for the Italian *L. vulgaris* populations, based on a Bayesian approach using the same haplotypes analysed in this study. The only exception was haplogroup M10, represented by a single sample (KM262132-179), which clustered within haplogroup M9—possibly due to long branch attraction (Figure 2). The specimen found in Sicily belongs to haplogroup M5 (Veneto, northeastern Italy) and shares the same ND2 and ND4 haplotypes with a sample from "Collesel di San Rocco" (Province of Treviso; KM262106-153) (Figure 2). This sampling site, reported by Maura et al. (2014), is located approximately 14 km north of the nursery where the pond plants were purchased.

4. Discussion

Our results indicate that the newt was likely introduced via plants purchased from a nursery in Veneto. This is strongly supported by the shared haplotypes with the M5 clade (see Maura et al., 2014), which includes the geographic area where the nursery is located. Plant trade is a significant vector for the spread of alien amphibian species (Kraus, 2009), as demonstrated for both arboreal (Borroto-Páez et al., 2015) and terrestrial species (Kraus, 2007; Laorden-Romero et al., 2024). Furthermore, plant nurseries provide suitable habitats for the survival of small amphibian populations due to the presence of artificial waterbodies, irrigated soils, suitable shelters, and other resources (Kraus, 2007, 2009). Nurseries are therefore ideal both as primary sources of dispersal for propagules of native species and as "stepping stones" for alien species introduced into these environments. Our findings strongly support the role of the plant nurseries in the spread of nonnative newts in Europe (Brede et al., 2000; Bogaerts, 2002) and additionally enable a precise temporal reconstruction of the case presented here.

Approximately two months elapsed between the introduction of the plants into the pond and the discovery of the newt at a near-metamorphic stage—an interval consistent with the larval development period of *L. vulgaris* (Razzetti et al., 2016 and references therein). This suggests that the newt was likely introduced either as an egg or during an early larval stage. This timing also aligns with the reproductive phenology of *L. vulgaris* in the Veneto region (Fracasso, 2007). It is more likely that the newt was transported with *Utricularia australis* at the egg stage, as the packaging of this plant could maintain constant humidity and provide protection against pressure (Figure 1D). Moreover, *L. vulgaris* typically lays its eggs attached to aquatic plants (Razzetti et al., 2016 and references therein). *L. vulgaris* eggs require approximately 8–20 days from deposition to hatching (Razzetti et al., 2016 and references therein), allowing sufficient time for exposure to collection and transport processes. Additionally, eggs may be more resilient to transport-related stress than larval stages. Amphibian eggs may represent a suitable stage for passive dispersal, as they are highly resistant to desiccation, transport, and other adverse conditions (Marin da Fonte et al, 2019 and references therein). The spread of amphibians during their egg and early larval stages has been speculated in relation to both human-mediated passive dispersal (e.g., trade of aquatic plants) (de Villiers, 2006) and zoochory processes (Marin da Fonte et al., 2019 and references therein), but direct evidence and detailed records have been lacking.

Our results, although based on a single case study that does not, in itself, represent a direct risk for reproduction and spread, provide further clear evidence of the translocation and survival of amphibian egg and early stages as contaminants of aquatic plants. This underscores the need to strengthen prevention and early detection programs related to plant trade and nursery management

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[2] IQ-TREE (2024). IQ-TREE web server: fast and accurate phylogenetic trees under maximum likelihood [online]. Website http://iqtree.cibiv.univie.ac.at/ [accessed 18 December 2024].

(Kraus, 2009; Laorden-Romero et al., 2024). Prophylactic procedures aimed at minimising the risk of introducing alien species are crucial, particularly in regions such as Sicily, where a notable richness of nonnative freshwater species is documented (Marrone and Naselli-Flores, 2015; Vecchioni et al., 2025). A more cautious approach, involving not only institutions, regulatory bodies, and traders (Pergl et al., 2017), but also buyers of aquatic plants, should be adopted. In this context, it is essential to disseminate warnings, guidelines, and biosecurity measures (e.g., quarantine and plant monitoring) to hobbyists as well, to minimise the risk of unintentionally introducing alien species as contaminants of aquatic plants.

Acknowledgment

The collection of the newt was carried out in line with the provisions of Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora, since the specimen was "outside its natural range."

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