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Short Note

Alien amphibian introductions via the plant trade: a breeding population of the Catalanian midwife toad (*Alytes almogavarii*) in Central Spain

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Abstract. The plant-trade is among the main sources of accidental introduction of alien biodiversity. This includes amphibians, although effective colonization pathways have rarely been reported except from tropical areas. We document the discovery of an established breeding population of the Catalanian midwife toad, *Alytes almogavarii almogavarii* Arntzen and García-París, 1995, in a plant nursery in Madrid (Central Spain), 400 km away from its natural distribution. During surveys in 2023 we found adults, juveniles, and tadpoles, confirming reproduction. We used mtDNA sequences to genotype six individuals for taxonomic assignment and tracing their putative origin. Two haplotypes were found, one of them showing a broad distribution, precluding precise identification of the source of the introduction. We discuss conservation concerns, including the potential spread of pathogens. Our results suggest unintentional amphibian introductions via the plant-trade may be more frequent than perceived, and call for rigorous monitoring and education efforts.

Keywords: biological invasions, conservation, introduced species, mitochondrial DNA, plant nursery.

The introduction and establishment of plant and animal species far beyond their native ranges as a result of human activities have been commonplace for centuries, but in the last decades their frequency has increased (Hulme, 2009).

These introductions impact native biodiversity in many ways, for example through predation and competition with native species, habitat alteration, hybridization with related taxa, or the spread of infectious diseases (Abbott et al.,

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2003; Gurevitch and Padilla, 2004; Crowl et al., 2008; Bucciarelli et al., 2014). Some of the main introduction sources and pathways are related to commercial trade: around 100 million plants and animals are transported across the world every year to supply the food, pet and gardening markets (Harfoot et al., 2018; Hughes et al., 2023). It is thus essential to invest in early detection and characterization of introduction pathways to facilitate implementation of successful eradication programs (Parkes and Panetta, 2009).

The nursery-trade, or plant-trade, is responsible for numerous cases of introduction of invasive plant species (Dehnen-Schmutz et al., 2007; Beaury, Patrick and Bradley, 2021), but also of the accidental spread of animal species associated with plants (Kraus, 2009; Bergey et al., 2014). This is for example the case of some continental Iberian snakes (especially *Hemorrhois hippocrepis* (Linnaeus, 1758)), that have colonized the Balearic Islands (Spain) via ornamental olive trees, resulting in rapid declines of the local endemic lizard *Podarcis pityusensis* (Boscá, 1883) (Silva-Rocha et al., 2015; Montes et al., 2022). The introduction of amphibians through the plant nursery trade has also increased in the last 50 years (Kraus, 2007). Most cases occur in tropical areas, involving different tree frog species which travel undetected hidden in tree trunks or leaves (Powell et al., 2011). Other species have been reported to travel as eggs and tadpoles in aquatic vegetation, such as the guttural toad *Sclerophrys gutturalis* (Power, 1927) in South Africa (Measey and Davies, 2011). In addition to direct impacts like predation, hybridization and competition, translocated individuals can spread viable spores of the causal agent of chytridiomycosis, a lethal amphibian disease (Johnson and Speare, 2005). Thus, accidental amphibian introductions through the plant nursery trade deserve serious attention, even in areas with few reported cases, like Europe (Kraus, 2007), where however intentional introductions

of amphibians have been frequently described (Kark et al., 2009).

The establishment of allochthonous populations depends on the form of introduction, the frequency of introduction events, and the bioclimatic similarity of the native and alien range (Mahoney et al., 2015). Some characteristics of the plant nursery trade increase the risk of successful establishment of viable populations of exotic species: (1) plant nurseries are often located at short distances from each other, which implies short travels and little mortality effects; (2) the climate is similar between the origin and destination of ornamental plants, thus facilitating the acclimation of associated amphibians; and (3) nurseries offer suitable terrestrial habitat and trophic resources to sustain small populations (Kraus, 2007, 2009).

We report the finding of an established breeding population of the Catalanian midwife toad, *Alytes almogavarii almogavarii* Arntzen and García-París, 1995, in Central Spain. The population was located in the neighbourhood of Sanchinarro (fig. 1; 40.50°N, 3.65°W; municipality of Madrid). In this area, the climate is “Bsk Cold steppe” according to the Köppen classification, with annual average temperature and precipitation of 13.8°C and 410 mm, respectively (<http://worldclim.org>). The landscape is highly anthropized and features zones with bare vegetation, recent constructions, roads and urban parks, which include several artificial ponds and a stream connecting with natural areas (open fields) 3 km downstream. Several plant nurseries settled in the area and have been operating at least since 2002.

On January 17th, 2023, we found two juveniles of *Alytes* in one of these plant nurseries during a casual visit (fig. 1). In the following days we performed additional surveys and found three *Alytes* tadpoles in a sewer, confirming successful reproduction. The nearest native *Alytes* populations in the area are 10 km (*Alytes cisternasii* Boscá, 1879) or 25 km (*Alytes obstetricans* (Laurenti, 1768)) away from the nursery (Martínez Solano, 2006).

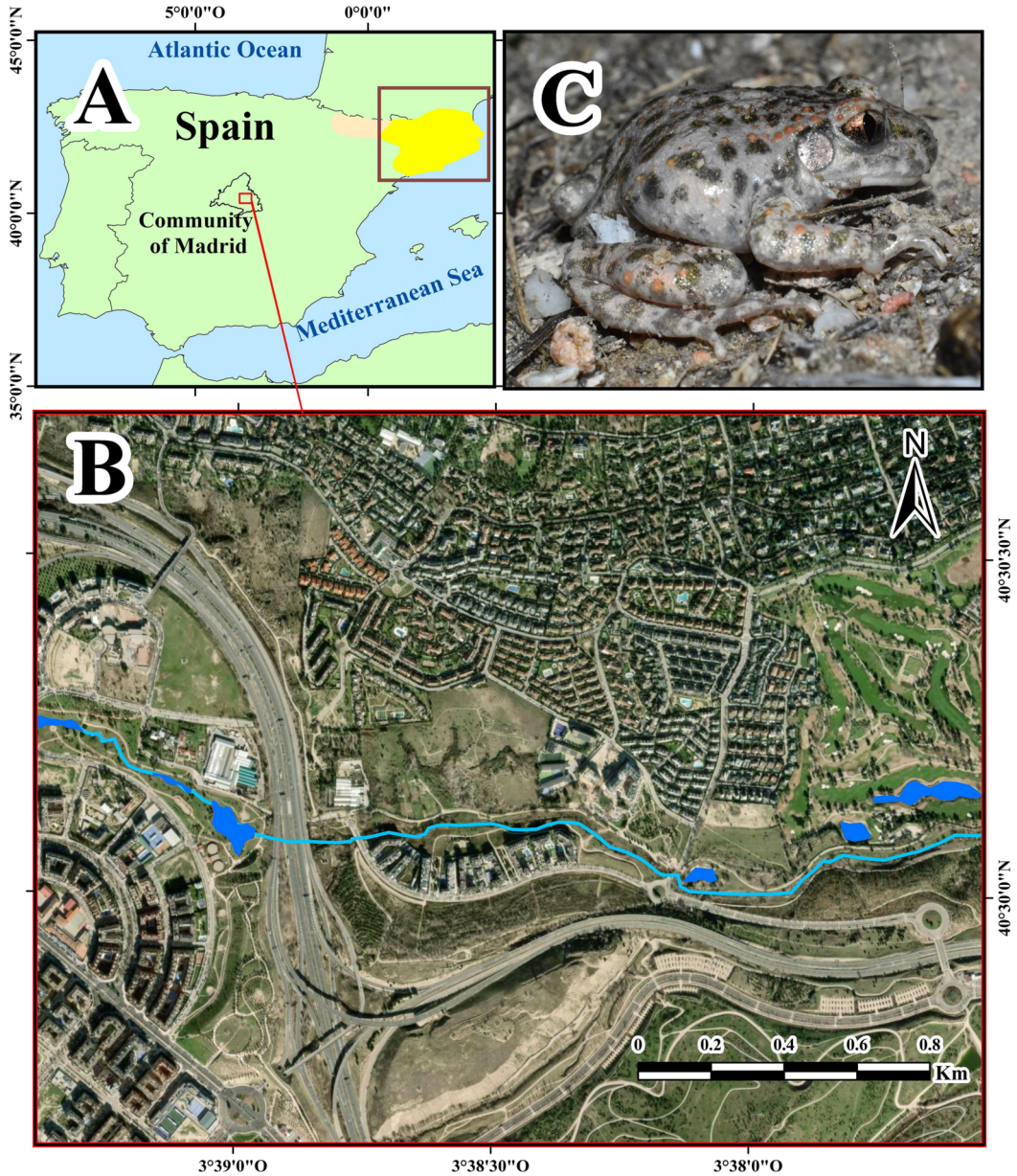


Figure 1. (A) Inset of the Iberian Peninsula with the natural distribution range of the Catalanian midwife toad (*Alytes algogavarii*). The subspecies *A. a. algogavarii* and *A. a. inigoii* are drawn on yellow and cream colours respectively, following fig. 1 in Ambu et al. (2023). The brown rectangle refers to the area highlighted in fig. 2. (B) Satellite image of the area where the nursery is located, highlighting the stream (light blue) and the artificial ponds (dark blue). (C) An *A. algogavarii* individual found in the surroundings of the plant nursery. Photo credit: Carlos Caballero-Díaz.

Caballero-Díaz et al., 2020, 2022), raising suspicions on the introduced origin of the nursery population. During a nocturnal survey in late spring (June 12th, 2023, 22:00 CEST, temperature 20°C, no rainfall), we counted mating calls

from >17 individuals within the nursery, and observed and photographed two adult individuals of unknown sex in its surroundings (fig. 1). We also sampled the nearby stream along a 350 m transect and the closest artificial ponds,

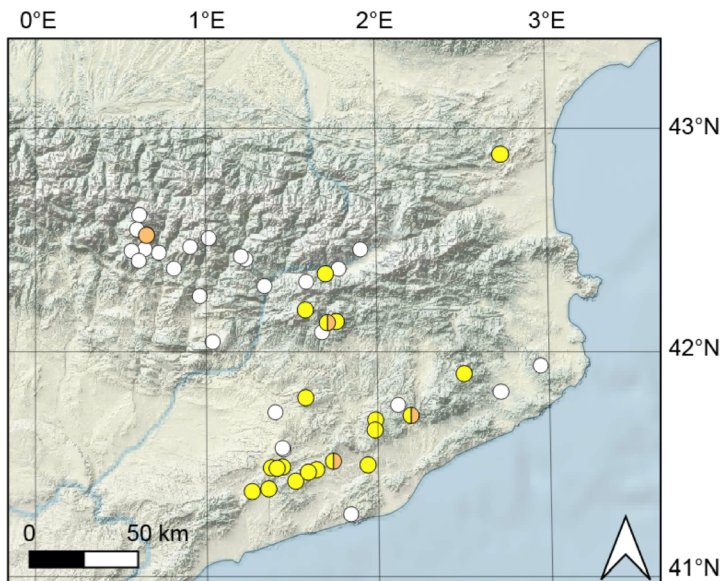


Figure 2. Localities where the two ND4 haplotypes found in Madrid were detected across the native range of *A. a. almogavarii*, according to Gonçalves et al. (2015), Dufresnes and Martínez-Solano (2020) and Lucati et al. (2022). Yellow: H68; Orange: H66; White: only other haplotypes.

without detecting the presence of *Alytes*. We toe-clipped two adults and two juveniles, and tail-clipped two tadpoles to obtain tissue samples for genetic analyses (stored in absolute ethanol).

For molecular taxonomic identification of the sampled individuals, we isolated DNA from tissue samples using commercial kits (Promega, Madison, WI, USA). We targeted the mitochondrial gene NADH dehydrogenase subunit 4 gene (ND4), which distinguishes all species and subspecies of midwife toads and has been extensively sampled across their range, including to trace the origin of introduced populations (Vliegenthart et al., 2023). We used primers *Alytes*ND4_196F – *Alytes*ND4_814R (Vliegenthart et al., 2023) to amplify a fragment of about 600 base pairs (bp) of the target gene (methods as in Vliegenthart et al., 2023), which was then sequenced in the forward and reverse directions at Macrogen (Madrid, Spain). Sequences were edited using MEGA X (Kumar et al., 2018), manually aligned in Seaview 5 (Gouy et al., 2021), and compared to the *Alytes*

ND4 sequences available in GenBank (originating from Gonçalves et al., 2015; Dufresnes and Martínez-Solano, 2020; Lucati et al., 2022). We recovered two haplotypes identical to the haplotypes previously documented in the subspecies *A. a. almogavarii*: H66 (2 samples) and H68 (4 samples) – haplotype labels as in Vliegenthart et al. (2023). These haplotypes are found across most of the distribution of the taxon, being particularly frequent in the province of Barcelona (fig. 2): H66 was previously reported in four different localities scattered in NE-Spain; H68 is the most common ND4 haplotype of *A. a. almogavarii* and was reported in 20 localities from Spain and in nearby France (fig. 2). The two haplotypes are found together in three localities (fig. 2).

Amphibians have been shown to rapidly establish in new areas after long-distance translocation events through the transport of commercial plants, as best seen from arboreal species in tropical areas (Powell et al., 2011; Borroto-Páez et al., 2015). However, these events have been seldom reported in European amphibians (Kraus, 2007; Dufresnes and

Alard, 2020), and it remains unclear whether this is because their frequency is lower, or because their incidence is underreported. In our case, *Alytes* individuals could have been transported buried in soil plots or hidden in cracks of big tree trunks, which provide suitable microhabitat for *Alytes* (Gosá, 2003). While the precise timing of the original introduction is unknown, the fact that local plant nurseries have been operating for only two decades suggests a relatively recent event. The observation of tadpoles, juveniles and adults confirms the presence of a small but successfully breeding population of *A. a. almogavarii* in Madrid, at least 400 km away from the nearest native populations (fig. 1; Dufresnes and Martínez-Solano, 2020). Its putative origin is probably the province of Barcelona (NE Spain), where the two ND4 haplotypes are mostly found, and which corresponds to the primary origin of the plants in the nursery (pers. obs.). However, the broad distribution of both haplotypes across the range of *A. almogavarii* precludes precise identification of the potential source population. Furthermore, we cannot rule out the possibility of multiple introductions from several localities. We also observed one adult individual of the tree frog *Hyla meridionalis* (Boettger, 1874) in the nursery, a species which is also common in north-eastern Spain but not present around Madrid. In the region, the closest native population occurs about 90 km SW of Madrid, in the municipality of Cenicientos (Martínez-Solano, 2006; Caballero-Díaz, unpubl. data). *Hyla meridionalis* is a known human commensal and appears to be recurrently translocated outside its native range, notably through the trade of commercial vegetables (reviewed by Dufresnes and Alard, 2020).

The population described herein entails a new alien amphibian species for the region, in addition to the alpine newt *Ichthyosaura alpestris* (Laurenti, 1768), which was introduced from the Cantabrian Mountains (Northern Spain) into the Guadarrama mountains (Peñalara) in the 1980's (Arano et al., 1991; Palomar, Vörös and

Bosch, 2017). In Peñalara, *I. alpestris* is considered a threat for native amphibians because of its role as predator, competitor, and vector and reservoir of the pathogen *Batrachochytrium dendrobatidis* Longcore, Pessier and Nichols, 1999. Paradoxically, *I. alpestris* is listed as Endangered in the regional catalogue of threatened species in Madrid, where it was included before evidence of its introduced status was unveiled. In our case, although we did not screen individuals of *A. almogavarii* for pathogens, it would be desirable to test for the presence of chytrid fungi or ranaviruses, to which *Alytes* species are highly sensitive (Bosch, Martínez-Solano and García-París, 2001; Balseiro et al., 2009). Additionally, in case of expansion, eventual hybridization of *A. almogavarii* with native *Alytes obstetricans* may be a concern, although both species are known to be partly reproductively isolated (Dufresnes and Martínez-Solano, 2020). Dispersal could proceed through colonization of additional water bodies in the area (although it mainly comprises marginal, suboptimal habitat for the species) and via additional entries from the plant trade, for instance, in other nurseries or private and public gardens. We recommend close monitoring of this population to assess potential impacts on the local fauna, and call for systematic efforts to rigorously assess the role of the plant nursery trade in the accidental transport of alien species. These introductions may often remain undetected, especially when local and alien species are morphologically similar, as in our case, but can have serious impacts on native biodiversity. Monitoring efforts should be accompanied by educational activities informing the general public about the negative impacts of biological introductions, whether intentional or not, for which early detection reports like this offer good opportunities.

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