Title: European newts establish in Australia, marking the arrival of a new amphibian order

Running title: European newts establish in Australia

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Abstract

We document the successful establishment of a European newt (*Lissotriton vulgaris*) in south-eastern Australia, the first recorded case of a caudate species establishing beyond its native geographic range in the southern hemisphere. Field surveys in south-eastern Australia detected *L. vulgaris* at six sites, including four sites where the species had been detected 15 months earlier. Larvae were detected at three sites. Individuals had identical ND2 and *cyt*b mtDNA gene sequences, and comparisons with genetic data from the species' native range suggest that these individuals belong to the nominal subspecies *L. v. vulgaris*. Climatic conditions across much of southern Australia are similar to those experienced within the species' native range, suggesting scope for substantial range expansion. *Lissotriton vulgaris* had been available in the Australian pet trade for decades before it was declared a 'controlled pest animal' in 1997, and thus the invasion documented here likely originated via the release or escape of captive animals. *Lissotriton vulgaris* is the sole member of an entire taxonomic order to have established in Australia, and given the potential toxicity of this species, further work is needed to delimit its current range and identify potential biodiversity impacts.

Keywords Caudata, climate match, *Lissotriton vulgaris*, pet trade, potential distribution, *Triturus vulgaris*, Urodela

Introduction

In many systems, biodiversity impacts of exotic species are closely linked to their phylogenetic relatedness to the native community. On average, exotic species with only distant relatives in their invaded ranges tend to have greater impacts (Ricciardi and Atkinson 2004; Strauss et al. 2006). Closely related species typically share similar predators, competitors, and pathogens, and thus phylogenetically distinct invaders are more likely to encounter species that lack co-evolved defences against them. Here we document an invasion that involves the establishment of a distantly-related phylogenetic lineage in Australia: the amphibian order Caudata (salamanders).

There are approximately 230 anurans (frogs) in Australia (Tyler and Knight 2011), but representatives from the other two extant amphibian orders (Gymnophiona and Caudata) are absent. At least four caudate species have been available in the pet trade in Australia over the last century but, to the best of our knowledge, none of these species has established wild populations (Tyler 2001; Kraus 2009). However, in June 2011, an individual European newt (*Lissotriton vulgaris*, formerly *Triturus vulgaris*) was discovered in the wild in an outer suburban area of Melbourne, Victoria, by a member of the public. Follow-up surveys conducted between June and November 2011 captured 15 *L. vulgaris* in an adjacent drainage basin. A further 73 *L. vulgaris* were subsequently captured at 6 sites approximately 4 km south of the initial detection site in September and October 2012 (Fig. 1). *Lissotriton vulgaris* therefore appears to have established at a number of sites in Melbourne. However, it is unclear whether these populations have persisted, and the geographic origin and potential distribution of *L. vulgaris* in Australia are unknown.

Our objectives were to: (i) determine the geographic origin of Melbourne *L. vulgaris* by sequencing two mitochondrial DNA fragments, (ii) confirm the persistence and reproduction of the species through additional field surveys, (iii) estimate the species' potential distribution in Australia using climatic data from the species' native range, and (iv) identify the potential impacts of this species on biodiversity in south-eastern Australia.

Methods

Study species

Lissotriton vulgaris is a widespread species, ranging from Ireland, through western and central Europe and Scandinavia, south to Italy, the Balkans and Turkey, and east into Ukraine and Russia (Artzen et al. 2009). The species inhabits a wide range of vegetation types, including woodlands, meadows, bushlands, and a range of disturbed habitats. Like many amphibian species, *L. vulgaris* has a biphasic life cycle in which aquatic eggs and larvae metamorphose into air-breathing semiaquatic juveniles. In the species' native range, adults spend most of the breeding season in the water, but return to land

soon afterwards. Breeding occurs in static and slow-moving shallow waters, where females lay 200–300 eggs per season, usually on broad-leaved aquatic plants. Development is temperature dependent, but eggs typically hatch in 2–3 weeks, whereas larvae take approximately 10 weeks to metamorphose. Males become sexually mature at 2–3 years of age, whereas females mature approximately one year later (Griffiths 1996). There are seven named subspecies of *L. vulgaris*, although the taxonomic status of several is a matter of contention (Dubois and Raffaëlli 2009).

Field surveys

In 2013, we resurveyed four of the six sites (roadside drains) where *L. vulgaris* was detected in 2012, as well as three additional sites in the immediate vicinity of the original detection sites (Fig. 1). From August–December 2013 (the suspected breeding season), 11 equidistantly spaced traps constructed from 2 L plastic soda bottles baited with 10 x 100 mm glow sticks (Glowstix Australia Pty Ltd, New South Wales, Australia) were placed at each of six of these sites (Griffiths 1985; Bennett et al. 2012). The seventh site was considerably smaller than the others, and so only four traps were placed at that site. Traps were set for four consecutive nights each month, providing a total of 20 nights of trapping at each site to assess the abundance of *L. vulgaris*. All animals were euthanased on site in accordance with The University of Melbourne animal ethics protocols (Permit ID 1212627.1).

Genetic analyses

Fragments of two mitochondrial genes were amplified to determine the subspecific status of the Melbourne *L. vulgaris* samples. Ten whole *L. vulgaris* samples were collected from the study area and stored in 95% ethanol. DNA was extracted from a single whole leg from each specimen using a QIAGEN DNA Easy kit (Qiagen Pty Ltd, Victoria, Australia). PCR amplifications were then performed using primers Ile3700L and COI5350H (Zhang et al. 2008) to amplify ~1600 bp of the NADH dehydrogenase subunit 2 (ND2) gene, and primers Glu14100L and Pro15500H (Zhang et al. 2008) to amplify ~1400 bp of the *cytochrome* subunit b (*cyt*b) gene. For each gene, amplifications were prepared in 20 µl volumes each containing 11.14 µl ddH_2O, 2 µl 1 x reaction buffer, 0.16 µl dNTP's (25 mM), 0.8 µl $MgCl_2$ (50 mM), 0.8 µl each primer pair (10 µM), 0.25 units Immolase taq (Bioline), and 4 µl DNA extract. Amplifications were undertaken using the cycling conditions from Zhang et al. (2008) on an Eppendorf Gradient *S* Master Cycler. PCR products for each sample were directly sequenced in a single direction using Ile3700L (ND2) and Glu14100L (*cyt*b). Sequences were aligned and manually edited in Sequencher version 5.1 (Gene Codes Corporation, Michigan, USA). Unique haplotypes were submitted to Genbank (accession numbers; *cyt*b - KJ676771 and ND2 - KJ676772).

The *cyt*b and ND2 sequences were compared between individuals to determine haplotype diversity. For ND2, a phylogenetic comparison was also undertaken with sequences in Babik et al. (2005) to determine the geographic origin of the Melbourne samples. Unique sequences for 143 haplotypes of *L. vulgaris* and *L. montandoni* representing each of the 12 haplotype groups were downloaded from Genbank (accession numbers: AY951337-347, 351-379, 382-414, 416-419, 425-429, 432-437, 439-446, 449-462, 464, 466-476, 478-489 and 493-501). The aim of the analysis was not an exhaustive phylogenetic reconstruction, but merely to determine the haplotype group to which the Melbourne *L. vulgaris* samples are most closely related. For this purpose, we used distance (Kimura's 2-parameter model) and maximum-likelihood (Tamura and Nei model with a nonzero proportion of invariant sites) methods to infer phylogenies in MEGA 5.2 (Tamura et al. 2011). Both methods inferred identical phylogenies, and therefore only the maximum-likelihood phylogeny with bootstrap support for nodes (1000 replicates) is shown.

Potential distribution

To estimate the potential distribution of *L. vulgaris* in Australia, we used the 'closest standard score' algorithm in the software CLIMATE (Bureau of Rural Sciences 2006). CLIMATE contains data on 8 precipitation and 8 temperature variables from meteorological stations across the globe, and is routinely used as a risk-assessment tool in Australia. The 'closest standard score' algorithm is based on the maximum Euclidian distance between each individual climate variable at meteorological stations within a species' native distribution and 50-km grid cells in Australia. Climate match scores range from 10 (suitable) to 0 (unsuitable). Here, we used data on all 16 variables from 1026 weather stations within the native geographic range of *L. vulgaris* (taken from the IUCN extent of occurrence range map: Arntzen et al. 2009). While more sophisticated methods for modelling species distributions exist, previous analyses have shown that CLIMATE predictions are capable of successfully discriminating successful and unsuccessful introductions of exotic vertebrates, including amphibians (Bomford et al. 2009).

Results and Discussion

Lissotriton vulgaris was present at six of the seven sites that we surveyed in 2013, including all four sites where the species was detected in 2012 (Fig. 1). Larvae were captured at three sites (4 individuals overall) in October, November and December, 2013. Across all six sites, the male-to-female sex ratio was ~2.5:1 (n = 27 males, 11 females). Abundance was highest in the smallest, most ephemeral site (23 trapped individuals), and relatively low and uniform at the other five sites where *L. vulgaris* was detected (median = 4 trapped individuals, range = 2–5). This low overall abundance could be interpreted as evidence that establishment has occurred only recently, but we feel this is

unlikely for two reasons. First, all 69 individuals that were detected in 2012 at the four sites that we resurveyed in 2013 were removed from the wild, artificially lowering abundance estimates in 2013. Second, the highly disjunct distribution of *L. vulgaris* across the study area (Fig. 1) suggests that the species has spread considerably, and is much more widespread than our initial surveys have revealed (although the possibility of separate releases cannot be ruled out). Collectively, these findings suggest that populations of *L. vulgaris* are capable of persisting and successfully reproducing in Melbourne.

All ten *L. vulgaris* individuals from the study area had identical sequences for each of the ND2 and *cyt*b mtDNA gene regions, indicating that all individuals belong to the same subspecies. Phylogenetic analysis of 863 bp of the ND2 sequence with haplotypes from Babik et al. (2005) shows the Melbourne *L. vulgaris* haplotype to be unique but places this haplotype within the L2 haplotype group (Fig. 2). Only two base pair differences separate the Melbourne haplotype (L-Melb) from other L2 haplotypes. Individuals from the L2 group have been identified in Germany, the Czech Republic, Slovakia, and Hungary (Babik et al. 2005). Babik et al. (2005) identified all individuals from the L2 haplotype group as being from the *L. vulgaris vulgaris* subspecies. *Lissotriton v. vulgaris* is widespread throughout Europe and Russia, and has by far the broadest geographic range of all known subspecies. Widespread amphibian species generally have larger population sizes and are more likely to be encountered by humans (Tingley et al. 2010); however, it is unclear from this single introduction whether availability is the underlying reason why *L. v. vulgaris*, and not one of the other six subspecies, was transported to Australia.

Regardless of why *L. v. vulgaris* in particular was transported, its eventual release into the wild is most likely tied to its historical presence in the pet trade. *Lissotriton vulgaris* had been available in the Australian pet trade for decades before it was declared a 'controlled pest animal' in 1997 under the *Catchment and Land Protection Act 1994* (CaLP Act), prohibiting importation, keeping and trading of the species without a permit. *Lissotriton vulgaris* was later upgraded to 'prohibited' in 2010 but has not yet been classified an 'established' pest. The results of our field surveys suggest that this species has indeed established viable populations in Victoria but in order to be upgraded to 'established' under the CaLP Act, there must be sufficient evidence that the species is widespread and poses a significant threat to the environment. Importantly, upgrading a species to 'established' also means accepting that eradication from the state is unachievable. Therefore, the current challenge for managers is to determine whether eradication of this species is required and feasible, or whether efforts should focus on containing the species to its current extent.

Our prediction of the potential distribution of *L. vulgaris* suggests that large proportions of New South Wales, Victoria, eastern Tasmania, southern South Australia, and south-western Western Australia are particularly suitable (Fig. 3a). Importantly, broad-scale climatic conditions at the site of establishment in Victoria are extremely similar to those present within the native range of *L. vulgaris*,

and our model suggests that there is suitable climate space in regions neighbouring the site of establishment (also see Parsons and Have 2013).

Some authors consider several of the subspecies of *L. vulgaris* distinct species (Dubois and Raffaëlli 2009), and different subspecies can occupy distinct climatic niches (Pearman et al. 2010). To account for this taxonomic uncertainty and potential niche differentiation, we reran our climate-match analyses solely on the native distribution of the subspecies present in Melbourne (*L. v. vulgaris*), based on distribution maps contained in Babik et al. (2005). This refined analysis produced more modest predictions (Fig. 3b), particularly in South Australia and Western Australia, but overall, predictions were broadly concordant between the two approaches. However, our range predictions should be treated with caution, as models trained on native-range data assume that the native climatic niche of a species is conserved in its invaded range (Hill et al. 2011). Additionally, within the potential range dictated by coarse climatic conditions, habitat connectivity will be a critical determinant of spread. Observed migration distances of *L. vulgaris* in its native range are typically <500 m/year (Kovar et al. 2009); however, the high density of artificial water sources in the immediate vicinity of sites where *L. vulgaris* has been detected in Melbourne could partially negate this lack of mobility.

Lissotriton vulgaris breeds in standing water of variable size and quality, occupies a range of terrestrial habitats, and is a carnivorous generalist that preys on invertebrates, crustaceans and the eggs and larvae of amphibians and fish (Parsons and Have 2013). As such, *L. vulgaris* may directly compete with and prey upon a wide range of terrestrial and freshwater species in Australia. Our field surveys demonstrate that *L. vulgaris* is sympatric with a number of frog and invertebrate species that share a similar trophic niche, and thus *L. vulgaris* may pose a competitive threat to these taxa. There is also potential for *L. vulgaris* to fatally poison native predators, as some members of the family Salamandridae produce a neurotoxic skin secretion (tetrodotoxin) (Wakely et al. 1966). *Lissotriton vulgaris* from Europe either tested negative for tetrodotoxin, or possessed the toxin in very low concentrations (Wakely et al. 1966; Yotsu-Yamashita et al. 2007). However, terrestrial Australian predators have no evolutionary history of exposure to tetrodotoxin, and thus the effect of even low doses of this toxin on Australian frog-eating predators remains unclear. The only other exotic amphibian species that has become established in Australia, the cane toad (*Rhinella marina*), also produces a novel toxin, and this species has had catastrophic impacts on native predators (Shine 2010). Numerous Australian taxa including invertebrates, wading birds, snakes, lizards, turtles and mammals prey on species that occupy similar environments or are morphologically similar to *L. vulgaris* (e.g. amphibians, fish, skinks), and thus *L. vulgaris* has the potential to impact a wide range of native taxa. Additionally, *L. vulgaris* may serve as a vector for the chytrid fungus *Batrachochytrium dendrobatidis*, a pathogen that has caused widespread amphibian declines in Australia (Berger et al. 1999). Although the presence of *B. dendrobatidis* has not been confirmed in *L.*

vulgaris, a close relative, *Ichthyosaura alpestris*, is an asymptomatic vector in the UK (Arntzen et al. 2013).

Interestingly, the invasion documented here represents the first recorded case of a caudate species establishing beyond its native geographic range in the southern hemisphere (Kraus 2009). Given the lack of evolutionary history of exposure to caudates (and tetrodotoxin) among terrestrial Australian predators, further work is needed to identify the potential impacts of *L. vulgaris* on Australian biodiversity. Our analyses also suggest that climatic conditions across much of southern Australia are similar to those experienced within the species' native range, and thus delimiting the current extent of the species' Australian range should be considered a top management priority.

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294 **Figure legends**

295 **Fig.1** Sites where *Lissotriton vulgaris vulgaris* was detected from 2011-2013 in an outer suburb of
296 Melbourne, Victoria, Australia. Site size is proportional to the number of individuals captured (shown
297 in six classes: 1, 2, 3, 4, 5, and 23 individuals). Sites labelled '2012/2013' were surveyed in both years
298 (capture numbers at these sites represent 2013 values). Also shown is a site that we surveyed by
299 trapping over 20 nights in 2013 but where the species was not detected. Distinctive landscape features
300 have been removed to reduce the probability of illegal collection.
301
302 **Fig. 2** Maximum likelihood phylogenetic tree of *Lissotriton vulgaris* haplotypes generated from an
303 863 bp fragment of the mitochondrial NADH dehydrogenase subunit 2 (ND2) gene. Haplotype groups
304 are as those from Babik et al. (2005). Bootstrap support for haplotype groups is indicated above
305 branches. Presence of unique haplotypes within groups is represented by triangles (except haplotype
306 group L2), with their height corresponding to the number of unique haplotypes (as found in Babik et
307 al. 2005). Haplotype group L2 shows some unique haplotypes from Babik et al. (2005) (L28, L30,
308 L31, L34, L35, L36, L37, L38) and the unique haplotype identified from ten individuals in this study
309 from Melbourne, Australia (L-MELB).
310
311 **Fig. 3** Potential distribution of *L. vulgaris* in Australia according to the software CLIMATE. The
312 climate-matching score was calculated using meteorological data from within the entire native
313 geographic range of *L. vulgaris* (a), or only from within the range of the subspecies (*L. v. vulgaris*)
314 that is present in Melbourne, Australia (b). The black star in south-eastern Australia represents the
315 location of establishment.