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

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15

16 **Abstract**

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

30

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

### 33 **Introduction**

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

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

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

58

### 59 **Methods**

60

#### 61 **Study species**

62

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

69 soon afterwards. Breeding occurs in static and slow-moving shallow waters, where females lay 200–  
70 300 eggs per season, usually on broad-leaved aquatic plants. Development is temperature dependent,  
71 but eggs typically hatch in 2–3 weeks, whereas larvae take approximately 10 weeks to metamorphose.  
72 Males become sexually mature at 2–3 years of age, whereas females mature approximately one year  
73 later (Griffiths 1996). There are seven named subspecies of *L. vulgaris*, although the taxonomic status  
74 of several is a matter of contention (Dubois and Raffaelli 2009).

75

#### 76 Field surveys

77

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

87

#### 88 Genetic analyses

89

90 Fragments of two mitochondrial genes were amplified to determine the subspecific status of the  
91 Melbourne *L. vulgaris* samples. Ten whole *L. vulgaris* samples were collected from the study area and  
92 stored in 95% ethanol. DNA was extracted from a single whole leg from each specimen using a  
93 QIAGEN DNA Easy kit (Qiagen Pty Ltd, Victoria, Australia). PCR amplifications were then  
94 performed using primers Ile3700L and COI5350H (Zhang et al. 2008) to amplify ~1600 bp of the  
95 NADH dehydrogenase subunit 2 (ND2) gene, and primers Glu14100L and Pro15500H (Zhang et al.  
96 2008) to amplify ~1400 bp of the *cytochrome* subunit b (*cytb*) gene. For each gene, amplifications  
97 were prepared in 20 µl volumes each containing 11.14 µl ddH<sub>2</sub>O, 2 µl 1 x reaction buffer, 0.16 µl  
98 dNTP's (25 mM), 0.8 µl MgCl<sub>2</sub> (50 mM), 0.8 µl each primer pair (10 µM), 0.25 units Immolase taq  
99 (Bioline), and 4 µl DNA extract. Amplifications were undertaken using the cycling conditions from  
100 Zhang et al. (2008) on an Eppendorf Gradient S Master Cycler. PCR products for each sample were  
101 directly sequenced in a single direction using Ile3700L (ND2) and Glu14100L (*cytb*). Sequences were  
102 aligned and manually edited in Sequencher version 5.1 (Gene Codes Corporation, Michigan, USA).  
103 Unique haplotypes were submitted to Genbank (accession numbers; *cytb* - KJ676771 and ND2 -  
104 KJ676772).

105 The *cytb* and ND2 sequences were compared between individuals to determine haplotype  
106 diversity. For ND2, a phylogenetic comparison was also undertaken with sequences in Babik et al.  
107 (2005) to determine the geographic origin of the Melbourne samples. Unique sequences for 143  
108 haplotypes of *L. vulgaris* and *L. montandoni* representing each of the 12 haplotype groups were  
109 downloaded from Genbank (accession numbers: AY951337-347, 351-379, 382-414, 416-419, 425-  
110 429, 432-437, 439-446, 449-462, 464, 466-476, 478-489 and 493-501). The aim of the analysis was  
111 not an exhaustive phylogenetic reconstruction, but merely to determine the haplotype group to which  
112 the Melbourne *L. vulgaris* samples are most closely related. For this purpose, we used distance  
113 (Kimura's 2-parameter model) and maximum-likelihood (Tamura and Nei model with a nonzero  
114 proportion of invariant sites) methods to infer phylogenies in MEGA 5.2 (Tamura et al. 2011). Both  
115 methods inferred identical phylogenies, and therefore only the maximum-likelihood phylogeny with  
116 bootstrap support for nodes (1000 replicates) is shown.

117

118 Potential distribution

119

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

132

### 133 **Results and Discussion**

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

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

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

160 Regardless of why *L. v. vulgaris* in particular was transported, its eventual release into the  
161 wild is most likely tied to its historical presence in the pet trade. *Lissotriton vulgaris* had been  
162 available in the Australian pet trade for decades before it was declared a ‘controlled pest animal’ in  
163 1997 under the *Catchment and Land Protection Act 1994* (CaLP Act), prohibiting importation,  
164 keeping and trading of the species without a permit. *Lissotriton vulgaris* was later upgraded to  
165 ‘prohibited’ in 2010 but has not yet been classified an ‘established’ pest. The results of our field  
166 surveys suggest that this species has indeed established viable populations in Victoria but in order to  
167 be upgraded to ‘established’ under the CaLP Act, there must be sufficient evidence that the species is  
168 widespread and poses a significant threat to the environment. Importantly, upgrading a species to  
169 ‘established’ also means accepting that eradication from the state is unachievable. Therefore, the  
170 current challenge for managers is to determine whether eradication of this species is required and  
171 feasible, or whether efforts should focus on containing the species to its current extent.

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

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

178         Some authors consider several of the subspecies of *L. vulgaris* distinct species (Dubois and  
179 Raffaelli 2009), and different subspecies can occupy distinct climatic niches (Pearman et al. 2010). To  
180 account for this taxonomic uncertainty and potential niche differentiation, we reran our climate-match  
181 analyses solely on the native distribution of the subspecies present in Melbourne (*L. v. vulgaris*),  
182 based on distribution maps contained in Babik et al. (2005). This refined analysis produced more  
183 modest predictions (Fig. 3b), particularly in South Australia and Western Australia, but overall,  
184 predictions were broadly concordant between the two approaches. However, our range predictions  
185 should be treated with caution, as models trained on native-range data assume that the native climatic  
186 niche of a species is conserved in its invaded range (Hill et al. 2011). Additionally, within the  
187 potential range dictated by coarse climatic conditions, habitat connectivity will be a critical  
188 determinant of spread. Observed migration distances of *L. vulgaris* in its native range are typically  
189 <500 m/year (Kovar et al. 2009); however, the high density of artificial water sources in the  
190 immediate vicinity of sites where *L. vulgaris* has been detected in Melbourne could partially negate  
191 this lack of mobility.

192         *Lissotriton vulgaris* breeds in standing water of variable size and quality, occupies a range of  
193 terrestrial habitats, and is a carnivorous generalist that preys on invertebrates, crustaceans and the  
194 eggs and larvae of amphibians and fish (Parsons and Have 2013). As such, *L. vulgaris* may directly  
195 compete with and prey upon a wide range of terrestrial and freshwater species in Australia. Our field  
196 surveys demonstrate that *L. vulgaris* is sympatric with a number of frog and invertebrate species that  
197 share a similar trophic niche, and thus *L. vulgaris* may pose a competitive threat to these taxa. There is  
198 also potential for *L. vulgaris* to fatally poison native predators, as some members of the family  
199 Salamandridae produce a neurotoxic skin secretion (tetrodotoxin) (Wakely et al. 1966). *Lissotriton*  
200 *vulgaris* from Europe either tested negative for tetrodotoxin, or possessed the toxin in very low  
201 concentrations (Wakely et al. 1966; Yotsu-Yamashita et al. 2007). However, terrestrial Australian  
202 predators have no evolutionary history of exposure to tetrodotoxin, and thus the effect of even low  
203 doses of this toxin on Australian frog-eating predators remains unclear. The only other exotic  
204 amphibian species that has become established in Australia, the cane toad (*Rhinella marina*), also  
205 produces a novel toxin, and this species has had catastrophic impacts on native predators (Shine  
206 2010). Numerous Australian taxa including invertebrates, wading birds, snakes, lizards, turtles and  
207 mammals prey on species that occupy similar environments or are morphologically similar to *L.*  
208 *vulgaris* (e.g. amphibians, fish, skinks), and thus *L. vulgaris* has the potential to impact a wide range  
209 of native taxa. Additionally, *L. vulgaris* may serve as a vector for the chytrid fungus  
210 *Batrachochytrium dendrobatidis*, a pathogen that has caused widespread amphibian declines in  
211 Australia (Berger et al. 1999). Although the presence of *B. dendrobatidis* has not been confirmed in *L.*

212 *vulgaris*, a close relative, *Ichthyosaura alpestris*, is an asymptomatic vector in the UK (Arntzen et al.  
213 2013).

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

221

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228

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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.