

Molecular identification of the exotic slug *Arion subfuscus* sensu stricto (Gastropoda: Pulmonata) in California, with comments on the source location of introduced populations

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Abstract *Arion subfuscus* sensu lato (s. l.) refers to a cryptic slug species complex that is native to Europe and which, from there, has been introduced into the northeastern U.S. The species complex was detected in California for the first time in 2005 and recently 12 specimens were collected during state-wide surveys. The genital morphology of these specimens suggested that only *A. subfuscus* sensu

stricto (s. s.) was present and partial sequences of mitochondrial 16S rDNA (443 bp) showed that they all belonged to a single haplotype of the mitochondrial lineage, S1 sensu Pinceel et al. (Genetica 125: 155–171, 2005). This result was corroborated by a parallel analysis of a 655 bp COI DNA barcode. The 16S rDNA S1 haplotype (S1-03) of the Californian specimens is hitherto known only from the northeastern U.S. Hence, it is likely that populations may have been introduced to California from the northeastern states, rather than directly from the native area of the S1 haplogroup in Europe.

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Introduction

The slug, *Arion subfuscus*, was originally described from the Montagne Noire in southern France by Draparnaud (1805: as “*Limax*”). Since then, the species has traditionally been regarded as highly polymorphic, both in external morphology (e.g., Taylor 1907; Chichester and Getz 1973; Wiktor 1983), and in allozyme variation (McCracken and Selander 1980; Foltz et al. 1982), resulting in a long list of synonyms (Hesse 1926). Hence, several authors have suggested that the name *A. subfuscus*

actually refers to a complex of cryptic species (e.g., Waldén 1976; Foltz et al. 1982; Backeljau et al. 1996; Falkner et al. 2002). In this context, Garrido et al. (1995) redescribed a series of species from the Iberian Peninsula including *A. subfuscus* and selected a neotype to definitively fix the name of the latter. Conversely, Pinceel et al. (2004) disentangled the species complex in northwest Europe by combining anatomical, allozyme and DNA sequence data, showing that in this region *A. subfuscus* comprises at least two distinct species, *Arion subfuscus* s. s. (based on the DNA analysis of the neotype) and *Arion fuscus* (Müller 1774). The gonad of the latter is small, deep dark and completely embedded in the digestive gland, while in the former the gonad is large, pale violet and located on the periphery of the gland. In addition, Pinceel et al. (2004, 2005) reported that *A. subfuscus* s. s. actually consists of, at least, five strongly divergent mitochondrial DNA lineages (=haplogroups; provisionally referred to as S1 to S5).

In the U.S., *A. subfuscus* s. l. was first reported from Boston in 1842 (Pilsbry 1948) and has since gradually colonized most of northeastern North America. It is now found in anthropogenic and natural habitats (Chichester and Getz 1969, 1973), where it is sometimes even more abundant than native slug and snail species (Ferguson 2004). Combining morphology, DNA (mitochondrial and nuclear) and allozyme variation, Pinceel et al. (2005) found that only one of their five European haplogroups was represented in specimens collected from northeastern states, the S1 haplogroup. In addition, a large loss in genetic variation was observed which was tentatively attributed to founder effects. The authors also found that the likely source area of the invasions comprised the British Isles and adjacent regions on the European mainland (e.g., Belgium, The Netherlands and northwest France), as based on the known distribution of the S1 lineage. Finally, Pinceel et al. (2005) suggested that of both species, *A. subfuscus* may be the more obvious invader, but *A. fuscus* may also occur in the U.S. because allozyme data indicated that certain North American strains of *A. subfuscus* s. l. were even more divergent than European *A. subfuscus* s. s. and *A. fuscus* (McCracken and Selander 1980; Foltz et al. 1982). This was confirmed by Barr et al. (2009) who relied on genital anatomy and mitochondrial 16S rDNA

sequences to demonstrate the presence of *A. fuscus* in the northeast of the U.S. i.e., Vermont and Massachusetts (Norman Barr, Personal Communication).

In 2005, *Arion subfuscus* s. l. was collected in California (Half Moon Bay, San Mateo Co.) for the first time (Heike Reise and Janet Leonard, Personal Communication). The current investigation aims to (1) determine which of the cryptic taxa of the *A. subfuscus* complex are present in the state and (2) provide preliminary evidence about the possible source areas of this introduction. To this end we conducted a DNA sequence analysis of Californian specimens of *A. subfuscus* s. l. and used these data in a DNA barcoding approach by comparing them with existing sequence data from Pinceel et al. (2005) and Barr et al. (2009).

Materials and methods

Specimen collection and morphology-based identifications

During statewide invasive slug surveys of California in 2006, 12 specimens of *A. subfuscus* s. l. were discovered in San Mateo Co., Sonoma Co. and Humboldt Co. (Table 1). Specimens were collected by direct searching and kept alive in small plastic containers lined with damp paper towels and fed on carrots. On return to the laboratory, a small piece of tissue was snipped from the tails of the living slugs and preserved in 100% ethanol for DNA analysis. Next, the slugs were killed by placing them overnight in a sealed container filled with boiled water that had been allowed to cool. The following morning, the stretched slugs were dissected and preserved in 70% ethanol. Specimens were identified using the diagnostic genital characteristics given by Pinceel et al. (2004).

Molecular analysis

DNA was extracted from the tail tissue pieces of 12 specimens, using the QIAamp Tissue Kit (Qiagen Inc., Valencia, CA). Cell lysis was achieved overnight in a 56°C constant temperature shaker with proteinase K and Qiagen lysis buffer. The QIAamp Tissue Kit then adsorbed the DNA onto a silica membrane in a spin column that was cleaned with

Table 1 Date locality information and accession numbers for the specimens of *Arion subfuscus* s. s. collected in California

Specimen number	County	Location	Date	Accession number (16S)	Accession number (COI)
1–6	San Mateo	Golden Nursery, San Mateo City	23rd June 2006	GU214712–GU214717	GU249576–GU249581
7	San Mateo	Central Nursery, San Mateo City	23rd June 2006	GU214718	GU249582
8	Sonoma	Sonoma Mission Gardens, Sonoma City	24th June 2006	GU214719	GU249583
9–10	Humbolt	Pierson Nursery, Eureka	26th June 2006	GU214720–GU214721	GU249584–GU249585
11–12	Humbolt	Shafer Nursery and Hardware, Eureka	26th June 2006	GU214722–GU214723	GU249586–GU249587

two wash buffers. Finally, purified template DNA was eluted in AE storage buffer. Polymerase chain reaction (PCR) was used to amplify a 443 bp fragment of the mitochondrial 16S rDNA gene and a 655 bp fragment of cytochrome c oxidase (COI; Table 2). Amplified DNA was cleaned using the Wizard[®] PCR Preps DNA Purification System (Promega, Madison, WI). The PCR product was first purified using a purification buffer and resin, and then washed using 80% isopropanol. Clean DNA was eluted in double distilled water and then directly sequenced in both directions at the University of California, Riverside Genomics Institute Core Instrumentation Facility using an Applied BioSystems 3730 DNA Analyser with a Big-Dye[®] V3.1 kit (Applied Biosystems, Foster City, CA). Raw sequences were trimmed by removing the primers and aligned manually in Bioedit v 7.0.5.3 (Hall 1999). The aligned 16S and COI sequences were then compared with those for the different mitochondrial haplogroups of *A. subfuscus* s. s. published by Pinceel et al. (2005) and Barr et al. (2009). Neighbor-Joining (NJ) trees based on Kimura-2-parameter distances, 1000 bootstrap replicates and using *Arion lusitanicus* and *Arion rufus* as outgroups were used to confirm the association of the sequences with haplogroups. The NJ tree reconstruction and analysis were carried out with MEGA v. 4 software (Kumar et al. 2008). The COI and 16S sequences (Table 1) for the *A. subfuscus* specimens were deposited in GenBank (Benson et al. 2008) and DNA extracts of the 12 specimens have been deposited in the collections of the Royal Belgian Institute of Natural Sciences under the general inventory number I.G. 31556.

Results and discussion

The gonads of all of the specimens collected during this study were large, pale violet and located on the periphery of the digestive gland which indicated that only *A. subfuscus* s. s. was collected in California. This was confirmed by the DNA data, which further specified that the Californian specimens belonged to haplogroup S1, since for the 16S rDNA they had one and the same haplotype that was identical to S1-03 as defined by Pinceel et al. (2005) (cf. GenBank accession number AY861459), while for COI, they shared a single haplotype that differed in only one nucleotide from the COI haplotype of *A. subfuscus* with GenBank accession number AY987905 (a specimen from Kortrijk Belgium). In the NJ tree of the COI sequences, both the sequence AY987905 and that of the Californian specimen fell in the S1 haplogroup with a 100% bootstrap support (Fig. 1).

A. subfuscus s. l. is native to Europe and the S1 lineage has been collected only in Ireland, Great Britain, Belgium and France, while it was introduced into northeastern North America (Pinceel et al. 2004). It is, therefore, logical to assume that the Californian specimens of *A. subfuscus* s. l. should originate from one of these two areas. However, since the S1-03 16S rDNA haplotype is hitherto known only from the northeastern U.S. (Pinceel et al. 2005), it seems most likely that the Californian specimens were introduced from that area and not directly from Europe. Given that (1) this introduction appears to be very recent i.e., the first specimens of the species were collected in the state during 2005 and (2) transport within the U.S. has been responsible for the spread of several other

Table 2 Primer detail, reaction ingredients, and cycling conditions used for PCR amplification of the mitochondrial 16S (443 bp) and COI (655 bp) gene fragments for *Arion subfuscus* s. s.

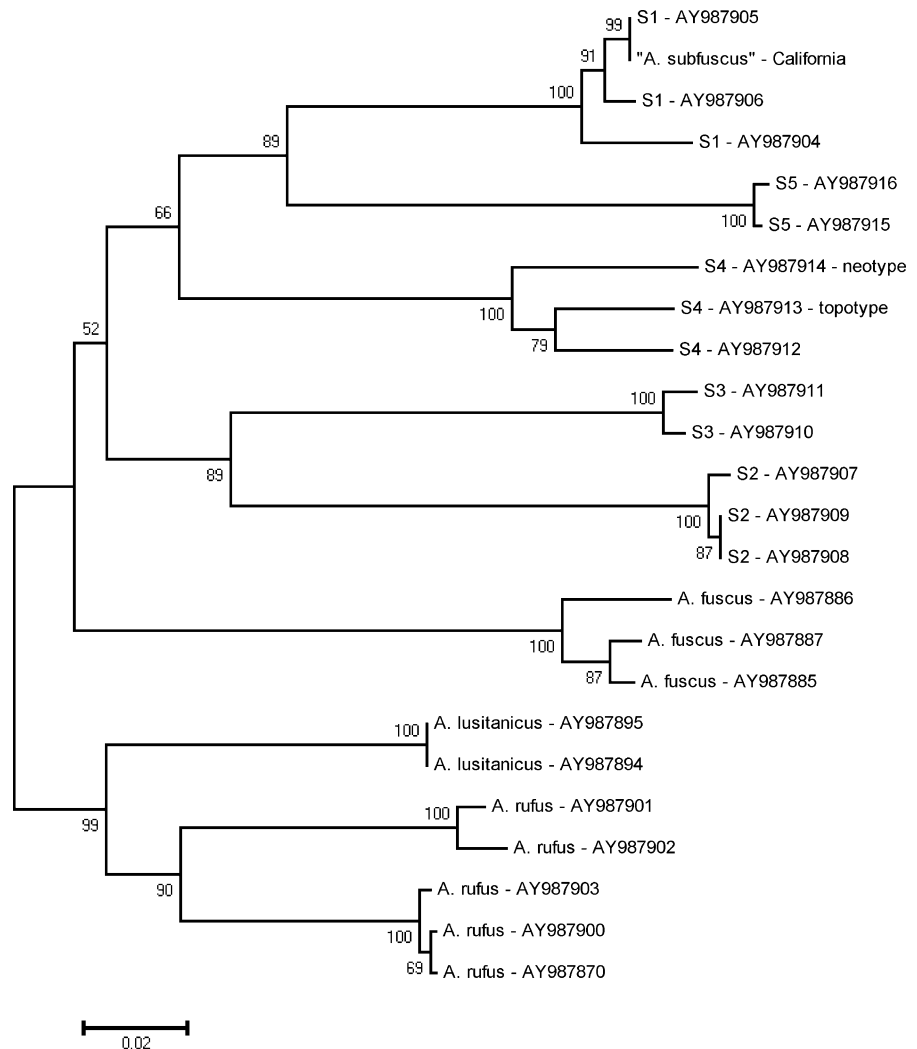
Gene	Primer	Primer sequence 5'–3'	PCR Reaction ingredients (in 25 µl)	PCR Cycling conditions
16S	16SAR (Modified from Palumbi and Benzie 1991)	CGCCTGTTTAWCAAAAACAT	1 × PCR buffer (contains 2 mM·MgSO ₄ ; New England BioLabs, Ipswich, MA, USA)	(1) 5 min at 95°C (2) 30 cycles of 1 min at 95°C, 1 min at 46°C and 2 min at 72°C (3) 5 min at 72°C
	16SBR (Modified from Palumbi and Benzie 1991)	CCGGTYTGAAGCTCAGATCAGATCAAYGT	0.2 mM of each dNTP 0.2 µM of each primer 1 U <i>Taq</i> polymerase (NEB) 5% (v/v) BSA (NEB)	
COI	LCO1490 (Folmer et al. 1994)	GGTCAACAAATCATAAAGATATTGG	1 × PCR buffer (contains 2 mM·MgSO ₄ ; New England BioLabs, Ipswich, MA, USA)	(1) 5 min at 95°C (2) 40 cycles of 1 min at 95°C, 1 min at 40°C and 2 min at 72°C (3) 5 min at 72°C
	HCO2198 (Folmer et al. 1994)	TAAACTTCAGGGTGACCAAAAAATCA	20 µM of each dNTP 0.2 µM of each primer 1 U <i>Taq</i> polymerase (NEB) 1 mM·MgCl ₂ 1.25 µl BSA detergent 2 µl Template DNA (concentration undetermined)	

alien terrestrial gastropods (e.g., Chichester and Getz 1969; Dundee 1970; Mead 1971), it is possible that other taxa of *A. subfuscus* s. l. may show up in California. Indeed Barr et al. (2009) recently demonstrated that *A. fuscus* and *A. subfuscus* S2 are also present in the U.S. Nevertheless, the current study illustrates how molecular techniques (i.e., DNA barcoding) can be easily and successfully applied to identify cryptic taxa, even if these are not yet fully defined. Moreover, the study emphasizes the need for continuous monitoring to determine how and where exotic gastropod species might spread within new regions should they evade detection at ports of entry and establish viable populations in the wider environment.

Finally, the 12 Californian specimens of *A. subfuscus* in this study were all identical both in their 16S rDNA and their COI, thus representing only one single

composite haplotype. This may be the result of a founder effect acting upon the already impoverished gene pool of *A. subfuscus* s. l. in the northeastern U.S., where Pinceel et al. (2005) observed a significant reduction in genetic diversity among 219 American specimens of this species compared to European populations. In fact Pinceel et al. (2004) reported in Europe no less than 53 different 16S rDNA haplotypes distributed over five haplogroups, while in the U.S. they found only two haplotypes, both of haplogroup S1. Even with the addition of the recent *A. subfuscus* s. s. data of Barr et al. (2009), the reduction in genetic diversity in the American populations compared to the European ones, remains overwhelming. The further reduction of genetic diversity in the Californian material is, hence, no surprise considering their probable recent introduction and the likely very small number of founders.

Fig. 1 Assignment of the partial COI gene sequences (655 bp) of Californian specimens of *Arion subfuscus* s. s. to the S1 haplogroup (100% bootstrap support) using a neighbor-joining tree based on Kimura-2-parameter distances, 1000 bootstrap replicates and both *Arion lusitanicus* and *Arion rufus* as outgroups



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