Towards the unravelling of the slug *A. ater–A. rufus* complex (Gastropoda Arionidae): new genetic approaches

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Abstract. The genus *Arion* includes several slug species, some of which are considered to be a pest to both cultivated and wild flora. Within this genus, the *Arion ater* complex comprises two different morphological forms: *Arion rufus* and *A. ater*, but there is no consensus about their species status. Their phylogenetic relationships have been recently solved, both of them belonging to different phylogenetic clades, but their species status is still unclear (as different clades are not always different species). For this reason, the aim of this study was to precisely identify these species status by employing the up-to-date multi-rate Poisson tree processes (mPTP) methodology as well as the classic methodology of genetic distances, both of which have three different mitochondrial genes. Results confirmed that both *A. ater* and *A. rufus* are independent evolutionary clades, and the high genetic distances between them (K2P distances ranged between 9.1 and 16.4 %, depending on genes) together with mPTP analyses, supported the idea that the clades correspond to different species. Results will be useful for the classification of these specific species as well as for developing proper pest control methodologies and conservation policies in both cultivated and wild plants.

1 Introduction

The genus *Arion* includes up to 50 slug species, several of which are of considerable agricultural and environmental concern (Barr et al., 2009). Some of the species in this genus are highly invasive, for example *Arion vulgaris* (Moquin-Tandon 1855), which is one of the worst invading species in Europe (Gismervik et al., 2014). Key in any strategy for pest control and conservation purposes is species identification (Amstrong and Ball, 2005), and in the genus *Arion* it is important to distinguish between closely related alien and native species. The correct identification of all *Arion* species must be a priority in order to properly face the diverse agricultural and conservation problems related to them but also to improve the taxonomy in the group.

Within the genus *Arion*, the *Arion ater* complex comprises two different morphological forms, *Arion rufus* and *A. ater*,

but there is no general consensus about their species status (Hatteland et al., 2015). Some researchers consider them to be subspecies (e.g. Burnet, 1972), and others suggest that they could be species (e.g. Bank et al., 2007). In addition, there are some indications of hybrid forms (e.g. Hagnell et al., 2003) and introgression has occurred between them (e.g. Hatteland et al., 2015; Noble and Jones, 1996; Rowson et al., 2014). This problem probably comes from their very similar external morphology and their distinct internal morphology, as well as life history and ecological traits (Blattmann et al., 2013). For example, *A. ater* is in general black coloured and *A. rufus* is orange, while another species of the genus (*A. vulgaris*) is brown; thus specimens of dark brown can be misidentified (Roth et al., 2012), although the morphology of their genitalia separates clearly these species (Hatteland et al., 2015). Genetically, their phylogenetic relationships were initially not clear and they were even proposed to be eco-

types (Quinteiro et al., 2005), but more recently it has been suggested that *A. ater* and *A. rufus* are probably independent monophyletic clades (Rowson et al., 2014). But the fact remains that they belong to different phylogenetic clades and does not establish that they are actually different species. For this reason, the different genetic species identification methods that are currently available, and that are especially useful in these kinds of problematic cases (e.g. García-Jiménez et al., 2017), must be employed here.

Species identification techniques have been greatly improved in the last decades thanks to the increasing availability of genetic information (i.e. DNA taxonomy; Goldstein and De Salle, 2011; Vogler and Monagan, 2007). In fact, the use of DNA characters (e.g. genetic distances) could be useful in the formal naming of species (Renner, 2016), and it has been frequently employed for all kinds of organisms during recent decades. However, the barcode scheme (Hebert et al., 2003) is not usually enough for diagnosis, but the coalescent model is a new tool for testing whether the clades involved in the taxa under study are evolutionary independent (Zhang et al., 2014). In all cases, the main goal is to identify known species and delimit new ones, which can be rather difficult in many cases (Vogler and Monagan, 2007). The phylogenetic species concept (PSC) approaches the species delimitation problem, relying on the fact that phylogenetic species are the smallest units for which phylogenetic relationships can be reliably inferred from DNA data (Baum and Shaw, 1995). Inspired by this concept, the coalescent Poisson tree processes (PTP) model was developed in order to infer putative species boundaries (Zhang et al., 2013). This method is more robust and less affected by restricted sampling (Agrens et al., 2016), a main constraint limiting the use of other phylogenetic methods. It overcomes, too, the difficulty of having to deal, in many cases, with a single specimen per species. This situation is more common than usually acknowledged, as a great number of taxa have been discovered from a unique specimen, and may be called the singleton methodological dilemma (Lim et al., 2012).

For all these reasons, the aim of this work was to unravel the species identity of *A. ater* and *A. rufus* with different genetic techniques. This study included the recently developed PTP estimations as well as the classic genetic distances method, all of which were performed with the information contained, for the first time, in three different mitochondrial genes. It may confirm the validity of these potentially two different species, which again motivates the search of morphological correlates and may lead to improved identification in the field.

2 Materials and methods

Because the phylogenetic information of a group of species can be contained in one or a few representative genes (Horreo, 2012), three mitochondrial genes were employed in this study (Table 1): 16s rRNA (16S), cytochrome oxidase subunit 1 (COI) and NADH dehydrogenase subunit 1 (ND1). They included sequences from different areas of Europe and America and their species identification was different depending on the authors. Sequences of both species (*Arion ater* and *A. rufus*) together with *A. vulgaris* and two outgroups (*A. iratii* and *A. subfuscus*) were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/, last access: 12 June 2018) and aligned with the programme AliView v.1.17.1 (Larsson, 2014) in a different data set for each gene. In total, 68 DNA sequences were analysed, with more than 20 sequences per gene (Table 2).

Genetic variability of the three genes was measured as the number of polymorphic sites, the number of haplotypes, and the haplotype and nucleotide diversity with DNAsp v.5 software (Librado and Rozas, 2009). The pairwise genetic distances (K2P distance) between groups (potential species *Arion ater* and *A. rufus*) were estimated with MEGA v.7 (Kumar et al., 2016).

Species delimitation was done online (http://mptp.h-its.org/#/tree, last access: 12 June 2018) with the multi-rate Poisson tree process (mPTP; Kapli et al., 2017), a Bayesian implementation of the PTP model for species delimitation. This software has been shown to be the most accurate method for genetic species delimitations (better than previous PTP and popular distance-based methods), especially with the information on only one gene (Kapli et al., 2017). As mPTP is a method for species delimitation based on rooted phylogenetic trees, a phylogenetic input tree was previously done for each gene with BEAST v.2.4.5 (Bouckaert et al., 2014). Settings for the latter included the HKY substitution model, random local clock and a birth–death model under 20 million Markov chain Monte Carlo steps.

3 Results and discussion

The genetic diversity of the three mitochondrial genes in each data set (359–544 base pair length) can be shown in Table 2. The number of haplotypes was 10 in the genes COI, 14 in the ND1 and 18 in the 16S. The haplotype diversity ranged between 0.848 (COI gene) and 0.981 (16S gene), and the nucleotide diversity ranged between 0.0774 (16S gene) and 0.1158 (ND1 gene). The K2P genetic distances between *A. ater* and *A. rufus* ranged between 9.16 % (16S gene) and 16.42 % (ND1 gene), all of which were much greater than the previously suggested 3 % of Hebert et al. (2004) for species differentiation. This result clearly points towards the classification of *A. ater* and *A. rufus* as different species. Because the genetic threshold for species identification is highly variable, there were discussions on which boundary is appropriate for different clades. For instance, even distances of 11 % have been found to signal different bird species (Tavares and Baker, 2008) but distances of less than 2 % distinguish water mites (García-Jiménez et al., 2017). Nevertheless, our results

Table 1. Sample details of the employed data set including three mitochondrial genes (16s, COI and ND1) and five species *Arion ater*, *A. rufus*, *A. vulgaris*, *A. iratii* and *A. subfuscus* with their GenBank accession numbers.
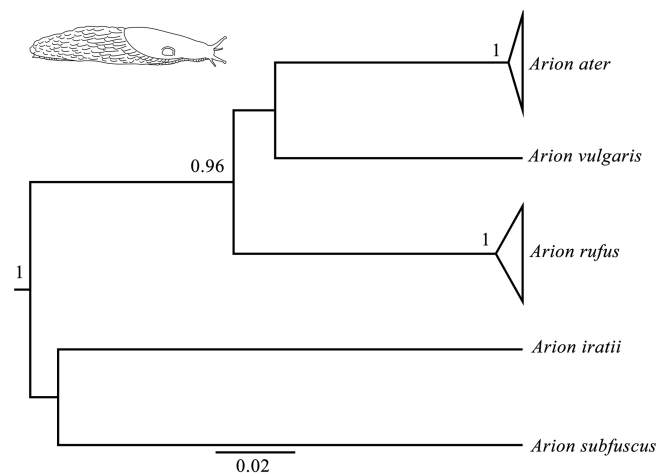
Gene	Species	GenBank	Location
16s	*A. subfuscus*	EU541951	Belgium
	A. iratii	AY947367	Spain
	vulgaris	KF754094	United Kingdom
	A. rufus	EU541919, EU541922, FJ896811, KP835195, KP835197, KP835196, KP835198, EU541920, EU541921	Canada, USA, Denmark, unknown
	A. ater	AY947349, KF894152, EU541904, KF894127, KF894147, KF894146, KF894149K EU541907, KF894137	Norway, England, Wales, Scotland, unknown
COI	*A. subfuscus*	KP976466	Unknown
	A. iratii	AY987892	Spain
	vulgaris	KX834815	United Kingdom
	A. rufus	AY987901, FJ481178, KX834735, KX834736, KX834737, KM611808, KX834707, KX834708, KX834743, KX834745, KX834744	Belgium, Finland, Switzerland, France, unknown
	A. ater	AY987870, KM612252, KX834784, KX834785, KX834739, KX834786, KM611936, KX834738	Norway, Faroe Islands, Poland, unknown
ND1	*A. subfuscus*	AY316248	England
	A. iratii	AY316249	Spain
	vulgaris	KX834693	United Kingdom
	A. rufus	KX834609, KX834610, KX834611, KX834619, KX834665, KX834666, KX834686, KX834617, KX834618, KX834631, KX834632, KX834633, KX834685, KX834620	Finland, France, Poland, Slovakia, Switzerland
	A. ater	AY316228, AY316229, KX834612, KX834664, KX834658, KX834659, KX834613, KX834663	Portugal, Spain, Faroe Islands, Poland, Norway

Table 2. Genetic variability of the three amplified genes in the total data set, measured as the number of polymorphic sites (S), the number of haplotypes (H), the haplotype diversity (Hd) and the nucleotide diversity (Nd). *n* is sample size, length is sequence length in base pairs, K2P is pairwise genetic distances between *A. ater* and *A. rufus* sequences.

Gene	*n*	Length	S	H	Hd	Nd	K2P
16S	21	403	109	18	0.981	0.0774	0.0916
COI	22	544	164	10	0.848	0.0833	0.1161
ND1	25	359	166	14	0.917	0.1158	0.1642

Figure 1. Bayesian phylogenetic tree done with the COI gene (the same topology was found also found for the 16S and ND1 genes). Node numbers show Bayesian posterior probabilities higher than 0.96. Slug drawn by Daniel Martinez.

are far beyond the proposed 1 % divergence for species identification within this genus in the USA (Barr et al., 2009). However, based on this classical criterion (genetic distance) alone and a conservative stance, we considered that it was not enough to reach a conclusive answer (maybe this species is more recent, so their genetic distance is smaller), and more analyses were needed to solve the complex *A. ater*–*A. rufus* dilemma.

Bayesian phylogenetic trees were carried out independently for each of the three genes (16s, COI and ND1) and consistently showed high posterior probabilities in all cases (several of them higher than 0.96). All three phylogenetic trees were identical in their main topology (Fig. 1), showing

three genetic lineages in addition to the outgroups (*A. iratii* and *A. subfuscus*): one with *A. vulgaris*, another comprising the sequences of *A. ater* and the last one including the sequences of *A. rufus*. The results obtained from these analyses confirm previous results, suggesting that both *A. ater*

and *A. rufus* are independent evolutionary clades (Pfenninger et al., 2014; Rowson et al., 2014; Zemanova et al., 2016). Moreover, the large genetic distances between them (Table 2) also imply that these independent clades are not very recent, strongly supporting the idea that both clades identify different species and not subspecies or morphotypes, and confirm that morphological variability within each of the species (in all cases specimens were taken from several different distant geographical areas) is actually reflecting the existence of such subspecies and morphotypes.

In the case of mPTP analyses, which infer putative species boundaries from phylogenetic information (Zhang et al., 2013), we have also found highly congruent results independently of which gene was used. These results finally included three different species in the data set (in addition to the outgroups): *Arion ater*, *A. rufus* and *A. vulgaris*. It is important to mention that, although heteroplasmy has been described in this complex (Roth et al., 2012), it does not seem to affect our results in the sense that all the three independent genes from different individuals (and authors) have yielded the same results in all analyses. Putative hybrids would not be detected here because mitochondrial DNA is only maternally inherited, but the fact that all specimens of species grouped among them suggest that no hybrids are included in the data set, or that hybrids morphology is determined by mitochondrial DNA. mPTP analyses also agree, therefore, with genetic and phylogenetic results when distinguishing *A. ater* and *A. rufus* as different animal species.

This work therefore validates *Arion ater* and *A. rufus* as separate species for the first time, with three different mitochondrial genes and two different approaches. In this way our results shed light on discussions about these taxa being considered different morphological forms (Quinteiro et al., 2005), subspecies (Evans, 1986) or species (Bank et al., 2007) because of their very similar morphological characteristics. Our results also highlight the usefulness of the mPTP species delimitation software in difficult cases such as the *A. ater*–*A. rufus* complex, confirming in this case previous phylogenetic results, which are not enough for species delimitation on their own. On this basis, as in the case of cryptic species, the search of additional morphological correlates will allow proper identification of slug species in the field. As a final remark, Zemanova et al. (2016) pointed out that having obtained several hundred specimens from central and southern Iberia, none were *A. ater* or *A. rufus*. We have included in this work a sample of *A. ater* from Leon, Spain (GenBank accession number AY316229) and another from Portugal (GenBank accession number AY316228). These sequences were identified as *A. ater* by experts (Quinteiro et al., 2005) and phylogenetically grouped here with all the other *A. ater* from northern areas, confirming they are the same species.

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