DNA barcodes and phylogenetic affinities of the terrestrial slugs *Arion gilvus* and *A. ponsi* (Gastropoda, Pulmonata, Arionidae)

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**Abstract**

The Iberian Peninsula is a region with a high endemicity of species of the terrestrial slug subgenus *Mesarion*. Many of these species have been described mainly on subtle differences in their proximal genitalia. It therefore remains to be investigated 1) whether these locally diverged taxa also represent different species under a phylogenetic species concept as has been shown for other *Mesarion* species outside the Iberian Peninsula, and 2) how these taxa are phylogenetically related. Here, we analysed DNA sequence data of two mitochondrial (COI and 16S) genes, and of the nuclear ITS1 region, to explore the phylogenetic affinities of two of these endemic taxa, viz. *Arion gilvus* Torres Mínguez, 1925 and *A. ponsi* Quintana Cardona, 2007. We also evaluated the use of these DNA sequence data as DNA barcodes for both species. Our results showed that ITS did not allow to differentiate among most of the *Mesarion* molecular operational taxonomic units (MOTUs) / morphospecies in *Mesarion*. Yet, the overall mean p-distance among the *Mesarion* MOTUs / morphospecies for both mtDNA fragments (16.7% for COI, 13% for 16S) was comparable to that between *A. ponsi* and its closest relative *A. molinae* (COI: 14.2%; 16S: 16.2%) and to that between *A. gilvus* and its closest relative *A. urbiae* (COI: 14.4%; 16S: 13.4%). Hence, with respect to mtDNA divergence, both *A. ponsi* and *A. gilvus*, behave as other *Mesarion* species or putative species-level MOTUs and thus are confirmed as distinct ‘species’.
Keywords
DNA barcoding, terrestrial slugs, Gastropoda, taxonomy, Iberian Peninsula, Arion ponsi, Arion gilvus

Introduction

The genus Arion Férussac, 1819 is the most species rich genus of the terrestrial slug family Arionidae (Mollusca, Pulmonata, Gastropoda). It comprizes approximately 40 species, grouped into four subgenera, viz. Arion s.s. Férussac, 1819, Kobeltia Seibert, 1873, Carinarion Hesse, 1926 and Mesarion Hesse, 1926. Species of the subgenus Mesarion (type species: Limax subfuscus Draparnaud, 1805) are characterized by 1) a medium body-size (up to 75 mm when extended), 2) an orange to dark brown dorsum, 3) two dark bands on the sides of the mantle, 4) (usually) yellow to orange body mucus, and 5) an enlarged free-oviduct with a long and V-shaped ligula (Kerney et al. 1983). Many Mesarion species are highly polymorphic with respect to body colour and genital anatomy. As a consequence, the species limits and phylogenetic relationships of taxa within this subgenus have been debated for decades (e.g. Garrido et al. 1995, Castillejo 1997, 1998, Pinceel et al. 2004, 2005a, b, Quinteiro et al. 2005). Arion subfuscus (Draparnaud, 1805) (type locality: Montagne Noire, France) is probably the most problematic “species” within Mesarion as it shows an overwhelming amount of variation in body pigmentation, genital anatomy, and reproductive behavior [see Garrido et al. (1995) and the references listed in their table 1]. This variation often has been interpreted as indicating reproductive isolation between geographically isolated populations, and A. subfuscus thus is considered a species complex (Wiktor 1973, Waldén 1976, De Winter 1986, Backeljau 1989, Altonaga et al. 1994, Backeljau et al. 1994, Garrido et al. 1995). Especially in the Pyrenees and the coastal regions of Spain there are local, morphologically diverged populations (e.g. Garrido et al. 1995, Castillejo 1998). Several of these have been described as endemic species on the basis of where the epiphallus, oviduct and pedunculus of the bursa copulatrix open into the atrium, in combination with differences in the relative lengths of the vas deferens and the epiphallus (e.g. Castillejo 1998, Garrido et al. 1995, Quintana Cardona 2007). Two of these endemic taxa occur in the eastern coastal region of Spain or the Balearic Islands, viz. Arion gilvus Torres Mínguez, 1925 and A. ponsi Quintana Cardona, 2007.

Arion ponsi (Figure 1) was described from Menorca (Balearic Islands, type locality: Barranc d’Algendar). The species has a medium body size (range: 54–66 mm), an orange to beige dorsal body colour with dark lateral bands that can be blurry in the posterior parts, a foot sole that is cream coloured with a greyish hue, and a transparent body mucus (Quintana Cardona 2007). Its genital anatomy is very similar to that of A. gilvus, A. iratii Garrido, Castillejo & Iglesias, 1995, A. molinae Garrido, Castillejo & Iglesias, 1995 and A. lizzarrustii Garrido, Castillejo & Iglesias, 1995, but its epiphallus is shorter than the vas deferens (as in A. molinae) and opens into the genital atrium in
between the oviduct and the pedunculus of the bursa copulatrix (unlike in *A. molinae*, where the pedunculus is positioned in between the epiphallus and oviduct) (figures 3–5 in Quintana Cardona 2007).

*Arion gilvus* (Figure 2) was described from ‘Mandol’ in the Spanish Province of Tarragona. However, the toponym ‘Mandol’ seems to be erroneous (e.g. Bech 1990) and therefore Castillejo (1990) assigned eight specimens with an *A. gilvus* morphology from Serra de Pandols near Gandesa (Province of Tarragona) as topotypes [see also Castillejo and Rodríguez (1991)]. Subsequently, *A. gilvus* was redescribed by Garrido (1992). Afterwards, the species has also been found in the Provinces of Valencia, Teruel and Albacete [Borredà (1994), figure 15 in Castillejo (1997), figure 1 in Quinteiro et al. (2005)]. *Arion gilvus* reaches a length of up to 65 mm when extended. It has a yellowish to brown dorsum that gets lighter downwards at the sides and dark lateral bands that have a yellowish grey line on their upper side (Figure 1). The sole is white or evenly yellowish and the mucus is pale yellow (Torres Mínguez 1925, Bech 1990, Garrido 1992, Castillejo 1997). The epiphallus, the pedunculus of the bursa copulatrix, and the free oviduct join the atrium on a single line with the pedunculus of the bursa copulatrix in the middle, as in *A. molinae*, but in contrast to the latter, the epiphallus is longer than the vas deferens (Torres Mínguez 1925, Borredà 1994, Castillejo 1997, and figures 26–28 in Garrido et al. 1995).
As illustrated by *Arion ponsi* and *A. gilvus*, the alleged species-specific genital differences among the Iberian species of the *A. subfuscus* complex are very subtle and little is known about their intraspecific variation. Moreover, genital differences among arionid taxa do not necessarily imply reproductive isolation (Dreijers et al. 2013). Hence, if alleged species-specific phenotypic differences in arionids are to be interpreted under a phylogenetic species concept, then their correlation with reproductive isolation should be corroborated by molecular data. Molecular markers have been very effective in this respect (e.g. Pinceel et al. 2005a, b; Quinteiro et al. 2005; Geenen et al. 2006; Jordaens et al. 2010). As such, Quinteiro et al. (2005) investigated the taxonomic affinities of Iberian *Mesarion* species using DNA sequence data. Their analysis of the nuclear ribosomal internal transcribed spacer 1 region (ITS1) showed a polytomy of *Mesarion*
species, yet, the analysis of the mitochondrial NADH dehydrogenase I (ND1) gene suggested a strongly bootstrap supported group of Iberian Mesarion species with a continental-Mediterranean distribution (A. paularenisis, A. baeticus, A. urbiae, A. anguloi, A. wiktori, and A. gilvus), and an unsupported group of species with an Atlantic distribution (A. lusitanicus, A. nobrei, A. fuligineus, A. hispanicus and A. flagellus). In addition, the positions of three Pyrenean species (A. lizarrustii, A. iratii, A. molinae) remained unresolved. More specifically, the ND1 data placed A. gilvus as sister taxon of A. urbiae and A. anguloi. Quinteiro et al. (2005) did not study individuals from the Balearic Islands and thus probably did not include A. ponsi.

Because DNA sequence data do not only provide phylogenetic information, but can also serve as DNA barcodes for species identification (Hebert et al. 2003, 2004), we here expand on the work of Quinteiro et al. (2005) by 1) characterizing A. gilvus and A. ponsi using mitochondrial COI and 16S rDNA gene fragments, and the larger part of the nuclear ITS1 region, 2) exploring the phylogenetic affinities of A. gilvus and A. ponsi within the subgenus Mesarion, and 3) providing diagnostic COI barcodes for both species.

**Material and methods**

Information on the species and specimens included here is provided in Table 1. In total, we screened 45 specimens (Table 1). DNA was extracted from small parts of the foot using a NucleoSpin Tissue Kit (Macherey-Nagel, Düren) following the manufacturer’s instructions. PCR reactions were done in 25 µl reaction volumes that contained 1.5 mM MgCl₂ in 1 x PCR buffer (Qiagen), 0.2 mM of each dNTP, 0.2 µM of each primer and 0.5 units of Taq polymerase (Qiagen). A fragment of the mitochondrial COI and 16S genes was amplified using primer pairs LCO1490 and HCO2198 (Folmer et al. 1994) and 16Sar and 16Sbr (Palumbi 1996), respectively. The nuclear ITS1 region (except the ± first 30 bp) was amplified using the primer pair ITS1L and 58C (Hillis and Dixon 1991). The PCR profile was an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of 45 s at 95 °C, 45 s at an annealing temperature of 40 °C (COI), 42 °C (16S) or 55 °C (ITS1) and 1.5 min at 72 °C, and ending with a final extension step of 5 min at 72 °C. PCR products were purified using the GFX PCR DNA Purification Kit (GE Healthcare) following the manufacturer’s instructions. Purified DNA was diluted in 15 µl of sterile water. PCR-products were bidirectionally sequenced using the ABI PRISM BigDye® Terminator v1.1 Cycle Sequencing Kit and run on an ABI3130xl Genetic Analyzer. Sequences were assembled in SeqScape v2.5 (Life Technologies) and inconsistencies were checked by eye on the chromatogram. Sequences were submitted to GenBank under accession numbers KF305196–KF305225 for COI, KF356212–KF356245 for 16S and KF385449–KF385469 for ITS1. These datasets were supplemented with DNA sequences from GenBank [including a few species of the other Arion subgenera (Table 1)]. We used those of Carinarion as outgroup.

Sequences were aligned in ClustalW (Thompson et al. 1994) with default settings and without subsequent manual adjustments. In each alignment sequences were trimmed
Table 1. List of specimens used in this study with specimen ID, sampling locality, GenBank accession numbers for the COI, 16S and ITS1 sequences, and collection number at the museums (if available). Neo-, para- and topotypes have been indicated. Specimen codes with an asterisk are data taken from Quinteiro et al. (2005); NA = not assessed. The specimen ID and GenBank accession numbers of newly sequenced specimens are given in bold.

| Species/ID | Locality, country | COI    | 16S   | ITS1   | Collection number |
|------------|-------------------|--------|-------|--------|-------------------|
| **Subgenus Mesarion Hesse, 1926** |
| Arion anguloi Martin and Gómez, 1988 |
| ang-SU2777 | Torralba del Rio, Spain | AY987869 | AY947348 | AY947386 | RBINS Brussels, I.G. 32471 |
| ang-115 (topotype) | Osma, Álava, Spain | **KF305196** | **KF356212** | AJ509055 | RBINS Brussels, I.G. 32471 |
| AANG 73A* | Burgos, Spain | NA | NA | AY316291 |
| Arion baeticus Garrido, Castillejo and Iglesias, 1995 |
| ba-556 (paratype) | Malaga, Spain | AY987871 | AY947350 | AJ509054 | MNCN Madrid 15.05/6969 |
| Arion flagellus Colligne, 1893 |
| fla-130 | Glasgow, UK | AY987880 | AY947359 | AJ509053 | RBINS Brussels, I.G. 32471 |
| fla-161 | Glasgow, UK | AY987881 | AY947360 | AJ509052 | RBINS Brussels, I.G. 32471 |
| fla-SU672 | Salamir, Spain | AY987882 | AY947361 | AJ509054 | RBINS Brussels, I.G. 32471 |
| AFLA 44A* | Croydon, UK | NA | NA | AY316278 |
| Arion fuliginosus Morelet, 1845 |
| AFUL 43A* | São Silvestre, Portugal | NA | NA | AY316277 |
| Arion fuscus (Müller, 1774) |
| fus-SU155 | Grudki, Poland | AY987885 | AJ786721 | AY947390 | RBINS Brussels, I.G. 32471 |
| fus-2320 | Predel, Bulgaria | AY987886 | AJ786722 | AY947391 | RBINS Brussels, I.G. 32471 |
| fus-SU1335 | Steinegg, Austria | AY987887 | AJ786726 | AY947392 | RBINS Brussels, I.G. 32471 |
| fus-SU2188 | Kreuzen, Austria | NA | **KF356221** | NA | RBINS Brussels, I.G. 32471 |
| Arion gilvus Torres Mínguez, 1925 |
| gil-46 | Serra de Pandols, Valencia, Spain | NA | NA | **KF385450** | RBINS Brussels, I.G. 32471 |
| gil-47 | Serra de Pandols, Valencia, Spain | **KF305199** | **KF356222** | **KF385451** | RBINS Brussels, I.G. 32471 |
| gil-73 | Serra de Pandols, Valencia, Spain | **KF305200** | **KF356223** | **KF385452** | RBINS Brussels, I.G. 32471 |
| AGIL 49A* | Serra de Pandols, Valencia, Spain | NA | NA | AY316282 |
| Arion hispanicus Simroth, 1886 |
| AHIS 52B* | Cáceres, Spain | NA | NA | AY316285 |
| Species/ID | Locality, country | COI    | 16S    | ITS1    | Collection number            |
|-----------|------------------|--------|--------|---------|-----------------------------|
| Arion iratii Garido, Castillejo and Iglesias, 1995 |
| ira-559 (paratype) | Navarra, Spain | AY987892 | AY947367 | AJ509042 | MNCN Madrid, 15.05/18705 |
| Arion lizarrustii Garido, Castillejo and Iglesias, 1995 |
| liz-562 (paratype) | Navarra, Spain | AY987893 | AY947368 | AJ509046 | MNCN Madrid, 15.05/18706 |
| ALIZ 47C* | Lizarrusti, Spain | NA | NA | | AY316280 |
| Arion lusitanicus Mabille, 1868 |
| lus-1613 | Feitos, Portugal | KF305203 | KF356224 | NA | RBINS Brussels, I.G. 32471 |
| lus-1631 | Currais, Portugal | KF305204 | KF356225 | NA | RBINS Brussels, I.G. 32471 |
| lus-1641 | Cacia, Portugal | KF305205 | NA | NA | RBINS Brussels, I.G. 32471 |
| lus-1647 | Cacia, Portugal | KF305206 | NA | NA | RBINS Brussels, I.G. 32471 |
| lus-1652 | Forjães, Portugal | KF305207 | KF356226 | NA | RBINS Brussels, I.G. 32471 |
| lus-1654 | Currais, Portugal | NA | NA | NA | RBINS Brussels, I.G. 32471 |
| lus-1655 | Forjães, Portugal | KF305208 | KF356228 | NA | RBINS Brussels, I.G. 32471 |
| lus-79 | Ursel, Belgium | AY987894 | AY947369 | AJ509062 | RBINS Brussels, I.G. 32471 |
| lus-181 | Terceira, Azores, Portugal | NA | NA | KF385453 | RBINS Brussels, I.G. 32471 |
| lus-186 | Namur, Belgium | AY987895 | AY947370 | AJ509061 | RBINS Brussels, I.G. 32471 |
| lus-465 | Görlitz, Germany | NA | NA | AJ509063 | RBINS Brussels, I.G. 32471 |
| lus-509 | Emptinne, Belgium | KF305209 | KF356229 | NA | RBINS Brussels, I.G. 32471 |
| ALUS 42A* | Serra de Arrábida, Portugal | NA | NA | | AY316273 |
| ALUS 42B* | Serra de Arrábida, Portugal | NA | NA | | AY316274 |
| ALUS 42C* | Serra de Arrábida, Portugal | NA | NA | | AY316275 |
| ALUS 42G* | Alpi Carniche, Rivolato, Italy | NA | NA | | AY316276 |
| ALUS 62E* | Montagne Noire, France | NA | NA | | AY316289 |
| ALUS 70C* | Girona, Spain | NA | NA | | AY316290 |
| Arion molinae Garido, Castillejo and Iglesias, 1995 |
| mol-565 (paratype) | La Molina, Spain | AY987896 | AY947371 | AJ509043 | MNCN Madrid, 15.05/18707 |
| AMOL 48A* | Serra del Cadí, Barcelona, Spain | NA | NA | | AY316281 |
| Arion nobrei Pollonera, 1889 |
| ANOB 41A* | Luso, Portugal | NA | NA | | AY316271 |
| Species/ID | COI | ITS1 | Locality, country | Collection number |
|------------|-----|------|-------------------|-------------------|
| ANOB 41B*  | NA  | Y316277 | Luso, Portugal | NA |
| Arion paularensis | NA  | AY978909 | Sierra de Guadarrama, Spain | NA |
| pau-121 | NA  | AY978909 | Sierra de Guadarrama, Spain | NA |
| pau-224 | NA  | AY978909 | Sierra de Guadarrama, Spain | NA |
| pau-226 | NA  | AY978909 | Sierra de Guadarrama, Spain | NA |
| APAU 51A* | NA  | AY978909 | Sierra de Guadarrama, Spain | NA |
| Arion ponsi | NA  | AY978909 | Sierra de Guadarrama, Spain | NA |
| pon-1959 | NA  | AY978909 | Sierra de Guadarrama, Spain | NA |
| pon-1960 | NA  | AY978909 | Sierra de Guadarrama, Spain | NA |
| pon-1962 | NA  | AY978909 | Sierra de Guadarrama, Spain | NA |
| pon-1965 | NA  | AY978909 | Sierra de Guadarrama, Spain | NA |
| Arion subfuscus | NA  | AY978909 | Sierra de Guadarrama, Spain | NA |
| sub1-2312 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub1-2318 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub1-2317 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub1-1618 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub1-1633 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub1-2309 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub1-2314 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub2-SU2424 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub2-SU349 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub2-2309 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub2-2314 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub2-2313 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub2-2314 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub3-2322 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub3-2310 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub3-SU2401 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub4-123 (topotype) | NA  | KF305211 | Kuortinka, Finland | NA |
| sub4-568 (neotype) | NA  | KF305211 | Kuortinka, Finland | NA |
| sub4-2341 | NA  | KF305211 | Kuortinka, Finland | NA |
| sub4-SU1058 | NA  | KF305211 | Kuortinka, Finland | NA |
| Arion pulchellus Wokör and Parejo, 2007 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| pon-1959 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| pon-1960 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| pon-1962 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| pon-1965 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| Arion punctualis Quintana Cardona, 2007 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| pon-1959 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| pon-1960 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| pon-1962 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| pon-1965 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| Arion punctulatus Draparnaud, 1805 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| pon-1959 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| pon-1960 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| pon-1962 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| pon-1965 | NA  | AY316278 | Sierra de Guadarrama, Spain | NA |
| Species/ID | Locality, country          | COI         | 16S         | ITS1         | Collection number         |
|-----------|----------------------------|-------------|-------------|--------------|----------------------------|
| sub5-2321 | Villemont-Baubiat, France  | AY987915    | AY860681    | KF385468     | RBINS Brussels, I.G. 32471 |
| sub5-2311 | Villemont-Baubiat, France  | AY987916    | AY860679    | KF385467     | RBINS Brussels, I.G. 32471 |
| ASUB 45A* | Montagne Noire, France     | NA          | NA          | NA           | RBINS Brussels, I.G. 32471 |
|           |                            |             |             |              |                             |
| *Arion transylvanus* Simroth, 1885 |
| tra-SU1088 | Covasna, Romania            | AY943858    | AY860798    | AY947393     | RBINS Brussels, I.G. 30412 |
| tra-SU1203 | Lunca Vișagului, Romania    | AY943859    | AY860805    | AY947394     | RBINS Brussels, I.G. 30412 |
| tra-SU1296 | Hilda, Romania              | AY943860    | AY860799    | AY947395     | RBINS Brussels, I.G. 30412 |
| *Arion urbiae* De Winter, 1986 |
| urb-SU2755 | Saldaña, Spain              | AY987919    | AY947381    | AY947396     | RBINS Brussels, I.G. 32471 |
| urb-99    | Sierra da Urbia, Spain      | NA          | NA          | NA           | RBINS Brussels, I.G. 32471 |
| AURB 50A* |                            | NA          | NA          | KF385469     | RBINS Brussels, I.G. 32471 |
|           |                            |             |             |              |                             |
| *Subgenus Kobeltia* Seibert, 1873 |
| *Arion distinctus* Mabille, 1868 |
| dis-106   | Mortsel, Belgium            | AY987875    | AY947354    | AJ509040     | RBINS Brussels, I.G. 32471 |
| dis-14    |                            | AY987874    | AY947353    | AJ509038     | RBINS Brussels, I.G. 32471 |
| *Arion hortensis* Férussac, 1819 |
| hor-102   | Mortsel, Belgium            | AY987888    | AJ518061    | AJ509037     | RBINS Brussels, I.G. 32471 |
| hor-220   | London, UK                  | AY987889    | AY947364    | AJ509036     | RBINS Brussels, I.G. 32471 |
| *Arion intermedius* Normand, 1852 |
| int-104   | Rochefort, Belgium          | AY987891    | AY947366    | AJ509031     | RBINS Brussels, I.G. 32471 |
| int-52    | Flores, Azores, Portugal    | AY987890    | AY947365    | AJ509029     | RBINS Brussels, I.G. 32471 |
| *Arion obesoductus* Reischütz, 1973 |
| alp-1610  | Žďárské Vrchy, Czech Republic | DQ904249    | DQ904248    | NA           | RBINS Brussels, I.G. 32471 |
| alp-208   | Saxony, Germany             | AY987867    | AY947346    | AJ509041     | RBINS Brussels, I.G. 32471 |
| *Arion oweni* Davies, 1979 |
| owe-310   | Devon, UK                   | AY987897    | AY947372    | AJ509033     | RBINS Brussels, I.G. 32471 |
| owe-316   | Devon, UK                   | AY987898    | AY947373    | AJ509034     | RBINS Brussels, I.G. 32471 |
| *Arion wiktori* Parejo & Martín, 1990 |
| wik-SU2693 | Viniegra de Abajo, Spain    | AY987921    | AY947383    | AY947397     | RBINS Brussels, I.G. 32471 |
| Species/ID | Locality, country          | COI         | 16S         | ITS1        | Collection number             |
|-----------|---------------------------|-------------|-------------|-------------|------------------------------|
| wik-44    | Burgos, Spain             | AY987920    | AY947382    | AJ509060    | RBINS Brussels, I.G. 32471  |
| wik-94    | Burgos, Spain             |             |             |             |                              |
| AWIK 58A* | Demanda Sierra, Burgos, Spain | NA         |             |             |                              |
| AWIK 58C* | Uribión Mountains, Soria, Spain | NA         |             |             |                              |

**Subgenus Carinarion Hesse, 1926**

*Arion circunscriptus* Johnston, 1828

| Species/ID | Locality, country          | COI         | 16S         | ITS1        | Collection number             |
|-----------|---------------------------|-------------|-------------|-------------|-------------------------------|
| cir-151   | Aran Island, Kilmurvey, Ireland | AY987872    | AY947351    | AJ509071    | RBINS Brussels, I.G. 32471  |

*Arion fasciatus* (Nilsson, 1823)

| Species/ID | Locality, country          | COI         | 16S         | ITS1        | Collection number             |
|-----------|---------------------------|-------------|-------------|-------------|------------------------------|
| fas-144   | Görlitz, Germany          | AY987877    | AY947356    | AJ509068    | RBINS Brussels, I.G. 32471  |

*Arion siluaticus* Lohmander, 1937

| Species/ID | Locality, country          | COI         | 16S         | ITS1        | Collection number             |
|-----------|---------------------------|-------------|-------------|-------------|-------------------------------|
| sil-142   | Poulseur, Belgium         | AY987917    | AY947379    | AJ509070    | RBINS Brussels, I.G. 32471  |

**Subgenus Arion s.s. Férussac, 1819**

*Arion ater-rufus* complex

| Species/ID | Locality, country          | COI         | 16S         | ITS1        | Collection number             |
|-----------|---------------------------|-------------|-------------|-------------|-------------------------------|
| ate-SU157 | Musland, Norway           | AY987870    | AY947349    | AY947387    | RBINS Brussels, I.G. 32471  |
| ate/ruf-1602 | Manteigas, Portugal     | KF305219    |             |             |                              |
| ate/ruf-1619 | Santa Leocádia, Portugal | KF305220    | KF356213    |             |                              |
| ate/ruf-1620 | Gortmore, Ireland        | KF305221    | KF356214    |             |                              |
| ate/ruf-1624 | Olearinhos, Portugal     | KF305222    | KF356215    |             |                              |
| ate/ruf-1638 | Portulezo, Portugal      | KF305223    | KF356216    |             |                              |
| ate/ruf-1649 | Manteigas, Portugal      | KF305224    | KF356217    |             |                              |
| ruf-105   | St.-Katelijne Waver, Belgium | KF305225    | KF356234    |             |                              |
| ruf-15    | Santiago de Compostela, Spain | AY987900    | AY947375    | AJ509066    | RBINS Brussels, I.G. 32471  |
| ruf-155   | Brussels, Belgium        | AY987901    | AY947376    | AJ509064    | RBINS Brussels, I.G. 32471  |
| ruf-180   | Hoboken, Belgium         | AY987902    | AY947377    | AJ509065    | RBINS Brussels, I.G. 32471  |
| ruf-182   | Brecht, Belgium          | AY987903    | AY947378    | AJ509067    | RBINS Brussels, I.G. 32471  |
| ruf-624   | Nazareth , Belgium       |             |             |             |                               |
| AATE 39A* | Caldas de Gerês, Portugal | NA          |             |             |                               |
| AATE 39E* | Valporquero Cave, Leon, Spain | NA          |             |             |                               |
| ARUF 40G* | Montagne Noire, France   | NA          |             |             |                               |
to equal length. The final alignments had a length of 504 bp (COI), 408 bp (16S) and 587 bp (ITS1), and of 1499 bp after concatenating the three fragments. The COI sequences were translated to amino acid sequences to check for stop codons (but none were found). The ITS1 sequences were also analysed together with those of Quinteiro et al. (2005). In this way we could extend our taxon coverage to A. hispanicus Simroth, 1886, A. fuliginus Morelet, 1845 and A. nobrei Pollonera, 1889 (Table 1). Because Quinteiro et al. (2005) used other ITS1 primers, we had to trim this dataset to a length of 378 bp. For each gene fragment, and for the concatenated dataset, we constructed Neighbour-Joining (NJ) trees (Saitou and Nei 1987) using the Kimura 2-parameter (K2P) model in MEGA v5 (Tamura et al. 2011) with complete deletion of insertions and deletions (indels). Branch support was evaluated with 1000 bootstrap replicates (Felsenstein 1985). Only bootstrap values ≥ 70% were considered as indicating strong support (Hillis and Bull 1993). Uncorrected p-distances (hereafter simply referred to as p-distance) were calculated in MEGA v5 (Tamura et al. 2011). For these calculations we considered the following Molecular Operational Taxonomic Units (MOTUs): 1) the five 16S rDNA clades of A. subfuscus (S1 to S5) defined by Pinceel et al. (2005a), 2) A. anguloi and A. urbiae jointly as a single MOTU (Backeljau et al. 1994, Quinteiro et al. 2005), 3) A. wiktori and A. paularensis jointly as a single MOTU (Backeljau et al. 1996, Quinteiro et al. 2005), and 4) A. lusitanicus from Portugal vs. A. lusitanicus from elsewhere as two different MOTUs (Davies 1987, Castillejo 1998, Quinteiro et al. 2005). Standard errors of mean p-distances among taxa and MOTUs were calculated on 1000 bootstrap replicates.

Result

Overall

The alignments comprised 504 bp for COI (196 variable sites), 408 bp for 16S (121 sites with alignment gaps, 122 variable sites) and 587 bp for ITS1 (277 sites with alignment gaps, 64 variable sites). For the concatenated dataset, there was strong support for the subgenera Carinarion, Kobeltia (excluding A. wiktori) and Arion s.s., and for a clade of Arion s.s. + Mesarion (including A. wiktori) (Figure 3). The subgenus Mesarion was not monophyletic but consisted of (1) a clade of A. flagellus, A. wiktori, A. paularensis, A. baeticus, A. urbiae, A. anguloi, and A. gilvus, (2) two haplotypes of A. lusitanicus (lus-79 and lus-186) that formed a sister group of Arion s.s. [insofar A. lusitanicus is, of course, considered as a member of Mesarion; see e.g. Backeljau (1989)], and (3) a number of species/clades among which the relationships were mostly unresolved. Within A. subfuscus (for which the monophyly was not supported) there were five clades (S1 to S5), with strong support for (S1,S5),S4) and (S2,S3). The mean p-distance (± SE) among the Mesarion OTUs (including A. ponsi and A. gilvus) was 0.168 ± 0.011 (range: 0.11–0.22) for COI, 0.134 ± 0.012 (range: 0.058–0.195) for 16S, and 0.022 ± 0.004 (range: 0.000–0.048) for ITS1 (a minimum distance of zero means that the two sequences only differed in a number of indels). The mean p-distances (± SE) excluding A. ponsi and A.
Figure 3. Neighbour-Joining tree (Kimura 2-parameter model) of a 1499 bp concatenated fragment (504 bp of the mitochondrial cytochrome c oxidase subunit I (COI) gene, 408 bp of the mitochondrial 16S rDNA gene, 587 bp fragment of the nuclear internal transcribed spacer 1 (ITS1) region) for the land slug subgenus Mesarion. Bootstrap values ≥ 70% are shown at the nodes. For sample codes see Table 1.
gilvus were 0.167 ± 0.011 (range: 0.11–0.22) for COI, 0.130 ± 0.012 (range: 0.058–0.195) for 16S, and 0.023 ± 0.004 (range: 0.000–0.048) for ITS1. For the concatenated dataset these values were 0.108 ± 0.006 (range: 0.071–0.137) (including A. ponsi and A. gilvus) and 0.107 ± 0.006 (range: 0.071–0.137) (excluding A. ponsi and A. gilvus). The phylogenetic trees inferred from the three gene fragments and from the concatenated dataset are shown in Appendix, Supplementary Figures 1–4 and Figure 3, respectively.

**Arion ponsi**

The four individuals of *A. ponsi* yielded four COI and three 16S haplotypes (Appendix, Supplementary Figures 1–2), yet two 16S haplotypes only differed by an indel of two base pairs at positions 291–292. For both genes *A. molinae* showed the smallest p-distance with *A. ponsi* (COI: mean p-distance 0.142 ± 0.014; 16S: mean p-distance 0.162 ± 0.019), but a sister species relationship with *A. molinae* was only well-supported by 16S. There were three ITS1 haplotypes for *A. ponsi*; one of these had a deletion of a poly-T stretch of six base pairs at positions 556–561; the other two differed by a deletion of a G at position 554. These three ITS1 haplotypes of *A. ponsi* clustered within a clade of *A. subfuscus* S1–5, *A. lizarrustii*, *A. molinae*, *A. iratii* and *A. transsylvanus* (Appendix, Supplementary Figure 3). The ITS1 analysis with the sequences of Quinteiro et al. (2005), placed the single remaining *A. ponsi* haplotype in the same clade (mean p-distance with the other taxa of this clade = 0.046 ± 0.004), but without bootstrap support (Appendix, Supplementary Figure 4).

As for 16S, the concatenated tree of the three gene fragments showed a sister species relationship between *A. ponsi* and *A. molinae* (Figure 3).

**Arion gilvus**

The three *A. gilvus* specimens yielded two COI (one synonymous A-G substitution at position 366) and one 16S haplotypes. For both genes the smallest mean p-distances were observed relative to *A. urbiae* and *A. anguloi* (COI: mean p-distance = 0.145 ± 0.013; 16S: mean p-distance = 0.134 ± 0.016). The two *A. gilvus* ITS1 haplotypes reduced to one when considering the stretch that overlapped with the Quinteiro et al. (2005) sequences. In this stretch it differed from that of Quinteiro et al. (2005) by a deletion of a T at position 349. Separately, none of the three genes provided reliable evidence about the sister group relationships of *A. gilvus* (Appendix, Supplementary Figures 1–4). Yet, the concatenated tree showed a well-supported sister species relationship between *A. gilvus* and the *A. urbiae* / *A. anguloi* clade (mean p-distance = 0.021 ± 0.003) (Figure 3). Mean p-distances within this *A. urbiae* / *A. anguloi* clade (in which *A. anguloi* was paraphyletic) were *P* = 0.041 ± 0.006 for COI, *P* = 0.023 ± 0.006 for 16S, *P* = 0.004 ± 0.002 for ITS1 and *P* = 0.020 ± 0.003 for the concatenated dataset.
Discussion

The NJ-tree of the concatenated dataset confirms the major outcomes of previous phylogenetic studies, viz. 1) a strong support for the monophyly of the subgenus *Carinarion* (Geenen et al. 2006), 2) a clade of *Arion* s.s. and non-Portuguese *A. lusitanicus* (Quinteiro et al. 2005), 3) *A. wiktori* clustering with *Mesarion* species, in particular with *A. paularensis* (Quinteiro et al. 2005) instead of with *Kobeltia* species (Castillejo 1998), and 4) the strong differentiation within *A. subfuscus* s.s. that consists of, at least, five phylogenetic species (Pinceel et al. 2005a). It therefore seems that the analysis of COI, 16S and ITS1 DNA sequences yields relevant taxonomic information with respect to the characterisation of arionid species that have been described under the morphospecies concept.

Because *Arion gilvus* and *Arion ponsi* were originally described on morphological grounds they are to be interpreted as morphospecies. This phenetic morphological distinction, however, correlates well with a phenetic separation based on mtDNA distances. Indeed, the overall mean p-distance among the *Mesarion* MOTUs (excluding *A. ponsi* and *A. gilvus*) dealt with in this study is 16.7% for COI and 13% for 16S. As such, the mean p-distances between *A. ponsi* and *A. molinae* (COI: 14.2%; 16S: 16.2%) or between *A. gilvus* and *A. urbiae* (COI: 14.5%; 16S: 13.4%) are perfectly comparable with the mean p-distances among the other MOTUs and morphospecies in *Mesarion*. Hence, with respect to mtDNA divergence, both *A. ponsi* and *A. gilvus*, behave as other *Mesarion* species or putative species-level MOTUs.

Obviously, the strong COI differentiation among *Mesarion* taxa, and of *A. ponsi* and *A. gilvus* in particular, suggests that DNA barcoding may be a suitable identification tool for these animals. Yet, this may be a too simplistic conclusion, since stylommatophorans may show extremely high intraspecific mtDNA divergences of sometimes up to 27% (K2P-distances, but note the uncorrected p-distances are almost similar) (Thomaz et al. 1996, Chiba 1999). In addition, Davison et al. (2009) showed that in the Stylommatophora the mean interspecific K2P-distances (± 3%) can be substantially lower than the mean intraspecific K2P-distances (± 12%). Under these conditions, it becomes very difficult to define generally applicable thresholds that distinguish between intra- and interspecific sequence divergences. Such thresholds are normally associated with DNA barcoding gaps (Hebert et al. 2003), but Davison et al. (2009) were unable to detect DNA barcoding gaps in the taxa they studied. Nevertheless, Davison et al. (2009) suggested a pragmatic 4% threshold to separate intra- and interspecific values, but at the same time they also concluded that DNA barcoding in itself is insufficient to identify and/or detect stylommatophoran species. Unfortunately, our sample sizes were too small to explore eventual DNA barcoding gaps in *Mesarion*.

Because DNA barcoding on its own may be unreliable for identifying and detecting species-level taxa in stylommatophorans, it is necessary to backup this sort of data with, amongst others, phylogenetic analyses. As such, our phylogenetic trees of the DNA sequence data show that the morphospecies *A. ponsi* and *A. gilvus*, also represent phylo-
genetic species, since both form well-supported clades that are “significantly” associated with well-defined, but morphologically different sister species. For *A. ponsi*, the sister species appears to be *A. molinae*, the distribution range of which is located in NE continental Spain (Castillejo 1997), i.e. north of, and facing, the Balearic Islands. Conversely, the sister taxon of *A. gilvus* is the “tandem” of *A. urbiae* and *A. anguloi*, two species that have been synonymized by Backeljau et al. (1994) and that jointly should be referred to as *A. urbiae*. Our DNA sequence data on COI, 16S and ITS1 (e.g. Figure 3), as well as those on ND1 and ITS1 of Quinteiro et al. (2005) are in line with this. As such, the distribution range of *A. urbiae* is situated northwest of, and probably adjacent to, that of *A. gilvus*. Thus, for both the species pairs *A. ponsi* / *A. molinae* and *A. gilvus* / *A. urbiae*, the distribution ranges appear at least consistent with the suggested sister group relationships.

In conclusion, the present work shows that *A. ponsi* and *A. gilvus* clearly differ from *A. subfuscus* or any other currently recognized arionid species. As such, former records of *A. subfuscus* from Menorca (e.g. Gasull and van Regeren Altena 1970, Mateo 1993, Beckmann 2007) almost certainly refer to *A. ponsi*. Similarly, probably all reports of *A. subfuscus* in the regions of Valencia and Albacete involve *A. gilvus* (e.g. Borredà 1994, Borredà and Collado 1996). Finally, Borredà (1994) wondered about the eventual relationship between *A. subfuscus* from Menorca and *A. gilvus*. The current data confirm unambiguously that these are two different species, with the former being *A. ponsi*. Yet, the overall phylogenetic relationships within *Mesarion* and many other *A. subfuscus*-like taxa remain to be resolved. In this context, one of the main questions is whether *Mesarion* in its present use is a monophyletic taxon. At the same time one may wonder about the relationships with the subgenus *Arion* s.s., with which *Mesarion* seems to form a well-supported clade (Figure 3).

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Appendix

Supplementary Figure 1. Neighbour-Joining tree (Kimura 2-parameter model) of a 504 bp fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene for the land slug subgenus Mesarion. Bootstrap values ≥ 70% are shown at the nodes. For sample codes see Table 1.
Supplementary Figure 2. Neighbour-Joining tree (Kimura 2-parameter model) of a 408 bp fragment of the mitochondrial 16S rDNA gene for the land slug subgenus *Mesarion*. Bootstrap values ≥ 70% are shown at the nodes. For sample codes see Table 1.
Supplementary Figure 3. Neighbour-Joining tree (Kimura 2-parameter model) of a 587 bp fragment of the nuclear internal transcribed spacer 1 (ITS1) region for the land slug subgenus Mesarion. Bootstrap values ≥ 70% are shown at the nodes. For sample codes see Table 1.
Supplementary Figure 4. Neighbour-Joining tree (Kimura 2-parameter model) of a 378 bp fragment of the nuclear internal transcribed spacer 1 (ITS1) region for the land slug subgenus *Mesarion*. This figure also includes the Iberian *Mesarion* ITS1 sequences of Quinteiro et al. (2005) Bootstrap values ≥ 70% are shown at the nodes. For sample codes see Table 1.