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One town's invasion by the pest slug *Arion vulgaris* (Gastropoda: Arionidae): microsatellites reveal little introgression from *A. ater* and limited gene flow between infraspecific races in both species

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Short title: Introgression and variation in *Arion* slugs

ABSTRACT

The terrestrial slug *Arion vulgaris* has recently spread across most of Europe, often causing the local extinction of resident populations of *Arion ater* s.l. The species hybridise, which leads to the prediction of massive introgression of *A. ater* genes into *A. vulgaris*. To test this, we utilised 16 microsatellite markers applied to samples of both species collected around Görlitz, Germany, during the invasion. Amongst *A. vulgaris* individuals with typical genitalia, an analysis using STRUCTURE suggested that only 6% were appreciably admixed with local *A. ater*; admixture did not increase over the course of the invasion. Amongst the c. 4% of slugs with intermediate genitalia, microsatellites confirmed that often they were hybrids, their anatomy correlating with the estimated share of ancestry from each species. The microsatellites also distinguished the three subspecies of *A. ater* previously recognised on the basis of genital anatomy and mitochondrial DNA. The subspecies were not well mixed spatially, with *A. a. ater* in wilder places, and *A. a. rufus* never found in the Polish part of the town; nevertheless hybridisation between them was occurring. Unexpectedly, the microsatellites indicated three genetic races amongst *A. vulgaris*; these occurred in different districts and are only slowly mixing spatially and genetically.

ADDITIONAL KEYWORDS: ADEGENET – *Arion ater ruber* – *Arion rufus* – COI – Germany – hybrid – introduced species – Poland – Spanish slug – STRUCTURE program

INTRODUCTION

The large slug *Arion vulgaris* Moquin-Tandon, 1855 (formerly known as *A. lusitanicus* auct. non Mabille: see Castillejo, 1998; Kadolsky *et al.*, 2018) causes considerable damage in gardens and to crops (von Proschwitz, 1996; Kozłowski & Kozłowski, 2011). Over the last 60 years or so, it has spread northwards and eastwards across Europe, reaching Scandinavia and eastern Europe, and is now in North America (Hatteland *et al.*, 2013; Păpureanu *et al.*, 2014; Zemanova *et al.*, 2018, Araiza-Gómez *et al.*, 2021). In contrast to the claims of Pfenninger *et al.* (2014), Zemanova *et al.*'s (2016) more thorough study convincingly demonstrated a likely origin in southwestern France; the subsequent work of Zając *et al.* (2020) does not contradict this.

In many places, a few years after *A. vulgaris* arrived the resident, similarly-sized congener *Arion ater* (Linnæus, 1758) disappeared from synanthropic habitats (von Proschwitz, 1996; Rüetschi *et al.*, 2012; Reise *et al.*, 2020). Here we consistently take *A. ater* to include *A. ater rufus* (Linnæus, 1758), which has often been considered a separate species (Reise *et al.*, 2020). *Arion vulgaris* and *A. ater* have clearly different genitalia and show differences in mating behaviour (Dreijers *et al.*, 2013; Reise *et al.*, 2020), yet the occurrence of intermediate forms has suggested hybridisation. This has been supported by observations of spermatophore exchange in mixed-species matings (Dreijers *et al.*, 2013), by the detailed description of these intermediate forms (Reise *et al.*, 2020) and by genetic studies (Hatteland *et al.*, 2015). Using microsatellite markers, Zemanova *et al.* (2017) showed a progressive dilution of *A. vulgaris* alleles with alleles of *A. rufus* across three hybrid zones in the Swiss Alps.

General theoretical models of an invasion process accompanied by even rare hybridisation suggest that the invader will be genetically transformed by successively picking up the resident's genes as it spreads (Currat *et al.*, 2008). The argument involves two components. First, at the invasion front the invader is in the minority and thus each colonist is particularly likely to interbreed with the commoner resident species because it rarely encounters conspecifics. Secondly, these introgressed, invasion-front populations will reproduce disproportionately because they suffer least from intraspecific competition (or other forms of density-dependent population regulation) and because their geographic edge position makes them most likely to seed the next step of the invasion. These models apply to neutral alleles. If we suppose that in *A. vulgaris* there is stabilising selection on the form of the genitalia to function well with those of a mating partner, then, despite massive introgression, we need not expect change in the genital characters used to identify the species.

Hagnell *et al.* (2003) further suggested that *A. vulgaris* may have benefitted from accumulating locally adapted genes from the resident species. This process could also favour closely linked genes, but we expect that its major consequence on the introgression of neutral genes would arise through locally adapted genes increasing the fitness of hybrids in the first one or two generations, an effect similar in consequence to increasing the rate of hybrid formation through interbreeding. Conversely, if hybrids are at a selective disadvantage they may, at the extreme, be evolutionary dead ends that fail to lead to further introgression.

Zemanova *et al.*'s (2017) study rejected this latter scenario for *A. vulgaris* and *A. ater*, since a full range of admixture was observed, including individuals that the microsatellites implied were not admixed but which had the mitochondria of the other species. However, the hybrid zones that Zemanova *et al.* (2017) studied may be static arrangements (the species replace each other along an altitudinal gradient), for which the population genetics are different than at a dynamic invasion front (Currat *et al.*, 2008); for instance, asymmetry in the direction of introgression is expected in the invasion scenario, but not necessarily in a static cline. In our study we made use of samples taken over a succession of years from the same localities to examine introgression during a stepwise invasion that was complete within a few

years. To do this, we utilised mostly the same microsatellite markers as Zemanova *et al.* (2017).

Besides addressing the question of whether *A. ater* genes introgress into *A. vulgaris*, we utilise the microsatellites to score the reliability of our identification of *vulgaris–ater* hybrids based on genital characters (Reise *et al.*, 2020). In the same paper we identified three subspecies of *A. ater* present in and around Görlitz. This was based on the similarities with the black form found in northern Europe (*A. ater ater*), a form from the British Isles, NW France and occasionally elsewhere on the Continent (*A. ater rufus*), and one almost absent from the British Isles but widespread in central France, eastwards to Poland and nowadays Scandinavia (*A. ater ruber* (Garsault, 1764)). These subspecies are known to hybridise (Noble, 1992; Hatteland *et al.*, 2015). Although the subspecies have been treated by some authors as distinct species (e.g. Rowson *et al.*, 2014; reviewed in Reise *et al.*, 2020), in this paper *A. ater* is consistently used to refer to all three subspecies, not just to *A. a. ater*. We distinguished the subspecies on the basis of subtle genital differences besides distinct mitochondrial DNA sequences, but in the Görlitz area the agreement between these character sets was not perfect, as expected given the likelihood of hybridisation where the subspecies co-occur. The nuclear microsatellite markers are used here (1) to provide a more stringent test of whether three genetically distinct subspecies occur in this area, (2) to test how reliably the subspecies can be separated by the different anatomies we described, and (3) to quantify how much hybridisation between them has occurred. A further component of our work, not anticipated, is the discovery of genetic varieties within *A. vulgaris*.

MATERIAL

Görlitz is a town of about 60,000 inhabitants on the eastern border of Germany. The former suburb on the eastern side of the river Neiße became Polish in 1945, taking the name Zgorzelec. *Arion vulgaris* was first noted in Görlitz in 1994, which was also the first record for eastern Saxony (Reise *et al.*, 1996). Subsequently we regularly sampled large *Arion* from Görlitz and the surrounding area to monitor the ongoing invasion (Reise *et al.*, 2020). Four sites were sampled particularly intensely and regularly; these included a park mostly of trees and lawn in the middle of the town (called “Stadtpark”) and a landslip on the edge of an opencast coal mine, which succeeded from agricultural land to deciduous woodland over the course of the study (called “Rutschung P”). By 2007 *A. ater* was largely extinct within the town, except for some areas adjacent to woodland. *Arion vulgaris* was not recorded in Zgorzelec until 2001, but soon replaced *A. ater* also there. We ceased regular sampling after 2014, but again collected for this project in 2019. Some samples of *A. ater* collected before the arrival of *A. vulgaris* were also available. In total we have collected over 3,500 animals, nearly all of which have been identified morphologically, in some cases supplemented by standard barcoding sequences from the mitochondrial COI gene (Reise *et al.*, 2020). All specimens are stored in 70% methylated ethanol in the Senckenberg Museum of Natural History Görlitz.

From this collection, we selected several categories of slug for microsatellite analysis.
1) Anatomically typical *A. vulgaris* from Görlitz. We attempted to span a wide range of dates and locations and to include collections at different dates from similar locations.
2) Reference individuals of *A. ater* from the local area that were anatomically representative of each subspecies. Because *A. a. ater* is typical of wild areas and absent from synanthropic sites, the samples of this subspecies include individuals from tens of kilometres away from Görlitz. These reference individuals were to test whether microsatellites picked out the subspecies already recognised by anatomy and COI sequence (Reise *et al.*, 2020); thereafter we could use the microsatellites to infer the ancestry of animals whose anatomy was less

typical, perhaps because of hybridisation between subspecies. In selecting reference individuals we excluded candidates for which an existing COI sequence conflicted with the anatomy-based identification, although with others COI was sequenced subsequently, so in two reference individuals there was a conflict.

3) Additional *A. ater* collected from Görlitz, preferentially before the arrival of *A. vulgaris* at each site, even if the subspecific identification was uncertain (e.g. if juvenile). *Arion vulgaris* might have already accumulated many *A. ater* alleles as it spread across Europe. By widely sampling the local *A. ater*, we hoped to recognise whether its alleles in particular became incorporated into the *A. vulgaris* population over the course of the invasion.

4) A set of 25 individuals that anatomically appeared to be hybrids between *A. ater* and *A. vulgaris*. This is an over-representation of their proportion in mixed populations (c. 4%: Reise *et al.*, 2020). They had been classified on a scale of similarity to each species (Reise *et al.*, 2020; see Methods). We wished to learn whether these classifications reflected the ancestry.

In all we scored the microsatellites of 218 individuals.

METHODS

We extracted DNA from foot muscle of slugs preserved in 70% alcohol, following the method of Winnepenninckx *et al.* (1993). For some slugs the barcoding region of the COI mitochondrial gene was sequenced using primers and protocols described in Hutchinson *et al.* (2020).

We used 16 microsatellite loci (ALU_02_3, ALU_11_2, ALU_12_2, ALU_13_2, ALU_30_2, ALU_34_2, ALU_37_2, ALU_60_2, ALU_76_2, ALU_79_2, ALU_86_2, ALU_88_2, ALU_92_2, ALU_94_2, ALU_96_2, ALU_102_2); Zemanova *et al.* (2015) had developed the primers for *A. vulgaris*. We assessed but did not use their 17th locus (ALU_06_4) because the peaks were indistinct. Zemanova *et al.* (2016, 2017) did use ALU_06_4, but not ALU_94_2 or ALU_102_2.

PCRs were performed in 12.5 µl volumes as singleplex PCR. For each PCR we used 1.25 µl of 10× PCR Key buffer (VWR International), 1 unit of VWR *Taq* DNA polymerase, 2.5 nmol of each dNTP (peqlab, Erlangen, Germany), 25 nmol of $MgCl_2$, 0.5 pmol of forward primer with universal M13 tail (18 bp), 2 pmol of reverse primer, and 1 µl of template DNA. The fluorescent labelling of the resulting products was performed during the amplification using 2 pmol of dye-labelled universal M13 primer (following Schülke, 2000). The PCR thermal regime consisted of: 1 cycle of 5 min at 95 °C; 36 cycles of 1 min at 95 °C, 1 min at 57 °C, and 1 min at 72 °C; and a final cycle of 10 min at 72 °C. The resulting products were sent undiluted to Laborzentrum BIK-F for fragment analysis using GeneScan 600 LIZ Size Standard (Applied Biosystems) to determine the lengths of the PCR products.

Fragments were scored manually with PEAK SCANNER v. 1.0 (Applied Biosystems). At locus ALU_102_2 frequently no band appeared (highly associated with *A. ater* anatomies), which we interpreted as a homozygous recessive condition. At the other loci, we used the missing-value code for 1.8% of entries. The program STRUCTURE v. 2.3.4 (Pritchard *et al.*, 2000; Falush *et al.*, 2003, 2007) was used for further analysis. STRUCTURE is a clustering algorithm that identifies co-occurrences of alleles across the 16 microsatellite loci. The number of such clusters or populations (*K*) fitted to the data is specified by the user. Then STRUCTURE estimates the ancestry of each individual as a mixture from these populations. Each STRUCTURE run involved 2×10^6 iterations, half of them as burn-in. All models allowed admixture and correlated allele frequencies. Analyses examining different questions differed in which sets of individuals we included and whether we used some as a learning set, as explained in the Results section. Otherwise parameter values followed the defaults.

Heuristically, we consider slugs with estimated admixture greater than 1/16 as genetically intermediate, without expecting that this categorisation will necessarily be correct for every individual. This threshold of 0.0625 compares with 0.05 and 0.15 used by Zemanova *et al.* (2017).

To check for the robustness of the results from STRUCTURE, we repeated the main analyses using the R package ADEGENET v. 2.1.3 (Jombart, 2008; Jombart & Ahmed, 2011; R Core Team, 2021). Its functions find.cluster and dapc (Discriminant Analysis of Principle Components) use a different distance-based principle for clustering than does the model-based STRUCTURE. It was necessary to make the assumption of codominant alleles, even though one of our microsatellite markers has a recessive allele. ADEGENET does not estimate admixture, but the discriminant functions are used to estimate probabilities that an individual belongs to each cluster.

Details of the COI categorisation and anatomical scoring are provided in Reise *et al.* (2020). Based on five different aspects of the genital anatomy, each individual was placed into one of five categories: pure *A. vulgaris* or pure *A. ater*, intermediate but closer to one species or the other, or not closer to either species. For pure *A. ater* we also tried to identify the subspecies, but some individuals were scored as uncertain or as inter-subspecific hybrids. The variation is continuous, and different parts of the genitalia do not vary with perfect concordance, so the scoring is partly subjective, but the experience of examining thousands of individuals repeatedly over several years led to consistency. Anatomy was scored prior to learning the genetics. The genetic background of the anatomical characters is unknown but the phenotypic variation seems compatible with multiple genes each having small effects.

Maps were produced using QGIS v. 3.10.14 (QGIS.org, 2021).

DATA AVAILABILITY

The following three files are archived in the Zenodo repository at https://doi.org/10.5281/zenodo.4748540. Microsat_data.csv contains the raw microsatellite data (allele lengths). Other_data.csv contains anatomical identification, COI haplotype, locality, date of collection, as well as details of which individuals were used for each analysis and the estimated ancestries output by each STRUCTURE analysis. Data_explanations.txt explains these two files. Novel COI haplotypes are at Genbank MW659463–MW659465.

RESULTS

INTROGRESSION BETWEEN SPECIES

In our first STRUCTURE analysis we excluded all slugs scored as anatomically intermediate between the species or that were too juvenile for a confident identification. The program was specified to fit a model involving two populations ($K = 2$).

For every individual, STRUCTURE's estimate of the predominant ancestry matched our prior anatomical identification (Fig. 1). Amongst the 71 morphological *A. ater* only 2 scored greater than 1/16th admixture (0.21 and 0.08 admixed), and amongst the 120 morphological *A. vulgaris* only 7 (0.30 and 0.14, with the other 5 < 1/8 admixed). Some of the cases of apparent mild admixture are liable to be generated by noise (genetic variation and errors from the PCR or in the scoring of alleles) rather than really representing introgression. Indeed, the 90% probability intervals for all but one slug in this analysis include zero admixture.

One prediction of Currat et al. (2008) is that there will be more introgression into the invader than into the resident. Our data in Figure 1 cannot meaningfully test this because (1)

many of the *A. ater* samples predate the arrival of *A. vulgaris* at the site of collection and (2) *A. ater* went extinct locally within a very few years after *A. vulgaris* arrived.

A second prediction is that, if hybridisation occurred in each generation as the invasion progressed stepwise through the town, *A. vulgaris* specimens collected later should show higher levels of admixture with local *A. ater* alleles. Figure 1 does not immediately suggest this, although STRUCTURE is not an ideal tool to detect gradual progressive change (Pritchard *et al.*, 2010). Nevertheless we performed a rank correlation between the admixture score and year of collection, using only the 120 anatomically typical *A. vulgaris*. The correlation is weak and negative (Kendall's $\tau = -0.15$); so there is no indication that admixture increased over the period.

The *A. ater* showing 0.21 admixture with *A. vulgaris* was collected in 1993. This suggests that *A. vulgaris* was present in Görlitz already in 1991 or 1992, whereas the earliest *A. vulgaris* identified anatomically were collected in 1994.

ANATOMICAL INTERMEDIATES BETWEEN *A. ATER* AND *A. VULGARIS*

In our next analysis, we added 25 animals scored as having genitalia intermediate between the species, to test whether their anatomy-based score agreed with the level of genetic admixture indicated by STRUCTURE. Again we specified $K = 2$ but this time we used 69 *A. ater* and 113 *A. vulgaris* from the first analysis (i.e. those with admixture <1/16) as learning samples, whose identity was included in the model specification (parameters USEPOPINFO = 1 and PFROMPOPFLAGONLY = 1). Thus the anatomically intermediate slugs did not contribute to calculating the allele frequencies associated with each species.

In 17 of the 25 anatomically intermediate slugs (i.e. 68%), the estimated admixture was > 1/16, much higher than the 6% proportion amongst the anatomically identified *A. vulgaris* in the earlier analysis. Furthermore, STRUCTURE's estimates of ancestry correlated quite well with our anatomical classification of these intermediates into three categories of resemblance to the parent species ($\tau = 0.41$, $p = 0.01$). Nevertheless, often the anatomy and microsatellites disagree (Fig. 2). Of the 13 slugs with intermediate genitalia more similar to *A. ater*, 3 were classified by STRUCTURE as close to pure *A. ater* (i.e. <1/16 admixture), 2 as close to pure *A. vulgaris*, with 6 of the remaining 8 closer to *A. ater* than *A. vulgaris*. Of the 7 slugs with genitalia equally similar to the two species, 2 were genetically close to pure *A. vulgaris* and the other 5 also closer to *A. vulgaris* than *A. ater* or nearly so. Of the 5 slugs with intermediate genitalia more similar to *A. vulgaris*, 1 was close to pure *A. vulgaris* and the other 4 closer to *A. vulgaris* than *A. ater*; slugs with anatomies classified in this category showed little difference in their estimated ancestries to those in the preceding category.

We know the COI sequences from 19 of these 25 anatomically intermediate slugs (Fig. 2). The majority (13/19) had mitochondrial haplotypes associated with *A. vulgaris* (reported already by Reise *et al.* (2020) for an overlapping sample). However, one anatomical intermediate (equally similar to both parent species) whose microsatellites implied a nearly pure *A. vulgaris* genome (admixture = 0.033) had a COI haplotype of *A. ater*. So the anatomy might sometimes reflect introgression undetected by our microsatellite markers.

INTRASPECIFIC VARIATION: *ARION VULGARIS*

We expected that when we specified more populations than two STRUCTURE would distinguish the three subspecies of *A. ater*, which we had earlier separated on the basis of both anatomy and COI sequence (Reise *et al.*, 2020). For this analysis we excluded only the slugs identified anatomically as interspecific hybrids. Unexpectedly, STRUCTURE continued to classify all *A. ater* as one cluster, instead splitting the *A. vulgaris* into further clusters as we

increased K up to $K = 9$. For $K = 10$, *A. ater* was split between *A. a. rufus* and the other two subspecies. Using the LOCPRIOR option in STRUCTURE to guide the placement of clusters within *A. ater* did not help. So we analysed each species separately.

To analyse *A. vulgaris*, we initially excluded all individuals for which the earlier analysis had suggested >1/16 admixture with *A. ater*. We then compared the fit to the data between versions specifying different numbers of populations (K). As a measure of fit, STRUCTURE reports $\ln[\Pr(X|K)]$, the logarithm of the estimated probability of the data given a hypothesised value of K. This increases considerably from $K = 1$ to $K = 2$, somewhat from $K = 2$ to $K = 3$, and then roughly levels off (Fig. 3A). With $K = 4$, independent runs produced inconsistent patterns of clustering. Evanno *et al.*'s (2005) heuristic chose $K = 2$, but we proceeded with $K = 3$ because the second and third populations exhibited meaningfully different distributions.

We will term each cluster within *A. vulgaris* a "race". Pooling over all sites, it appears that one race predominated initially (marked red in Fig. 4), then was replaced by the other two (yellow and blue), with admixed individuals becoming commoner. However, date and location are unavoidably confounded (because *A. vulgaris* arrived later at some localities), and much is revealed by considering areas within the city separately. Figure 5 shows how strongly the proportion of each race varied spatially, and how we accordingly categorised localities into six areas. To maximise the sample sizes we reanalysed the data to include all 218 individuals (i.e. including also *A. ater* and hybrids) and specified $K = 4$ (to separate *A. ater* + the 3 races of *A. vulgaris*). In the results presented in Figure 4 we omitted animals scored as being >50% *A. ater*, but considered the relative proportions of the three *A. vulgaris* races in animals less admixed with *A. ater* than this.

The two areas first colonised by *A. vulgaris*, Rauschwalde and Rutschung P, received the red race. Later, hybrids with the yellow and blue races also appeared at these sites, but this was 17 years later in the case of Rutschung P, perhaps because this is a rural area over 4 km from built-up areas of the town. In two other areas of the town pure red-race animals were the first to be found, but thereafter this race was found only admixed with the others. Although this is suggestive, it need not indicate a replacement because these two initial sites were a little way from the others and were not resampled. At most sites the yellow race predominated, but in Stadtpark, the predominant race was the blue. (That difference was our reason for not lumping these races.) Hybrids between all forms occurred. The tendency is probably for individuals scored as mixtures between the *A. vulgaris* races to be commoner later (this is clear in Rauschwalde and Rutschung P), but the first collections in Südstadt (already in 1998) have been scored as admixtures between yellow and blue. In contrast to the species' rapid spread across the city, the spatial mixing of its races is still incomplete.

The relationship between the microsatellite races of *A. vulgaris* and partial-COI sequences is consistent but slight. The 10 slugs with a red race that were sequenced had only two COI haplotypes, one point mutation apart (haplotypes 2 and 14 in fig. 4 of Reise *et al.*, 2020). None of the six other *A. vulgaris* sequenced had either of these two COI haplotypes, but all, with one exception, were only one or two bases different from one of them (haplotypes 4 and 7); this is the level of variation that might have arisen after arrival in Görlitz. The exceptional slug had a very different COI sequence (haplotype 8), 10 bases different from any others. Its microsatellite alleles do not suggest a further distinct race (all but one allele were found in multiple other individuals): STRUCTURE scored its ancestry as 77% yellow race and 23% blue.

INTRASPECIFIC VARIATION: *ARION ATER*

To analyse the intraspecific variation of *A. ater*, we considered only animals for which the earlier analysis had implied <1/16 admixture with *A. vulgaris*. In these 73 slugs, one locus was monomorphic (ALU_102_2: only the recessive null allele was present) and so was

excluded from the analysis. Based on anatomy and COI sequences (Reise *et al.*, 2020), we expected the appropriate model to be $K = 3$ corresponding to the three subspecies; this was indeed compatible with the diminishing changes of $\ln[\Pr(X|K)]$ as K was increased over the range 1 to 6 (Fig. 3B), although the Evanno *et al.* (2005) heuristic would have chosen $K = 2$.

To compare the genetic results with our anatomy-based identifications, we considered just the 47 of the 73 individuals for which we had made a firm prior identification (the rest had been scored with a "?" or as possible hybrids between *A. ater* subspecies). As shown in Figure 6, of the 15 slugs scored anatomically as *A. a. ater*, STRUCTURE scored 7 as this subspecies with <1/16 admixture and for another 6 this subspecies was the main component; the remaining 2 were predominantly *A. a. rufus*. Of the 19 slugs scored anatomically as *A. a. ruber*, STRUCTURE scored 10 as this subspecies with <1/16 admixture, for 8 this subspecies was the main component, or in one case nearly so, and 1 was a *rufus–ater* hybrid. Of the 13 slugs scored anatomically as *A. a. rufus*, STRUCTURE scored 8 as this subspecies with <1/16 admixture, and for the remaining 5 this subspecies was the main component. To summarise, only 3 out of 47 slugs were completely misidentified anatomically, but we had often not suspected from the anatomy that an individual was of mixed ancestry.

Note that the agreement of anatomy and microsatellites may be overestimated because we had avoided examining the microsatellites of some slugs for which anatomy and COI sequence disagreed. But this bias is not sufficient to invalidate a claim of accuracy. Suppose that we had additionally assessed microsatellites for all of the remaining 12 *A. ater* anatomically identified to subspecies for which we knew the COI haplotype. Then, even if all 8 of these in which there was a conflict between COI and anatomy also showed a clear conflict between the microsatellites and anatomy, still the proportion of individuals completely misidentified by anatomy would not exceed 0.22.

Of the 73 *A. ater* for which we have microsatellite data, 47 had yielded a COI sequence. All but three sequences matched one of the subspecies that STRUCTURE indicated was an appreciable part of the ancestry of that individual (i.e. >1/16). The three exceptions were cases of *A. a. ater* COI sequences persisting in individuals that STRUCTURE scored as almost pure *A. a. ruber* (one of these appears in Fig. 6; its anatomy also clearly indicated *A. a. ruber*).

Evidently, slugs that are a mixture of the subspecies occurred frequently (Fig. 7C). Nevertheless, in more than half of the 73 *A. ater* the estimated ancestry was predominantly (>15/16) one subspecies: 8 *A. a. ater*, 19 *A. a. ruber* and 12 *A. a. rufus*. This proportion is not representative, since part of our selection of slugs to analyse genetically was biased towards what we considered anatomically unambiguous examples, but it shows that such individuals commonly persisted. One reason might be that habitat or other geographic differentiation kept the subspecies apart. Figure 7 indeed shows that pure *A. a. ater* are restricted to sites away from the town (confirming what we believed from anatomy and external coloration). But there was sometimes admixture with the other two subspecies even at such sites. Conversely, in the built-up areas of Görlitz and Zgorzelec, *A. a. ater* genes persisted in animals of mixed ancestry.

Figure 7B further shows that within Görlitz and Zgorzelec *A. a. ruber* and *A. a. rufus* are not evenly mixed. In particular, none of 11 *A. ater* from Poland showed appreciable *A. a. rufus* ancestry. The absence holds if we add five hybrids with *A. vulgaris* from Poland or if we consider COI sequences. Of 15 Polish *A. ater* COI sequences from this region (11 from animals already considered for their microsatellites) none were *A. a. rufus*, compared with 15 out of 40 on the German side (significantly different proportions, $p = 0.03$, Fisher's exact test). (This corrects table 1 in Reise *et al.* (2020) which showed one Polish animal with an *A. a. rufus* sequence: the tissue sample had been mislabelled.)

GENETIC DISTANCES

We were interested to compare how much the three races of *A. vulgaris* differed from each other in comparison to the variation between the three subspecies of *A. ater*. To ensure that STRUCTURE recognised both types of cluster in a single analysis it was necessary to specify 6 sets of individuals as learning samples. We chose examples that earlier analyses implied were <1/16 admixed, so sample size was small for some groups. Table 1 shows STRUCTURE's measure of population divergence ("net nucleotide distance"; see Pritchard *et al.* 2010), together with a measure of diversity within each population (expected heterozygosity).

Genetic variation within each race of *A. vulgaris* tends to be greater than within each subspecies of *A. ater*, but the ranges overlap. The divergence between the races of *A. vulgaris* tends to be less (0.1–0.27) than between the subspecies of *A. ater* (0.21–0.34), but again the ranges overlap. The *A. vulgaris* races are consistently more similar to each other than to any of the *A. ater* subspecies. But, disconcertingly, *A. a. rufus* comes out as more similar to two of the *A. vulgaris* races than to one member of its own species, *A. a. ater* (0.29 and 0.31 vs 0.34). One might interpret this as evidence of past interspecific introgression, but we cannot judge the statistical significance of this variation, and the large *ater–rufus* distance may well be anomalous, consequent on the small sample sizes. If we summarise the genetic divergence values by constructing a two-dimensional map (Fig. 8A), the races do look to cluster together by species.

THE REANALYSIS WITH ADEGENET

Reanalysing the same data using ADEGENET has generally confirmed that our results with STRUCTURE were robust. For our first analysis, including all slugs except those identified anatomically as interspecific intermediates, the allocation to species agreed 100% with that of STRUCTURE. We considered the discriminant function separating the two species as a measure of admixture. Amongst the *A. vulgaris*, this score's rank correlation with year was again opposite to that predicted supposing a progressive increase in introgression. When the anatomical intermediates were assessed with the same discriminant function (i.e. fitted on the non-intermediates), their scores very closely correlated with the admixture scores from STRUCTURE ($r = 0.98$) so the correlation with anatomy is again significant ($\tau = 0.38$, $p = 0.02$). However, ADEGENET also calculates from the discriminant function a probability of belonging to each species, which has been recommended as a proxy for admixture (Jombart & Collins, 2017); only 7 out of the 25 anatomical intermediates were given a non-trivial probability of belonging to both species, and none were close to 50:50 (closest was 0.09:0.91).

Recall that when analysing *A. vulgaris* and *A. ater* together, STRUCTURE kept dividing up only *A. vulgaris* further as we increased *K*, which made us analyse the two species separately. This was not necessary with our alternative analysis using ADEGENET. Splitting into $K = 6$ groups was optimal according to the Bayesian information criterion used by the function find.clusters, and in their compositions these six groups closely matched the three races within each species that STRUCTURE had found. The exceptions were that some of STRUCTURE's yellow race of *A. vulgaris* were classified with the blue race by ADEGENET. Figure 8B plots these 6 clusters on the first two discriminant axes, where the individual dots represent single slugs (excluding only the anatomical between-species intermediates, as these were over-represented in our sample). The overlaps in the clouds of different coloured points disappear when including further discriminant functions (necessarily, because the colours are determined from this analysis). Reassuringly, the relative distances apart of the six races agree quite well with the output from the STRUCTURE analysis (Fig. 8A).

DISCUSSION

RELIABILITY OF IDENTIFICATION

The microsatellite markers developed to study introgression between Swiss populations of *A. ater* and *A. vulgaris* worked well also for our populations 700 km away. The results correlated with evidence from anatomy and COI sequences. Moreover, in a novel application, the same microsatellites differentiated the subspecies of *A. ater*. By providing a quantitative measure broadly representative of the nuclear genome, microsatellites are a tool of choice in providing an identification when the taxa hybridise.

Although distinguishing adult *A. ater* and *A. vulgaris* anatomically is quick and straightforward, it requires more experience to identify some hybrids between them (Reise *et al.*, 2020). STRUCTURE's analysis of the microsatellites indicated that anatomy does provide a quantitative indication of a hybrid's ancestry but is not fully reliable. Although ADEGENET produced virtually the same ranking of intermediacy as STRUCTURE, it seemed too prone to classify intermediates instead as a single species without uncertainty. The anatomical identification of the subspecies of *A. ater* is also demanding. Although our prior anatomical identifications of the subspecies were mostly correct (Fig. 6), prior knowledge of COI sequences had somewhat biased the specimen selection in favour of agreement, despite which we underestimated how many individuals were hybrids, a few identifications were completely wrong, and we were not confident of the identification of other individuals. At the level of subspecies, probably it is best to consider anatomy as sufficiently indicative only to identify local populations, based on examining several individuals. Realise, however, that Görlitz presented an unusually thorny identification problem in having three subspecies coexisting and hybridising; elsewhere, *A. ater* populations usually consist of only a single subspecies.

Mitochondrial COI sequences also are an imperfect identification tool when there has been a history of introgression, since they give information only on maternal ancestry and may represent an isolated occurrence of a rarely occurring hybridisation event many generations earlier. We have highlighted some rare cases in which COI and microsatellites would disagree about the identification of subspecies (3 cases) or species (1 case); Zemanova *et al.* (2017) found 5 similar examples of the latter.

INTROGRESSION BETWEEN *A. VULGARIS* AND *A. ATER*

Although the microsatellites confirmed many cases of hybridisation between *A. ater* and *A. vulgaris* amongst the sample anatomically identified as hybrids, only 4% of the population had such anatomies even at times when both species coexisted (Reise *et al.*, 2020). Amongst those *A. vulgaris* whose anatomy did not suggest that they were interspecific hybrids, STRUCTURE's analysis of the microsatellites implied that there was not much introgression (only 7/120 scored >1/16 admixture). Two slugs still showed >1/16 admixture in 2019, 12 years after *A. ater* had largely gone extinct in Görlitz, but these late-occurring admixed individuals were from the edge of the built-up area, beyond which some *A. ater* may have persisted in natural habitat. There was another admixed slug in 2016, from Stadtpark (in the middle of the town, although partly mature woodland), but it is just one individual, whereas our expectation was that admixture would be a general phenomenon within the population. The models of Currat *et al.* (2008) predict the process of introgression to occur most when the invader first arrives and is in the minority. Because the invasion progressed over several years in Görlitz, as the invader spread to new areas each year, we predicted a progressive increase in admixture with local *A. ater*, higher in regions invaded later. In contrast we found no evidence that the current *A. vulgaris* population has accumulated any more of the local *A. ater* alleles than the original colonists might already have had. Certainly our results do not suggest

the predicted "massive introgression" (Currat *et al.*, 2008). However, our study should not be considered an absolute rejection of the hypothesis, since 16 microsatellite loci would be insufficient to detect low levels of introgression, yet a little introgression might be nevertheless sufficient after multiple steps of the invasion process to influence the invader's genome considerably. Currat *et al.* (2008) presented quantitative predictions only for many more generations over larger spatial scales than we studied.

The predictions of introgression apply also to mitochondrial DNA. Currat *et al.* (2008) confirmed that occurrences of organelle replacement reported in the literature were predominantly in the predicted direction of resident to invader. Yet across Europe *A. vulgaris* appears to have retained mitochondrial sequences distinct from those of *A. ater* (e.g. Zając *et al.*, 2019), echoing the pattern we found for microsatellites. (Exceptionally, Zemanova *et al.* (2017) noted that two individuals from a hybrid zone that were judged as pure *A. vulgaris* on the basis of microsatellites and morphology had *A. ater* mitochondria.) *Arion ater* and *A. vulgaris* mitochondrial DNA has diverged rather much (Doğan *et al.*, 2020), so the lack of mitochondrial introgression is plausibly explicable by selection for host–organelle compatibility (Hill, 2019).

Why has more introgression of nuclear genes not occurred? Potentially one factor is that *A. ater* disappeared so soon after *A. vulgaris* appeared, limiting the opportunity for hybridisation. This is a situation included in Currat *et al.*'s (2008) modelling, yet the predictions still were of introgression if there were modest levels of interbreeding. Soon after the arrival of *A. vulgaris*, when it is in the minority, the colonists are particularly likely to interbreed because of the low probability of encountering conspecifics. Indeed, during the years of species coexistence, we commonly observed mixed-species couples mating in the field (E. Dreijers personal communication) and the microsatellite data confirm that they sometimes did produce viable hybrids. Laboratory observations showed that mixed-species couples exchange spermatophores at a lower rate than same-species couples (Dreijers *et al.*, 2013), but still at 37% of the rate in *A. vulgaris* couples, a reduction which Currat *et al.* (2008) predicted is insufficient to stop introgression. However, *A. vulgaris* can self-fertilise (Hagnell *et al.*, 2006) and also seems to mate frequently, so cryptic female choice for same-species sperm might have lowered this rate of hybridisation further.

Another possibility is that, although hybrids were produced, they were in some way unfit and contributed few offspring to future generations (cf. Wiwegweaw *et al.*, 2009). Various reasons for the competitive superiority of *A. vulgaris* over *A. rufus* have been investigated (e.g. Ryser *et al.*, 2011; Kappes *et al.*, 2012), but hybrids have not been included in such comparisons. However, it cannot be that the hybrids are completely sterile, since both we and Zemanova *et al.* (2017) found a broad range of degrees of admixture.

In Currat *et al.*'s (2008) model, because hybrids tend to arise particularly where population density of the invader is still low, low levels of competition with others of the invading species enhance their fitness relative to the invaders in denser, less-introgressed populations further back from the invasion front. Is this aspect realistic? The extraordinarily high densities observed in some *A. vulgaris* populations (Kozłowski, 2007) can be interpreted for or against the importance of intraspecific competition, but necessarily some form of density-dependent population regulation must operate sometimes. Indeed there is evidence of such competitive interactions in terrestrial gastropods (e.g. Baur, 1988; Pearce, 1997), including an effect on mortality in *A. ater* (Hamilton & Wellington, 1981). Another aspect is that, even though the hybrid offspring of new colonists may have had few *A. vulgaris* to compete with, they still faced the resident *A. ater*, which from its similar appearance one might naively have supposed to be an equivalent competitor. But the disappearance of *A. ater* implies that this species is actually a weaker competitor, and it is also often remarked that it did not attain the 'plague' densities subsequently observed in *A. vulgaris* (e.g. von Proschwitz, 1996).

A different sort of mismatch with the assumptions of Currat *et al.*'s (2008) models would be if the newest colonies of *A. vulgaris*, predicted to be the most introgressed populations, are not especially likely to be the ones seeding further colonies. Suppose, for instance, that a garden centre in another town were the direct source of most new colonies. However, that scenario does not fit well with the marked spatial structuring revealed by the races of *A. vulgaris* (Fig. 5) and nor can it apply to a wild area like Rutschung P.

THE GENETIC VARIETIES WITHIN *A. VULGARIS*

The three genetic clusters within *A. vulgaris* took us by surprise, although Zemanova *et al.* (2016, 2017), using the same microsatellite markers, had found genetic differences between more distant localities. The genetic distances between our clusters are comparable to those between the subspecies of *A. ater*, but it is important to realise that the distances in Table 1 are based on the proportion of shared alleles between individuals, so are sensitive to quantitative shifts in polymorphisms rather than necessarily indicating fixed genetic substitutions. The blue and yellow races are most similar to each other, and the earliest occurrences were scored as admixed individuals, so possibly these races have diverged through founder effects while they spread through Görlitz. But from the pattern of geographical and temporal occurrences, and from its greater genetic differentiation, it seems more likely that the red race has colonised Görlitz separately from a different source population. It is possible that the origin of the genetic differentiation is introgression with different *A. ater* populations as the species has expanded across Europe. Alternatives are that the microsatellite differences have arisen through mutation and drift as the species spread via different routes across Europe, or that the differences have persisted from more ancient geographic differentiation within the native range. Regardless of the source of the differentiation, the increase in genetic diversity when the races meet and hybridise may have fitness consequences (Rius & Darling, 2014) and may counteract the expected loss of intrapopulation genetic diversity through successive founder effects (Swaegers *et al.*, 2013).

In studies in Poland (Soroka & Kałuski, 2011), mitochondrial COI sequences also suggested heterogeneous origins of *A. vulgaris* at some sites. That applies to Görlitz, since one rare *A. vulgaris* haplotype (found twice) was 10 bases different from others (Reise *et al.*, 2020: fig. 4). Yet Görlitz COI sequences are otherwise so similar that we would have overlooked the further population divisions indicated by the microsatellite results. We have not noticed any anatomical differences associated with the different *A. vulgaris* races.

It seems paradoxical that *A. vulgaris* spread so rapidly across Görlitz, yet that subsequently this mobility has not caused the three races to mix fully spatially. The paradox has similarities with invasive species that have rapidly spread worldwide yet nevertheless retain differentiated geographical genetic structure ("isolation by distance") within their ancient ranges (e.g. the slug *Deroceras invadens*: Hutchinson *et al.*, 2020). The reason must be intraspecific competition or other sources of density-dependent population regulation; a single colonist is much more likely to survive and leave offspring when rare than when in the presence of a high density of conspecifics. The surprise is that other *A. vulgaris* individuals exert so much more competition than the superficially similar *A. ater* that were already occupying the "virgin territory". One explanation would be that the species have different enough niches not to compete much, but the subsequent extinction of *A. ater* suggests rather that it was a weaker competitor.

The slow population mixing spatially echoes the findings of a number of studies on other land molluscs (e.g. Pfenninger *et al.*, 1996; Arnaud *et al.*, 2003). Most reminiscent are the area effects in *Cepaea* snails, which are also now thought to reflect the "frozen" outcome of an invasion process: large areas are occupied by a different set of colour morphs than those in adjacent areas because they were colonised by different founders when the habitat became

suitable (Cameron & Dillon, 1984). As in our situation, the surprise is that migration has not broken down the geographical pattern, in the case of *Cepaea* even after decades.

THE SUBSPECIES OF *ARION ATER*

The microsatellites independently confirmed earlier proposals about the subspecific differentiation of *A. ater* based on mitochondrial sequences and anatomy (Reise *et al.*, 2020). Although the three subspecies *ater*, *rufus*, and *ruber* have different centres of distribution, with *A. a. rufus* mostly in the British Isles and north-west France (judging from mitochondrial sequences), they all occurred commonly around Görlitz. The microsatellites were particularly useful in establishing the frequent admixture between these subspecies, including at sites that seem undisturbed. This mixing supports our policy of considering these taxa subspecies rather than species. However, apparently non-admixed individuals were still to be found commonly. As we have seen with the three races of *A. vulgaris*, population mixing and introgression might take some time if the subspecies are strong competitors.

The absence of *A. a. rufus* from the Polish side of the river Neiße is intriguing. It suggests that this subspecies arrived in the area only after 1945, when the new international border and the wholesale replacement of the human population living east of the river had disrupted the close social and logistical connections across the river that hitherto existed. Despite restrictions progressively loosening since 1989, the disjunction caused by the border was still seen in the seven-year delay in *A. vulgaris* crossing from Görlitz to Zgorzelec, and a similar interruption to the spread of the slug *Deroceras invadens* (Hutchinson & Reise, 2015). Further evidence for a late arrival of *A. a. rufus* is the paucity of *rufus–ater* hybrids compared with those between the other two subspecies (Fig. 7C); we hypothesise that *A. a. rufus* arrived in the town, where it encountered mostly *A. a. ruber*. We collected *A. a. rufus* already in 1994, so it seems likely to have arrived during the lifetime of East Germany, which is surprising given the isolation from the West, but we know of another occurrence on Rügen and Hiddensee, also formerly within East Germany. The only other German record is from Paderborn (Genbank KJ843096.1), a major British Army base. If *A. a. rufus* indeed arrived in Görlitz only after 1945, the extent of its occurrences implies that it was outcompeting *A. a. ruber*.

We have also hypothesised that *A. a ruber* may be an earlier introduction (Reise *et al.*, 2020). In the eastern half of Poland, *A. a. ruber* is known only as a recent introduction in synanthropic habitats (Wiktor, 2004; Soroka *et al.*, 2009) and *A. a. ruber* is also a recent invader in Sweden (von Proschwitz, 1993). Near Berlin (250 km north of Görlitz), even in 1953 Frömming considered it worthwhile to publish about a population of orange *A. ater* (likely to be *A. a. rube*r or *A. a. rufus*), because the typical coloration at that time was black (likely in this region to be *A. a. ater*). In contrast, already in the mid-19th century both reddish and black populations were recorded in our area (Lusatia), but a difference with today is that both colours were reported as occurring in gardens and in woods, although never together (Scholz, 1853; see also Merkel, 1894).

Today *A. ater* is largely extinct in synanthropic habitats in this region, so we may never learn much more about these occurrences of *A. a. ruber* and *A. a. rufus*. We were only just in time to gather the present evidence. *Arion a. ater* persists in woods and in the uplands, but even there we encounter *A. vulgaris* increasingly encroaching (Reise *et al.*, 2020). Further west in Germany *A. a. ater* appears to be absent (except in the north) and it is *A. a. ruber* that still survives in the woods after *A. vulgaris* had driven it from synanthropic sites (Kappes & Kobialka, 2009; Allgaier, 2014). In the British Isles *A. vulgaris* has been present for decades and occurs widely but has not become dominant like in Central Europe (Rowson *et al.*, 2014). One might have hypothesised that its relative lack of success in Britain is because it is competing there against *A. a. rufus* rather than *A. a. ruber*. Our discovery that it drove extinct

the population of *A. a. rufus* in Görlitz makes that a less plausible explanation. If instead it is climatic differences in the British Isles that have protected *A. a. rufus* there, climate change may mean that another takeover is imminent.

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Table 1. Genetic distances within and between *Arion ater* subspecies and *A. vulgaris* races, based on a STRUCTURE analysis of 16 microsatellite loci.

subspecies/ race	*N*	Expected hetero-zygosity	Net nucleotide distance					
			A. ater subspecies			*A. vulgaris* races		
			ater	*ruber*	*rufus*	red	yellow	blue
ater	8	0.24	—	0.26	0.34	0.53	0.45	0.43
ruber	19	0.36	0.26	—	0.21	0.44	0.37	0.33
rufus	12	0.49	0.34	0.21	—	0.44	0.31	0.29
red	46	0.38	0.53	0.44	0.44	—	0.20	0.27
yellow	26	0.71	0.45	0.37	0.31	0.20	—	0.10
blue	12	0.70	0.43	0.33	0.29	0.27	0.10	—

N is the number of individuals in the learning samples, selected to exclude slugs appearing to be admixed. The measures of genetic distance are explained in Pritchard *et al.* (2010).

FIGURES

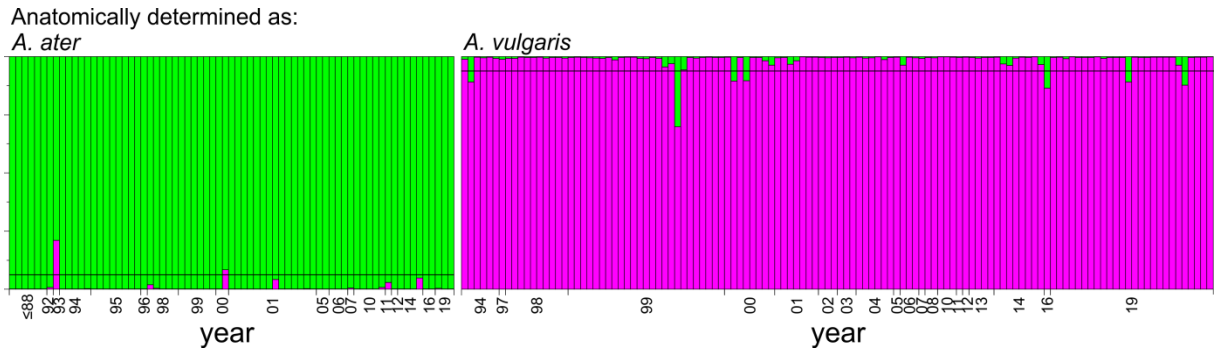

Figure 1. STRUCTURE analysis (K = 2) of slugs identified anatomically as pure *Arion ater* or *A. vulgaris*. Each column is an individual; the estimated ancestry is indicated by the relative lengths of the green (*A. ater*) and magenta (*A. vulgaris*) bars. Individuals are grouped by species and then ordered by year of collection ("≤88" means in or before 1988, etc.). The reference lines indicate admixture of 1/16.

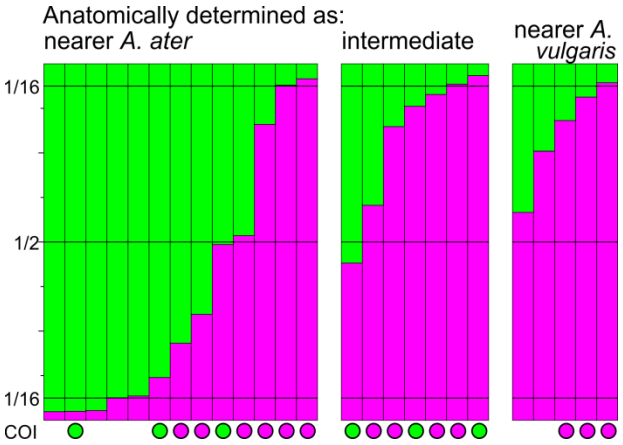

Figure 2. STRUCTURE analysis of slugs identified as anatomically intermediate between the species, categorised into those more similar to *A. ater*, those more similar to *A. vulgaris*, and those equally similar (Reise *et al.*, 2020). Each column is one individual. The estimated ancestry is indicated by the relative lengths of the green (*A. ater*) and magenta (*A. vulgaris*) bars. The genetic signature of each species was derived from an independent learning sample of minimally admixed individuals. The dots below show the species identity of the COI sequence (not all were sequenced).

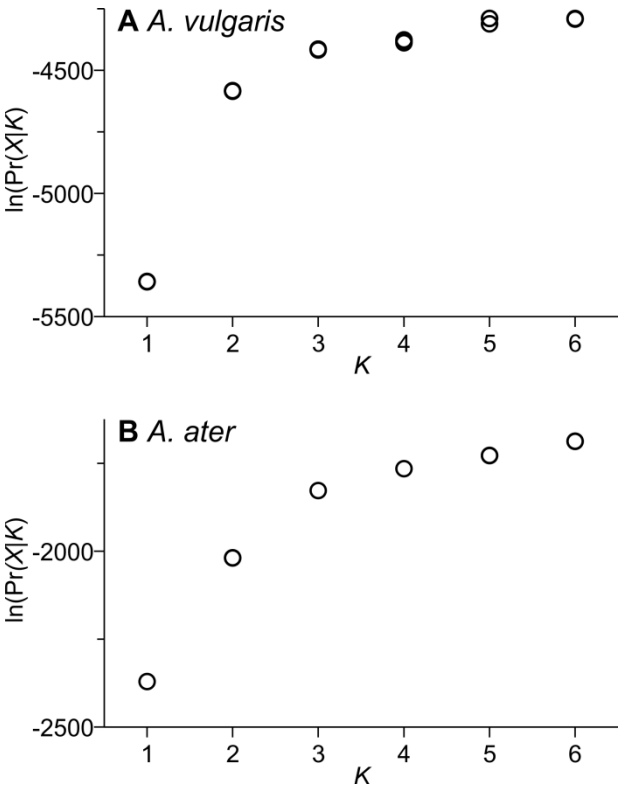

Figure 3. STRUCTURE analyses of (A) *Arion vulgaris* and (B) *A. ater*; in both cases animals suspected to be interspecific hybrids were removed. As the number of clusters specified (*K*) is varied, the vertical axis shows a measure of fit, the estimated probability (logged) of the observed pattern of microsatellites given this *K*. For each value of *K*, STRUCTURE was run three times: the resulting three values of ln[Pr(*X*|*K*)] are mostly so close as not to be distinguishable here. In both analyses, the levelling off of the increase at values of *K* above 3 suggests that *K* = 3 is an appropriate model.

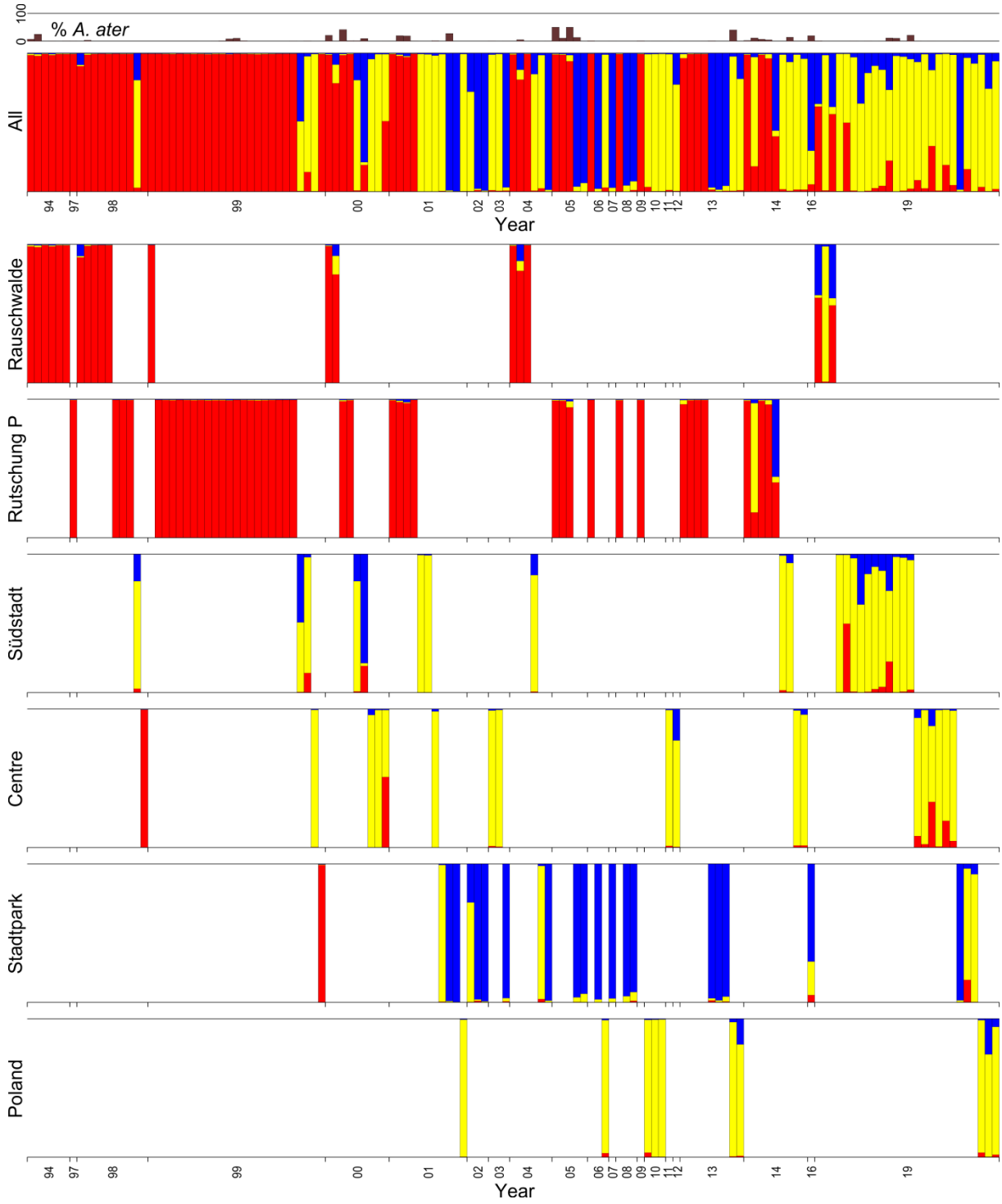

Figure 4. Genotypes of 137 *Arion vulgaris* and its hybrids, related to date and area of collection. Each column shows the ancestry of a single slug estimated by STRUCTURE using $K = 4$. Slugs are ordered by year of collection (1994–2019). The very top figure shows the estimated proportion of *A. ater* ancestry: only if this was < 50% is the slug included here. In the other diagrams the estimated share of ancestry amongst the three *A. vulgaris* races (red, yellow, blue) is rescaled to add up to 100%. All individuals are shown in the upper figure; below they are shown again, now split according to the site of collection (see Fig. 5).

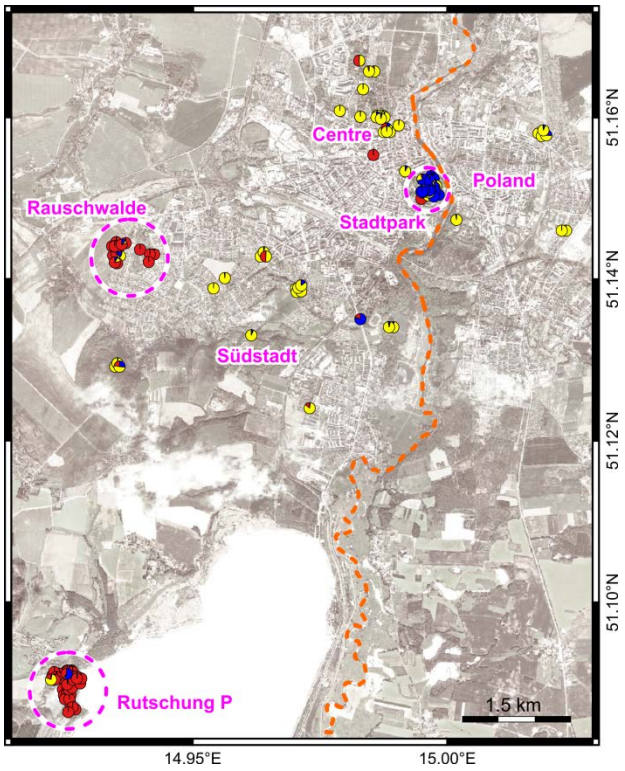

Figure 5. Collection sites within Görlitz (Saxony, Germany) of *Arion vulgaris* and its hybrids genotyped for this study. Each pie chart corresponds to a single slug, with the colours representing the estimated share of ancestry amongst the three races: see Fig. 4. Hybrids with *A. ater* ancestry > 50% were excluded, but otherwise *A. ater* ancestry is ignored in the pie charts. Because genotypes clearly differ between areas of the city, we subsequently group slugs according to the six districts indicated. The orange dashed line is the German–Polish border along the river Neiße. Two additional slugs from a village off the map to the east were included in the Poland sample. Satellite images: Esri, Earthstar Geographics, CNES/Airbus DS.

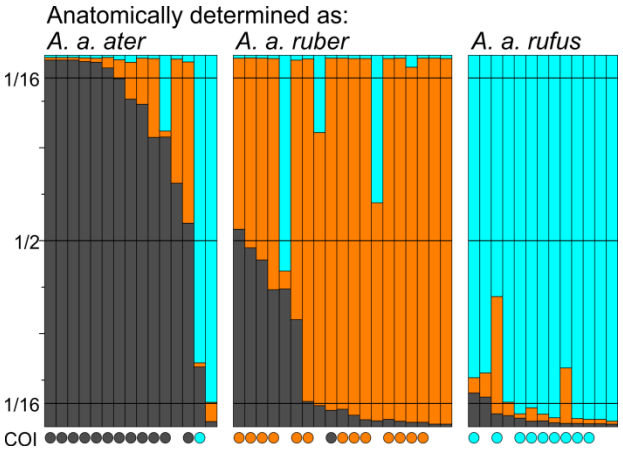

Figure 6. STRUCTURE analysis of *Arion ater* identified anatomically as one of its three subspecies. Each column is one individual; the estimated ancestry is indicated by the relative lengths of the grey (*A. a. ater*), orange (*A. a. ruber*) and cyan (*A. a. rufus*) bars. The dots below show the subspecies identity of the COI sequence (not all were sequenced).

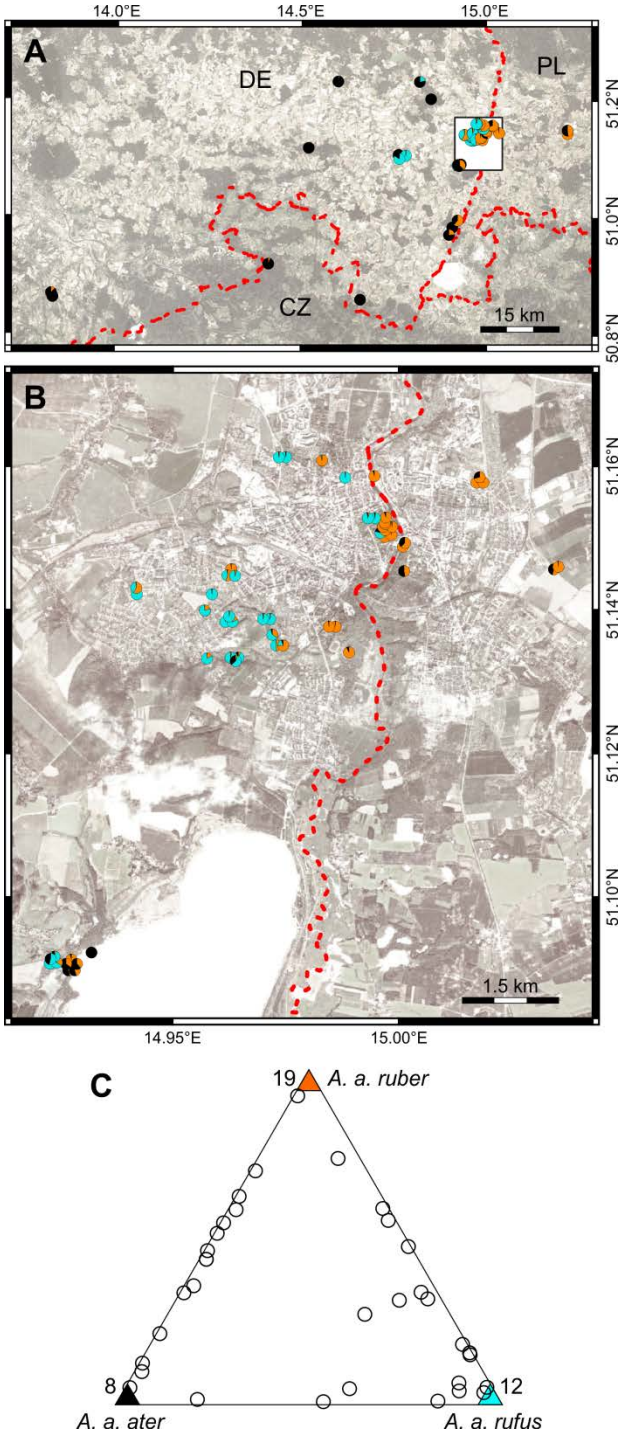

Figure 7. (A, B) Collection sites in SE Saxony (Germany) of *Arion ater* genotyped for this study; likely hybrids with *A. vulgaris* are excluded. Each pie chart corresponds to a single slug, with the colours representing the estimated share of ancestry amongst the three subspecies: black = *A. a. ater*, orange = *A. a. ruber*, cyan = *A. a. rufus*. The white rectangle in (A) shows the area around Görlitz represented in (B). The red dashed lines are borders between Germany, Poland and the Czech Republic. The purest *A. a ater* from within the town was excluded because it came from the garden of a malacologist who, we recently discovered from notes, used her garden to rear up *A. a. ater* collected elsewhere! Satellite maps: Esri, Earthstar Geographics, CNES/Airbus DS. (C) The same animals plotted so that the closeness to each corner of the triangles represents the estimated ancestry apportioned to that subspecies. The numbers at the corners are the numbers of "pure" individuals that would be plotted within the small shaded triangles (where admixture < 1/16).

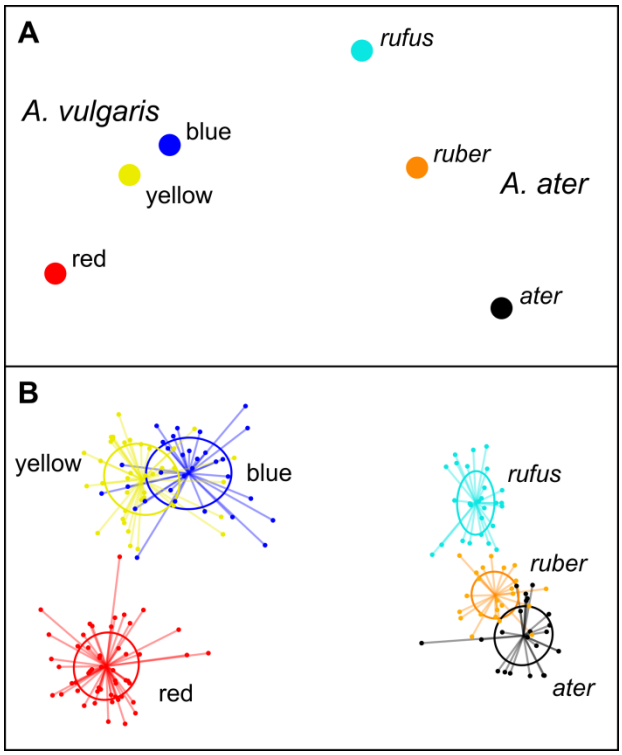

Figure 8. (A) A two-dimensional projection representing approximately the net nucleotide differences calculated by STRUCTURE (see Table 1) between pre-selected examples of the three subspecies of *Arion ater* and three races of *A. vulgaris*. (B) Scatterplot of the first two discriminant functions calculated by ADEGENET based on all individuals except the anatomical intermediates. Each dot represents an individual, with the cluster to which they belong derived *de novo*. (To avoid overfitting, the number of principal components was reduced to 6, indicated by both the xvalDapc and optim.a.score functions.)