Introgressive replacement of natives by invading Arion pest slugs

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Hybridization with invasive species is one of the major threats to the phenotypic and genetic persistence of native organisms worldwide. Arion vulgaris (syn. lusitanicus) is a major agricultural pest slug that successfully invaded many European countries in recent decades, but its impact on closely related native species remains unclear. Here, we hypothesized that the regional decline of native A. rufus is connected with the spread of invasive A. vulgaris, and tested whether this can be linked to hybridization between the two species by analyzing 625 Arion sp. along altitudinal transects in three regions in Switzerland. In each region, we observed clear evidence of different degrees of genetic admixture, suggesting recurrent hybridization beyond the first generation. We found spatial differences in admixture patterns that might reflect distinct invasion histories among the regions. Our analyses provide a landscape level perspective for the genetic interactions between invasive and native animals during the invasion. We predict that without specific management action, A. vulgaris will further expand its range, which might lead to local extinction of A. rufus and other native slugs in the near future. Similar processes are likely occurring in other regions currently invaded by A. vulgaris.

The displacement of native species by invasive ones is a serious threat to biodiversity worldwide, and is becoming more frequent due to the increasing number of species introductions1,2. Among the mechanisms of such displacement are very often competition or predation3,4. However, invasive species can also displace native organisms genetically, through hybridization and introgression5,6. This mixing of gene pools and potential loss of genotypically distinct species is especially problematic for rare organisms coming into contact with more abundant ones7,8. Hybridization might have different outcomes for the native and invasive species. Even though hybridization can in some cases contribute to generating species diversity8, the hybrid offspring could also be infertile or have reduced fitness, which may lead to decline and even extinction of the native species’ populations9,10. Moreover, hybridization can result in speciation reversal, and change the functional roles of organisms in the ecosystem12,13. For the invasive species, hybridization may provide advantages by increasing their invasiveness or adaptive potential to local environments14–16. The consequences of hybridization can be particularly challenging for conservation biology, because it is difficult to develop management strategies for hybrids of endangered species17. Therefore, to be able to protect native organisms and prevent spread of invaders, it is crucial to understand whether hybridization has occurred as well as what are its likely impacts.

Most studies on the impacts of hybridization between invasive and native species have been conducted in plants (e.g. ref.4,5), with only a few in animals (e.g. ref.15,18). Among the major animal taxonomic groups, molluscs have the highest number of documented extinctions, which has been attributed in part to interactions with other mollusc species invading native species ranges19. However, the role of hybridization with invasive species in molluscs remains poorly understood.

The hermaphroditic slug Arion vulgaris Moquin-Tandon 1855 (also referred to as non-topotype A. lusitanicus Mabille 1868) is considered one of the 100 most invasive species in Europe20, being a serious pest both in agriculture and gardening21,22. It has spread and become established in many European countries since the 1950’s23–25, but see ref.26 and represents an excellent case for studying the genetic impacts on native species, such as A. rufus (Linnaeus 1758). This closely related native slug used to be abundant in forests and meadows27, but recently has been listed as “vulnerable” on the Red List of endangered species in several European countries28,29. It has been reported that when A. vulgaris enters an area, the populations of A. rufus begin to decline, and hybridization is suspected to be one of the underlying mechanisms30,31.
The possibility and extent of hybridization among the three large *Arion* species—*A. vulgaris*, *A. rufus*, and *A. ater* (Linnaeus 1758)—has been sparked by and questioned following the observation of intermediate morphological phenotypes in the field and mating experiments under experimental laboratory conditions. Initial genetic investigations indicated hybridization between the invasive *A. vulgaris* and native *A. ater* or *A. rufus* in particular, but low-resolution genetic markers and sample sizes limited the conclusiveness. Thus, it remains unanswered how frequently hybridization among the invasive *A. vulgaris* and the native *A. rufus* occurs, and whether hybrid offspring can persist and have a lasting impact on natural populations.

In order to characterize the interactions between invasive *A. vulgaris* and native *A. rufus*, we examined the zone of contact of these two species in three different locations. In Switzerland, *A. vulgaris* has established a continuous distribution below 1000 m above sea level (a.s.l.) according to morphological assessment and given its synanthropic nature this species is often present in cities and villages. However, species identification based on external morphology may be misleading and genetic analyses of the slug invasion are lacking in Switzerland and elsewhere. Nonetheless, the most abundant large *Arion* slugs in cultivated areas in the Swiss lowlands are most likely *A. vulgaris*, while the native *A. rufus* is nowadays thought to be limited to higher altitudes. In order to characterize the interactions between invasive *A. vulgaris* and native *A. rufus*, we examined the zone of contact of these two species in three different locations. In Switzerland, *A. vulgaris* has established a continuous distribution below 1000 m above sea level (a.s.l.) according to morphological assessment and given its synanthropic nature this species is often present in cities and villages.

In this study we assessed i) whether there is evidence of past or recent hybridization between the two species, and ii) whether there is consistency between genetic and morphological species assignment, with the potential implications for management of the invasive and conservation of the native species.

### Results

**Species identification based on mtDNA and morphology.** Phylogenetic reconstructions clearly distinguished two clades representing the invasive and native species (Fig. S3). Forty-five individuals carried *A. rufus* and 60 *A. vulgaris* mtDNA haplotypes, with extensive sharing of haplotypes between the three altitudinal transects. Our sequencing yielded two different haplotypes for *A. vulgaris* and three haplotypes for *A. rufus*, and these differed in at least 60 nucleotides between the species (Fig. 2). Mitochondrial DNA from both species was
detected in pasture and forested habitats but there was strong variation between transects and altitude (Tables 1, 2 and 3).

Based on internal morphology we were able to classify 89 out of the 105 individuals sequenced for mtDNA. The remaining 16 specimens had immature genitalia or their preservation state did not permit a clear identification. Genital morphology allowed us to identify 59 individuals as *A. vulgaris*, 23 as *A. rufus*, whereas the remaining seven showed intermediate morphological characteristics (Tables 1, 2, 3 and 4).

**Signs of admixture and frequency of hybrids.** All microsatellite loci were highly polymorphic with an average of 15 alleles per locus (ranging from 9 to 26, Table S1). Using the threshold values obtained in HYBRIDLAB, we detected the presence of both non-admixed and admixed individuals in each transect with large differences regarding the frequency and distribution of hybrids. Out of the 183 individuals analysed in the Blumenstein transect, 49 were identified as non-admixed *A. vulgaris*, 54 as non-admixed *A. rufus* and 80 as hybrids with various levels of admixture. In the Salvan transect, 66 individuals were identified as pure *A. vulgaris*, 99 as pure *A. rufus* and 36 as hybrids. In the Filfalle transect, there were 25 and 31 pure *A. vulgaris* and pure *A. rufus*.
rufus, respectively, and 185 individuals identified as hybrids. Overall, we detected five cases (1–2 per transect) of mitonuclear discordance in both directions, i.e. species assignment for these individuals based on the autosomal loci was different from mtDNA (Table 4, Fig. S4). Genetic differentiation between non-admixed A. vulgaris and A. rufus in the three study regions was significant (p < 0.001) and relatively high in Blumenstein (FST = 0.425) and Salvan (FST = 0.475) but lower in Filfalle (FST = 0.186).

| Location | Latitude | Longitude | Elevation | nucDNA | ND1: AV | ND1: AR | M: AV | M: AR | M: H | M: np |
|----------|----------|-----------|-----------|--------|---------|---------|-------|-------|-------|-------|
| F-01     | 46.494   | 7.673     | 1173      | 14     | 3       | —       | 3     | —     | —     | —     |
| F-02     | 46.492   | 7.671     | 1174      | 11     | —       | —       | —     | —     | —     | —     |
| F-03     | 46.490   | 7.671     | 1175      | 7      | 3       | —       | 3     | —     | —     | —     |
| S-11     | 46.117   | 7.007     | 1123      | 14     | 3       | —       | 3     | —     | —     | —     |
| S-12     | 46.118   | 7.006     | 1187      | 8      | —       | —       | —     | —     | —     | —     |
| S-03     | 46.113   | 7.005     | 1244      | 10     | 2       | —       | 2     | —     | —     | —     |
| S-04     | 46.113   | 7.005     | 1273      | 8      | —       | —       | —     | —     | —     | —     |
| S-05     | 46.114   | 7.003     | 1291      | 8      | —       | —       | —     | —     | —     | —     |
| S-06     | 46.114   | 7.002     | 1315      | 15     | 2       | 2       | 1     | 1     | 2     | —     |
| S-07     | 46.114   | 7.001     | 1357      | 18     | —       | —       | 6     | 1     | 3     | 1     |
| S-08     | 46.114   | 7.000     | 1407      | 8      | 2       | —       | 2     | —     | —     | —     |
| S-09     | 46.115   | 6.999     | 1449      | 18     | 1       | 1       | 1     | —     | —     | 1     |
| S-10     | 46.116   | 6.999     | 1515      | 19     | —       | —       | 2     | 1     | 1     | —     |
| S-11     | 46.117   | 6.999     | 1559      | 13     | —       | —       | —     | —     | —     | —     |
| S-12     | 46.118   | 6.999     | 1623      | 13     | —       | —       | 2     | —     | 1     | —     |
| S-13     | 46.119   | 6.999     | 1674      | 17     | —       | —       | —     | —     | —     | —     |
| S-14     | 46.119   | 6.998     | 1720      | 14     | —       | —       | —     | —     | —     | —     |
| S-15     | 46.121   | 6.997     | 1767      | 11     | —       | 4       | —     | 3     | 1     | —     |
| S-16     | 46.122   | 6.995     | 1845      | 10     | —       | 5       | —     | 4     | 1     | —     |

Table 2. Sampling locations in the Salvan altitudinal transect with the number of samples analyzed for nuclear DNA (nucDNA) and identified with mitochondrial ND1 marker: A. vulgaris (ND1: AV) or A. rufus (ND1: AR). Morphological assessment is also listed (M: AV – A. vulgaris, M: AR – A. rufus, M: H – putative hybrid, M: np – morphological assessment not possible).

| Location | Latitude | Longitude | Elevation | nucDNA | ND1: AV | ND1: AR | M: AV | M: AR | M: H | M: np |
|----------|----------|-----------|-----------|--------|---------|---------|-------|-------|-------|-------|
| F-01     | 46.494   | 7.673     | 1173      | 14     | 3       | —       | 3     | —     | —     | —     |
| F-02     | 46.492   | 7.671     | 1174      | 11     | —       | —       | —     | —     | —     | —     |
| F-03     | 46.490   | 7.671     | 1175      | 7      | 3       | —       | 3     | —     | —     | —     |
| S-01     | 46.111   | 7.007     | 1123      | 14     | 3       | —       | 3     | —     | —     | —     |
| S-02     | 46.112   | 7.006     | 1187      | 8      | —       | —       | —     | —     | —     | —     |
| S-03     | 46.113   | 7.005     | 1244      | 10     | 2       | —       | 2     | —     | —     | —     |
| S-04     | 46.113   | 7.005     | 1273      | 8      | —       | —       | —     | —     | —     | —     |
| S-05     | 46.114   | 7.003     | 1291      | 8      | —       | —       | —     | —     | —     | —     |
| S-06     | 46.114   | 7.002     | 1315      | 15     | 2       | 2       | 1     | 1     | 2     | —     |
| S-07     | 46.114   | 7.001     | 1357      | 18     | —       | —       | 6     | 1     | 3     | 1     |
| S-08     | 46.114   | 7.000     | 1407      | 8      | 2       | —       | 2     | —     | —     | —     |
| S-09     | 46.115   | 6.999     | 1449      | 18     | 1       | 1       | 1     | —     | —     | 1     |
| S-10     | 46.116   | 6.999     | 1515      | 19     | —       | —       | 2     | 1     | 1     | —     |
| S-11     | 46.117   | 6.999     | 1559      | 13     | —       | —       | —     | —     | —     | —     |
| S-12     | 46.118   | 6.999     | 1623      | 13     | —       | —       | 2     | —     | 1     | —     |
| S-13     | 46.119   | 6.999     | 1674      | 17     | —       | —       | —     | —     | —     | —     |
| S-14     | 46.119   | 6.998     | 1720      | 14     | —       | —       | —     | —     | —     | —     |
| S-15     | 46.121   | 6.997     | 1767      | 11     | —       | 4       | —     | 3     | 1     | —     |
| S-16     | 46.122   | 6.995     | 1845      | 10     | —       | 5       | —     | 4     | 1     | —     |

Table 3. Sampling locations in the Filfalle altitudinal transect with the number of samples analyzed for nuclear DNA (nucDNA) and identified with mitochondrial ND1 marker: A. vulgaris (ND1: AV) or A. rufus (ND1: AR). Morphological assessment is also listed (M: AV – A. vulgaris, M: AR – A. rufus, M: H – putative hybrid, M: np – morphological assessment not possible).
Spatial patterns in nuclear DNA. The PCoA separated most non-admixed *A. vulgaris* in the Salvan transect clearly from non-admixed *A. rufus* (defined by the thresholds identified in HYBRIDLAB) with likely hybrids in between (Fig. 3). The first and second axis accounted for 26% and 4% of the variation, respectively. The separation between the species was less pronounced in the Blumenstein transect and practically non-existent in the Filfalle transect (Fig. S5).

Combining the three transect datasets for the Bayesian analysis, there was extensive structure among and within transects with gradual transitions between genotype clusters in each study region (Fig. S6). The Evanno approach suggested that the most likely number of genetic clusters was $K = 2$ for Blumenstein and Salvan, reflecting the results of mtDNA analysis regarding the general distribution of the two species and the PCoA. The genetic cluster representing *A. vulgaris* was present in the lowest parts of the sampling transects and *A. rufus* in the highest parts with different levels of admixture at approximately intermediate altitudes (Fig. 1). For the Filfalle transect, the Evanno approach suggested $K = 8$ as the most likely number of clusters. However, given the presence of mtDNA from two species only, we used $K = 2$ for further analyses of the Filfalle dataset. This showed that a majority of *A. vulgaris* genotypes were found at low altitudes. Despite different levels of admixture in the three study regions, all results were consistent among independent STRUCTURE runs.

When we estimated the position of the contact between species along the altitudinal transects with *hzar*, all fitted clines converged to the same values showing a single likelihood peak each. We observed relatively steep genetic clines of species transition with significant differences among the transects (Fig. 4). The cline centres were estimated to be located at 1,569 m a.s.l. in Blumenstein, 1,404 m a.s.l. in Salvan and at 1,186 m a.s.l. in Filfalle (Table S2). In addition to having the cline centre at the lowest altitude, Filfalle also displayed a very low estimated cline width (13 m of elevational difference) in comparison to Blumenstein (320 m) and Salvan (185 m; Table S2).

Discussion
In this study, we provided a landscape level perspective on the spatial genetic consequences of the early stages of contact between an invasive and native animal species. Genetically confirmed *A. vulgaris* was predominantly found in disturbed areas around settlements and *A. rufus* in more natural habitats at higher altitude which is consistent with a human contribution e.g. through inadvertent introduction at the origin of the invasion process. Considering the overlapping external morphology between the two species, we propose molecular techniques be used as the primary method of species identification for these taxa.

Our analyses provided the first large-scale evidence of hybridization between the invasive *A. vulgaris* and native *A. rufus* under natural conditions. This supports previous suggestions that hybridization is possible (e.g. ref.32,34), despite the divergence time among extant *Arion* spp. slugs estimated at approximately 5 Mya39. More importantly, this study shows that hybridization can be common where the two species co-occur, and through our design of small-scale landscape level transects in comparison with other studies spanning tens or hundreds of km (e.g. ref.40,41) we were able to precisely determine the position of the *A. vulgaris* invasion front.

The detection of individuals with different levels of admixture in all three regions suggests that interspecific mating is not limited to the first generation of hybrids. This is corroborated by the observation of a few individuals displaying mitonuclear discordance in both directions which requires several successful reproduction events of hybrids and backcrosses over multiple generations (Table 4; Fig. S4; ref.42,43).

Evidence of hybridization was observed in all three study regions but genetic admixture seems to be particularly extensive in the Filfalle transect. This could be explained by at least two scenarios. First, the two species have been in contact for more time than in the other transects. It is unknown when exactly the two species came...
into contact in the respective regions but the shared mtDNA haplotypes between regions (Fig. 2) – that are also frequent in the invaded ranges elsewhere in Europe – provide no indication of a substantially different local invasion history in Filfalle. Second, it is possible that the *A. vulgaris* slugs that invaded this region were already admixed which would explain the overall lower level of differentiation in nuclear DNA between the taxa in this region. A third possibility would be two consecutive invasions of the region. The first invasion could have led to extensive admixture even at high altitudes while the second one with mostly pure *A. vulgaris* is still ongoing. Very specific demographic analyses might be able to differentiate between these invasion scenarios but this would require vast genomic data sets.

Theoretical work by Currat *et al*. predicts that in the zone of contact, invaders could have experienced more introgression at selectively-neutral genes than the native species. These predictions have been supported in several empirical studies (e.g. ref.44), which however investigated hybrid zones that have existed for a long time. In our system, the invasion of *A. vulgaris* in Switzerland started approximately 60 years ago38, and we did not detect a pattern of asymmetrical introgression. It is unclear if this will build up over time and potentially lead to larger geographic regions with introgressed individuals (e.g. ref.42,47). Accordingly, very little pronounced patterns are expected in this regards for the regions of Europe that were more recently invaded or are currently being invaded by *A. vulgaris*25.

Because our study represents to our knowledge the first genetic assessment of the levels of introgression in *Arion* sp. slugs at the landscape scale, we do not have a baseline from an earlier time point to which these levels could be compared. We thus cannot determine with certainty whether the trend of introgression is declining or increasing. However, several studies have suggested that the propensity to hybridize might be influenced by relative abundance48,49, and if the more abundant *A. vulgaris* continues to spread, we predict that it will eventually lead to introgressive replacement of *A. rufus*. Such genetic replacement has been described for example in native newts8, crayfish50 and mussels51.

Nevertheless, additional factors could be contributing to the disappearance of *A. rufus*. Invasive species are often considered competitively dominant: they might use food resources more rapidly and/or efficiently than native species52, exhibit rapid adaptation and spread53, faster growth rates54 and higher reproductive output55. Indeed, *A. vulgaris* seems to be able to use food resources more efficiently than native species56,57, and shows a

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**Figure 4.** Results of the cline analyses for the three altitudinal transects (B – Blumenstein, S – Salvan, F – Filfalle) based on the average q-value for each sampling location obtained with STRUCTURE. Elevation in metres above sea level is on the x-axis and the probability of belonging to the *A. rufus* cluster (q-value) on the y-axis. Circles correspond to sampling locations and their size is proportional to the number of samples within a cline. The 95% credible cline region is indicated in grey. Filfalle shows signs of more extensive admixture between the invasive and the native *Arion* species also at the highest altitudes. The altitude of the centre of the cline is indicated by a dashed line.
more pronounced exploratory behaviour in novel environments\textsuperscript{58,59}. Further, \textit{A. vulgaris} seems to be able to cope with land use change and agricultural intensification better than the native species\textsuperscript{60}. Together with hybridization, these factors might cause local extinction of \textit{A. rufus} in the near future.

The two species mostly occurred in different habitats, as described by Rüetschi \textit{et al}.\textsuperscript{28} based on morphological assessments. The contact between \textit{A. rufus} and \textit{A. vulgaris} is currently at intermediate altitudes (Fig. 1) with the cline centres at different elevations in each region (Fig. 4, Table S2), which might indicate differences in local invasion histories of \textit{A. vulgaris}\textsuperscript{61,62} as mentioned above. While the analogous highest altitude of slug occurrence in all study regions suggests environmental factors (e.g. temperature) constraining further spread of both species\textsuperscript{61}, it would be interesting to implement long-term genetic monitoring to document any further expansion of \textit{A. vulgaris} over time\textsuperscript{62}.

Most of the individuals assigned by nucDNA as hybrids were morphologically identified as either species (Table 4). Species identification of \textit{Arion} taxa is notoriously difficult especially regarding differentiation based on external morphology\textsuperscript{63,64}, and hybridization adds another complication. Although morphological species assignment is still extensively used for \textit{Arion} spp. slugs (e.g. ref\textsuperscript{65,66}), our results stress that identification should be primarily based on molecular techniques, particularly in regions where multiple species co-occur. In order to preserve the native, often endangered species, we encourage the use of non-invasive DNA sampling methods (e.g. ref\textsuperscript{67,68}) whenever possible.

Some of the observed discrepancy between genetic and internal morphological species assignment (Table 4) might be caused by phenotypic overlap, i.e. hybrids after the F1 generation could be difficult to tell apart from parental species\textsuperscript{67,68}. In this case, the removal of morphologically indistinguishable hybrids from mixed populations would be impossible, and management efforts need to focus primarily on prevention of further spread in the landscape and new introductions of the invasive slug.

We have unequivocally established that \textit{A. vulgaris} – one of the most destructive agricultural pest slugs in Europe – is able to hybridize with \textit{A. rufus} and produce fertile offspring in the natural environment. Given the current rapid spread of \textit{A. vulgaris} through vast areas of Europe, similar processes might be acting in other countries and closely related species although the time in contact is shorter in most regions and there is no evidence of “de-speciation” at larger scales yet\textsuperscript{25}. Nevertheless, the invasion of the slug \textit{A. vulgaris} may constitute a larger threat to native \textit{Arion} species than previously considered.

**Materials and Methods**

**Field sampling and morphological species identification.** We chose three study regions (Blumenstein, Salvan and Filfalle) in the Swiss Prealps based on the occurrence records of \textit{A. rufus} in the last 10 years\textsuperscript{28} (Fig. 1). In each study region, sampling along an elevational transect started in a settlement area and continued through mostly forested locations and pastures to higher altitudes (Fig. 1; Tables 1, 2 and 3). As the home range size of \textit{A. vulgaris} has been estimated to be approximately 45 m\textsuperscript{2}\textsuperscript{69}, sampling locations within a transect were spaced at least 50 meters apart to increase the chance of capturing unrelated individuals. Along the transect Blumenstein, 183 individuals were sampled from 21 locations in the altitudinal range of 664–1,872 m a.s.l. (Table 1). The transect Salvan comprised 16 locations, yielding 201 individuals from altitudes between 1,123 and 1,845 m a.s.l. (Table 2), and in the transect Filfalle, we collected 241 individuals from 22 locations (altitude 1,173–1,801 m a.s.l.; Table 3).

| nucDNA     | mtDNA     | Morphology | Nr. |
|-----------|-----------|------------|-----|
| \textit{A. vulgaris} | \textit{A. vulgaris} | \textit{A. vulgaris} | 20  |
| \textit{A. vulgaris} | \textit{A. vulgaris} | hybrid     | —   |
| \textit{A. vulgaris} | \textit{A. vulgaris} | \textit{A. rufus} | —   |
| \textit{A. vulgaris} | \textit{A. rufus} | \textit{A. vulgaris} | 2   |
| \textit{A. vulgaris} | \textit{A. rufus} | hybrid     | —   |
| \textit{A. vulgaris} | \textit{A. rufus} | \textit{A. rufus} | 30  |
| hybrid      | \textit{A. vulgaris} | \textit{A. rufus} | 2   |
| hybrid      | \textit{A. rufus} | \textit{A. vulgaris} | 3   |
| hybrid      | \textit{A. rufus} | hybrid     | 2   |
| hybrid      | \textit{A. rufus} | \textit{A. rufus} | 2   |
| \textit{A. rufus} | \textit{A. vulgaris} | \textit{A. vulgaris} | 3   |
| \textit{A. rufus} | \textit{A. vulgaris} | hybrid     | —   |
| \textit{A. rufus} | \textit{A. vulgaris} | \textit{A. rufus} | —   |
| \textit{A. rufus} | \textit{A. rufus} | \textit{A. vulgaris} | —   |
| \textit{A. rufus} | \textit{A. rufus} | hybrid     | 3   |
| \textit{A. rufus} | \textit{A. rufus} | \textit{A. rufus} | 19  |

Table 4. Comparison of species assignment with nuclear DNA (nucDNA), mitochondrial DNA (mtDNA) and morphology across all three transects (see Figure S4 for further information). The number of slugs in each category is given.
The highest altitudes in the transects constituted the upper limit of where we detected Arion slugs in the respective locations. In total, 625 slugs were analysed.

Slugs were collected directly in the field during rainy weather or at night, or by placing a trap designed by the authors (described in detail in Fig. S1) overnight. Slugs were killed by freezing at −20 °C for at least eight hours and the whole specimens were preserved in absolute ethanol. Slugs that were analysed with both mitochondrial and nuclear markers (105 individuals) were dissected and classified as either A. vulgaris, A. rufus or an intermediate form based on internal morphological traits of reproductive organs37,64. DNA was extracted from a small piece of foot tissue using proteinase K and the high-salt extraction method70. After dilution in double-distilled water, the DNA was stored at −20 °C.

Mitochondrial DNA analyses. In order to confirm that both A. vulgaris and A. rufus were present in each region, we sequenced the mtDNA gene ND1 in 32–38 Arion sp. individuals per transect (Tables 1, 2 and 3), totalling 105 slugs. Locus specific primers MOL-NAD1F (5′-CGRAARGGMCCTAACAARGTTGG-3′ and MOL-NAD1R (5′-GGRCGACGATTWGTCTCNGCTA-3′) developed by Quinteiro et al.30 were used to amplify a 350 bp long fragment, following the protocol described in Zemanova et al.25. The sequencing reactions were visualized on an ABI Prism 3130 (Applied Biosystems).

To assign slug mtDNA to the native or invasive species, we aligned our novel ND1 sequences with reference sequences from our previous study25 covering the entire distribution range of the species: A. vulgaris sequences with the accession numbers KX834566, KX834594, KX834637 and A. rufus sequences with the accession numbers KX834609, KX834617, KX834665. We also used one A. subfuscus Draparnaud 1805 sequence (accession number AY316248) as outgroup for the phylogenetic analysis. DNA sequences were aligned in BIOEDIT 7.1.31 and the number of haplotypes was determined in DNAsP 52. The phylogenetic tree was produced following the methodology described in Zemanova et al.25. In order to visualize relationships between haplotypes in either of the two species, we reconstructed a statistical parsimony haplotype network in POPTART17.

Nuclear DNA analyses. Genotyping with microsatellite markers. We genotyped all 625 Arion sp. individuals with fifteen nuclear microsatellite markers (nucDNA) ALU_02_3, ALU_06_4, 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 and ALU_96_231 in three primer mixes. The 10 µl PCRs contained approximately 100 ng DNA, 1 µl of primer mix, 5 µl of Qiagen multiplex kit and 3 µl of H2O. The PCR temperature profile was as follows: 15 min initial denaturation at 96 °C, followed by 32 cycles of denaturation at 94 °C for 30 sec, annealing at 57 °C for 1 min 30 sec, extension at 72 °C for 1 min 30 sec, and the final extension step of 30 min at 60 °C. The PCR product was diluted with 20 µl of distilled H2O and 1.2 µl of the diluted product were mixed with 12 µl of the internal size standard (GeneScan 500 LIZ, Applied Biosystems) to determine the size of alleles. The amplified fragments were separated on an ABI Prism 3130 Genetic Analyzer and fragment lengths were scored in GENEMAPPER 3.7 (Applied Biosystems). Approximately 10% of the samples were re-amplified and genotyped independently to ensure genotyping consistency.

Genetic diversity and estimates of admixture. The number of alleles per locus was calculated in GENALEX 6.535. We also conducted a principal coordinate analysis (PCoA) in the same software to summarize major patterns of variation in the microsatellite genotype dataset. FSTAT 2.9.3.228 was used to measure the average pair-wise level of genetic differentiation (F ST ) between 50 individuals in each transect classified by the Bayesian analysis (see below) as either non-admixed A. vulgaris or A. rufus. We used Bayesian assignment implemented in STRUCTURE 2.3.427 to identify genetic clusters in the Arion sp. samples. Analyses were run using the admixture model with correlated allele frequencies and without sampling location priors, with a burn-in period of 100,000 iterations followed by 1,000,000 MCMC iterations. The number of clusters (K) was set to range from 1 to 10, with 10 replicates for each K value. The optimal number of clusters was estimated using the Evanno approach76 implemented in Structure Harvester 0.6.94.79. These analyses were run separately for each transect, as well as for all three transects together.

To identify the optimal threshold q-value (i.e. the probability of belonging to a genetic cluster) for distinguishing between non-admixed individuals and potential hybrids between the two species, we used Hybridlab 1.082 to simulate parental and hybrid genotypes in each transect. The parental genotypes used for the simulations consisted of 50 individuals assigned to their genetic cluster with a q-value above 0.8. We simulated 10 data sets each with 100 generated genotypes of each parental and hybrid class (F1, F2, backcross with A. vulgaris, backcross with A. rufus). The simulated data sets were evaluated in STRUCTURE with K = 2 and 50,000 MCMC iterations following a burn-in period of 100. The accuracy and efficiency of assignment of the generated genotypes was calculated using the thresholds of 0.01, 0.05, 0.1, 0.2 and 0.3, following the procedure described in Vähä & Primmer88. The HYBRIDLAB results suggested that the best thresholds for delimiting non-admixed and hybrid individuals were 0.05 for Blumenstein and Salvan, and 0.15 for Filfalle (Fig. S2).

Cline analyses. To estimate the location and the spatial extent of admixture along the altitudinal transects, we conducted cline analyses with averaged q-values per sampling location using the statistical package hisz2 imple-mented in R 3.1.161. We modeled the cline shape using three equations44,46 that describe a sigmoid shape at the centre of the transition with two exponential decay curves on either side of the transition. We estimated the centre and width of each cline, and fitted three sets of three cline models using the Metropolis–Hasting algorithm. Model one had no scaling, model two had fixed scaling, and model three allowed the Pmin and Pmax (i.e. the bottom and top of a cline) to vary. We compared these models to a null model of no clinal transition. We ran each model for
100,000 iterations and assessed convergence and stability by visualizing the MCMC traces. We then selected the best-fit model based on the comparison of corrected AIC values.

Data accessibility. DNA sequences are available in GENBANK under accession numbers MG253687-MG253791. Genotype data are available in Dryad under doi:10.5061/dryad.rb187.

References
1. Davis, M. A. Invasion Biology. (Oxford University Press, 2009).
2. Lowry, E. et al. Biological invasions: a field synopsis, systematic review, and database of the literature. Ecology and Evolution 3, 182–196 (2013).
3. Luque, G. M. et al. The 100th of the world’s worst invasive alien species. Biological Invasions 16, 981–985 (2014).
4. Mooney, H. A. & Cleland, E. E. The evolutionary impact of invasive species. Proceedings of the National Academy of Sciences of the United States of America 98, 5446–5451 (2001).
5. Ayres, D. R., Smith, D. L., Zaremba, K., Kohr, S. & Strong, D. R. Spread of exotic cordgrasses and hybrids (Spartina sp.) in the tidal marshes of San Francisco Bay, California, USA. Biological Invasions 6, 221–231 (2004).
6. Kovach, R. P. et al. Dispersal and selection mediate hybridization between a native and invasive species. Proceedings of the Royal Society B-Biological Sciences 282, 20142454 (2015).
7. Currat, M., Ruedi, M., Petit, R. J. & Excoffier, L. The hidden side of invasions: massive introgression by local genes. Evolution 62, 1908–1920 (2008).
8. Dufresnes, C. et al. Massive genetic introgression in threatened northern crested newts (Triturus cristatus) by an invasive congener (T. carnifex) in Western Switzerland. Conservation Genetics 17, 839–846 (2016).
9. Abbott, R. et al. Hybridization and speciation. Journal of Evolutionary Biology 26, 229–246 (2013).
10. He gek, G. S., Nason, J. D., Clegg, J. M., Ellstrand, N. C. & Solits, P. The evolution of California’s wild radish has resulted in the extinction of its progenitors. Evolution 60, 1187–1197 (2006).
11. Leary, R. E., Allendorf, F. W. & Forbes, S. H. Conservation genetics of bull trout in the Columbia and Klamath river drainages. Conservation Biology 7, 856–865 (1993).
12. Seehausen, O. Conservation: losing biodiversity by reverse speciation. Current Biology 16, R334–R337 (2006).
13. Vonlanthen, P. et al. Eutrophication causes speciation reversal in whitefish adaptive radiations. Nature 482, 357–362 (2012).
14. Abbott, R. J., Ashton, P. A. & Forbes, D. G. Introgressive origin of the radiate groundsel, Senecio vulgaris L. var. hibernicus Syme: AAT-3 evidence. Heredity 68, 425–435 (1992).
15. Facon, B., Tomiuk, J. & David, P. Hybridization and invasiveness in the freshwater snail, Helisoma trisulcatum: increase in genetic variance. Journal of Evolutionary Biology 18, 524–535 (2005).
16. Ridley, C. E. & Ellstrand, N. C. Evolution of enhanced reproduction in the hybrid-derived invasive, California wild radish (Raphanus sativus). Biological Invasions 11, 2251–2264 (2009).
17. Sakai, A. K. et al. The population biology of invasive species. Annual Review of Ecology and Systematics 32, 305–332 (2001).
18. Blum, M. J., Walters, D. M., Burkhead, N. M., Freeman, B. J. & Porter, B. A. Reproductive isolation and the expansion of an invasive hybrid swarm. Biological Invasions 12, 2825–2836 (2010).
19. Lydeard, C. et al. The global decline of nonmarine mollusks. BioScience 54, 321–330 (2004).
20. DAISIE. Handbook of alien species in Europe (Springer, 2009).
21. Briner, T. & Frank, T. The palatability of 78 wildflower plant species to the slug Arion lusitanicus. Annals of Applied Biology 133, 123–133 (1998).
22. Kozlowski, J. The distribution, biology, population dynamics and harmfulness of Arion lusitanicus Mabelle, 1868 (Gastropoda: Pulmonata: Arionidae) in Poland. Journal of Plant Protection Research 47, 219–230 (2007).
23. Engelke, S., Koempf, J., Jordana, K., Tomiuk, J. & Parker, E. D. The genetic dynamics of the rapid and recent colonization of Denmark by Arion lusitanicus (Mollusca, Pulmonata, Arionidae). Genetic 139, 709–721 (2011).
24. Rabitsch, W. Arion vulgaris (Moquin-Tandon, 1855) fact sheet. Online database of delivering alien invasive species inventories for Europe, http://www.europe-alien.org/pdf/Arion_vulgaris.pdf (2006).
25. Zemanova, M. A., Knop, E. & Heckel, G. Phylogeographic past and invasive presence of Arion pest slugs in Europe. Molecular Ecology 25, 5747–5764 (2016).
26. Pfenninger, M., Weigand, A., Bähl, M. & Klussmann-Kolb, A. Misperceived invasion: the Lusitanian slug Arion lusitanicus (Mollusca, Pulmonata, Arionidae) in Sweden: a comparison of occurrence, spread and naturalization of two alien slug species. Heldia 4, 137–138 (1997).
27. Dreijers, E., Reise, H. & Hutchinson, J. M. C. Mating of the slugs Arion lusitanicus auct. non Mabelle and A. ater (L.): different genitalia and mating behaviours are incomplete barriers to interspecific sperm exchange. Journal of Molluscan Studies 79, 51–63 (2013).
28. Roth, S., Hatteland, B. A. & Solbo, T. Some notes on reproductive biology and mating behaviour of Arion vulgaris Moquin-Tandon 1855 in Norway including a mating experiment with a hybrid of Arion rufus (Linnaeus 1785) x ater (Linnaeus 1758). Journal of Conchology 41, 249–257 (2012).
29. Hagnell, J., Schander, C. & von Proschwitz, T. Hybridisation in Arionides: the rise of a super slug? British Crop Protection Council Symposium Proceedings: Slugs & Snails: Agricultural, Veterinary & Environmental Perspectives 80, 221–226 (2003).
30. Hatteland, B. A. et al. Introgression and differentiation of the invasive slug Arion vulgaris from native A. ater. Malacologia 58, 303–321 (2015).
31. Rowson, B., Anderson, R., Turner, J. A. & Symondson, W. O. C. The slugs of Britain and Ireland: undetected and undescribed species increase a well-studied, economically important fauna by more than 20%. Plos One 9, e91907 (2014).
32. van der P. Weichtiere Europas: Ein Bildband für den Naturfreund. 288 (Mosaik-Verlag, 1990).
33. CSCE Database of the Centre Suisse de Cartographie de la Faune. http://www.csce.ch/ (2014).
34. Quinteiro, J., Rodriguez-Castro, J., Castillejo, J., Iglesias-Pineiro, J. & Rey-Mendez, M. Phylogeny of slug species of the genus Arion: evidence of Iberian endemics and of the existence of relict species in Pyrenean refuges. Journal of Zoological Systematics and Evolutionary Research 43, 139–148 (2005).
35. Arntzen, J. W. et al. Concordant morphological and molecular clines in a contact zone of the Common and Spined toad (Bufo bufo and R. spinosus) in the northwest of France. Frontiers in Zoology 13, 52 (2016).
41. Wilk, R. J. & Horth, L. A genetically distinct hybrid zone occurs for two globally invasive mosquito fish species with striking phenotypic resemblance. Ecology and Evolution 6, 8375–8388 (2016).
42. Bastos-Silveira, C., Santos, S. M., Monarca, R., Mathias, M. D. & Heckel, G. Deep mitochondrial introgression and hybridization among ecologically divergent vole species. Molecular Ecology 21, 5309–5323 (2012).
43. Beysard, M. & Heckel, G. Structure and dynamics of hybrid zones at different stages of speciation in the common vole (Microtus arvalis). Molecular Ecology 23, 673–687 (2014).
44. Malaspinas, A. S. et al. A genomic history of Aboriginal Australia. Nature 538, 207–214 (2016).
45. Owens, G. L., Baute, G. J. & Rieseberg, L. H. Revisiting a classic case of introgression: hybridization and gene flow in Californian sunflowers. Molecular Ecology 25, 2630–2643 (2016).
46. Tsuda, Y. et al. The extent and meaning of hybridization and introgression between Siberian spruce (Picea obovata) and Norway spruce (Picea abies); cryptic refugia as stepping stones to the west? Molecular Ecology 25, 2773–2789 (2016).
47. Kindler, E., Arletta, R. & Heckel, G. Deep phylogeographic divergence and cytonuclear discordance in the grasshopper Oedaleus decorus. Molecular Phylogenetics and Evolution 65, 695–704 (2012).
48. Rohde, K., Hau, Y., Weyer, J. & Hochkirch, A. Wide prevalence of hybridization in two sympatric grasshopper species may be shaped by their relative abundances. BMC Evolutionary Biology 15, 191 (2015).
49. Willis, P. M., Ryan, M. J. & Rosenhall, G. G. Encounter rates with conspecific males influence female mate choice in a naturally hybridizing fish. Behavioral Ecology 22, 1234–1240 (2011).
50. Perry, W. L., Feder, J. L., Dwyer, G. & Lodge, D. M. Hybrid zone dynamics and species replacement between Oreonectes crayfishes in a northern Wisconsin lake. Evolution 55, 1153–1166 (2001).
51. Saarman, N. P. & Pogson, G. H. Introgression between invasive and native blue mussels (genus Mytilus) in the central California hybrid zone. Molecular Ecology 24, 4723–4738 (2015).
52. Morrison, W. E. & Hay, M. E. Feeding and growth of native, invasive and non-invasive alien apple snails (Ampullariidae) in the United States: invasives eat more and grow more. Biological Invasions 13, 945–955 (2011).
53. Lockwood, J. L., Hoopes, M. E. & Marchetti, M. P. Invasion Ecology. (Blackwell Publishing, 2007).
54. Sargent, L. W. & Lodge, D. M. Evolution of invasive traits in nonindigenous species: increased survival and faster growth in invasive populations of rusty crayfish (Oreconetes rusticus). Evolutionary Applications 7, 949–961 (2014).
55. Gibson, M. R. et al. Reproductive biology of Australian acacias: important mediator of invasiveness? Diversity and Distributions 17, 911–933 (2011).
56. Blattmann, T., Boch, S., Tuerke, M. & Knop, E. Gastropod seed dispersal: an invasive slug destroys far more seeds in its gut than native gastropods. Plos One 8, e75243 (2013).
57. Knop, E. & Reusser, N. Jack-of-all-trades: phenotypic plasticity facilitates the invasion of an alien slug species. Proceedings of the Royal Society B-Biological Sciences 279, 4668–4676 (2012).
58. Kappes, H., Stoll, S. & Haase, P. Differences in field behavior between native gastropods and the fast-spreading invader Arion lusitanicus auct. non MABILLE. Belgian Journal of Zoology 142, 49–58 (2012).
59. Knop, E., Rindlisbacher, N., Kyser, S. & Gruebler, M. U. Locomotor activity of two sympatric slugs: implications for the invasion success of terrestrial invertebrates. Ecosphere 4, 92 (2013).
60. Kyser, S., Rindlisbacher, N., Gruebler, M. U. & Knop, E. Differential survival rates in a declining and an invasive farmland gastropod species. Agriculture Ecosystems & Environment 144, 302–307 (2011).
61. Aboim, M. A., Mavarez, J., Bernatchez, L. & Coelho, M. M. Intra-specific hybridization between two Iberian endemic cymprid fish: a comparison between two independent hybrid zones. Journal of Evolutionary Biology 23, 817–828 (2010).
62. Jenn, H. V. et al. Investigating temporal changes in hybridization and introgression in a predominantly bimodal hybridizing population of invasive sika (Cervus nippon) and native red deer (C. elaphus) on the Kimtyn Peninsula, Scotland. Molecular Ecology 19, 910–924 (2010).
63. Allgaier, C. How can too soft bodied animals be precisely connected? A miniature quick-connect system in the slugs, Arion lusitanicus and Arion rufus. Journal of Morphology 276, 631–648 (2015).
64. Papureanu, A.-M., Reise, H. & Varga, A. First records of the invasive slug Arion lusitanicus auct. non Mabille (Gastropoda: Pulmonata: Arionidae) in Romania. Malacologica Bohemoslovaca 276, 6–11 (2014).
65. Palmer, A. N. S., Styan, C. A. & Shearman, D. C. A. Foot mucus is a good source for non-destructive genetic sampling in Mediaegendes. Conservation Genetics 9, 229–231 (2008).
66. Regnier, C., Gargominy, O., Falkner, G. & Puillandre, N. Foot mucus stored on FTA (R) cards is a reliable and non-invasive source of DNA for genetics studies in molluscs. Conservation Genetics Resources 3, 377–382 (2011).
67. Holbeek, G., Maes, G. E., De Meester, L. & Vokcaert, F. E. M. Conservation of the introgressed European water frog complex using molecular tools. Molecular Ecology 18, 1071–1087 (2009).
68. Grimm, B. & Paill, W. Spatial distribution and home-range of the pest slug Arion lusitanicus (Mollusca: Pulmonata). Acta Oecologica-International Journal of Ecology 22, 219–227 (2001).
69. Haussner, J. Bestimmungsschlüssel der Gattung der Pdotshenen der Welt. Vol. 10 (Centre de cartographie de la Faune, 2000).
70. Aljanabi, S. M. & Martinez, I. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nuclear Acids Research 25, 4692–4693 (1997).
71. Hall, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95–98 (1999).
72. Librado, P. & Rozas, J. DnaSP: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452 (2009).
73. Leigh, J. W. & Bryant, D. POPART: full-feature software for haplotype network construction. Methods in Ecology and Evolution 6, 1110–1116 (2015).
74. Zemanova, M. A., Knop, E. & Heckel, G. Development and characterization of novel microsatellite markers for Arion slug species. Conservation Genetics Resources 7, 501–503 (2015).
75. Peakall, R. & Smouse, P. E. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6, 288–295 (2006).
76. Goudet, J. FSTAT (Version 1.2): A computer program to calculate F-statistics. Journal of Heredity 86, 485–486 (1995).
77. Pritchard, J. K., Stephens, M. & Donnelly, P. Inference of population structure using multilocus genotype data. Genetics 155, 945–959 (2000).
78. Evanno, G., Regnaut, S. & Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14, 2661–2663 (2005).
79. Earl, D. A. & vonHoldt, B. M. Structure Harvester: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4, 359–361 (2012).
80. Nielsen, E. E., Bach, L. A. & Kottlick, P. Hybridlab (version 1.0): a program for generating simulated hybrids from population samples. Molecular Ecology Notes 6, 971–973 (2006).
81. Vahra, J. P. & Primmer, C. R. Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. Molecular Ecology 15, 63–72 (2006).
82. Derryberry, E. P., Derryberry, G. E., Maley, J. M. & Brumfield, R. T. hzr: hybrid zone analysis using an R software package. Molecular Ecology Resources 14, 652–663 (2014).
83. R Core Team. R: a language and environment for statistical computing. http://www.R-project.org (2014).
84. Szymura, J. M. & Barton, N. H. Genetic analysis of a hybrid zone between the fire-bellied toads, Bombina bombina and Bombina variegata, near Cracow in southern Poland. Evolution 40, 1141–1159 (1986).
85. Szymura, J. M. & Barton, N. H. The genetic structure of the hybrid zone between the fire-bellied toads Bombina bombina and B. variegata - comparisons between transects and between loci. Evolution 45, 237–261 (1991).
86. Sutter, A., Beysard, M. & Heckel, G. Sex-specific clines support incipient speciation in a common European mammal. Heredity 110, 398–404 (2013).

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Author Contributions
All authors designed this study together. M.A.Z. collected the samples and conducted the molecular work and data analyses under the supervision of G.H. and E.K. The morphological assessment was conducted by M.A.Z. and E.K. All authors contributed to the writing of the manuscript and approved the final version.

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