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Population and conservation genomics of the adder (Vipera berus) in the UK

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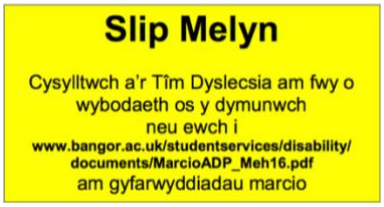

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Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

(Pozzi Andrea)

ABSTRACT

The intensification of human activities has led to a drastic loss of suitable habitats for various taxa, followed by patterns of population decline and isolation. During the past decades, in the UK, the adder (V*ipera berus*) has experienced a significant population decline. Once highly abundant, this reptile is now confined to small and fragmented populations, making it a national conservation concern. Despite this, data regarding the genetic status of the species in the UK are extremely scarce and limited to a single microsatellite-based study.

Therefore, for the first time, a whole-genome sequencing approach was here employed to assess patterns of population structure and genetic health in four adder populations from Wales and England. Despite strong population structure and a lack of recent gene flow between the investigated populations, the latter were characterised by extremely high levels of average genome-wide heterozygosity. The recovered heterozygosity levels turned out to be higher/comparable to those from higly genetically diverse vertebrates. However, further investigations revealed hidden extensive patterns of genomic erosion within the assessed adder's genomes, thus revealing overlooked inbreeding patterns. This discrepancy is likely to be the product of large and genetically diverse ancestral populations that have recently undergone rapid and extreme bottlenecks. This study highlights the efficiency of whole-genome sequencing in assessing patterns of population structure and genetic health. Moreover, the results call attention to the future persistence of the assessed adder populations, arguing for the potential necessity of human-mediated genetic conservation actions.

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1. INTRODUCTION

The Anthropocene period has been characterised by an unprecedented rate of biodiversity loss (Johnson *et al.,* 2017). This phenomenon, most commonly known as the "sixth mass extinction" (Wake and Vredenburg, 2008), has driven a decline in vertebrate populations of over 60% during the last fifty years (Turvey and Crees, 2019). The same negative trend has also strongly affected the abundance of global invertebrates (Wagner, 2020) and plant diversity worldwide (Vellend *et al.,* 2017). The decline of the Planet's biodiversity has negatively impacted various human-related sectors causing major economic losses across the globe (Sintayehu, 2018; Eyvindson *et al.,* 2018; Benton *et al.,* 2021).

Habitat loss and habitat fragmentation seem to represent some of the major drivers of the recent increase in biodiversity loss (Lino *et al.,* 2018; Chisholm *et al.,* 2018; Palmeirim *et al.,* 2020; Püttker *et al.,* 2020). Beyond the direct mortality caused by habitat loss and fragmentation, this phenomenon further leads to the formation of small populations, restricted within disconnected patches of suitable habitat (Trombulak and Frissell, 2000; Fahrig, 2002; Breininger *et al.,* 2012). Compared to large populations characterised by a sufficient levels of continuous gene flow, small and fragmented populations are generally affected by low to zero gene flow between them (Cushman, 2006; Walker *et al.,* 2008; Moore *et al.,* 2008). These conditions generally lead to inbreeding events causing a significant loss of genetic diversity, the fixation of potentially harmful alleles, and a decline in fitness and genetic adaptability (Templeton *et al.,* 1990; Dixo *et al.,* 2009; Rivera-Ortíz *et al.,* 2015; Lettoof *et al.,* 2021). Furthermore, small and fragmented populations are also more subject to stochastic demographic and environmental events, such as intensified genetic drift, compared to large populations and those characterised by moderate levels of gene flow (Rich *et al.,* 1979; Gabriel and Bürger, 1992; Lande, 1993; Hens *et al.,* 2017; van den Burg *et al.,* 2022). Thus, the sum of these factors makes small and fragmented populations more prone to extinction risk than larger and genetically diverse populations (Bijlsma *et al.,* 2000; O'Grady *et al.,* 2004; Frankham, 2005).

It has been estimated that, currently, circa 30% of the world's vertebrates occur in small and isolated populations characterised by inadequate levels of gene flow (Frankham *et al.,* 2019). Among them, ectotherm taxa, such as reptiles, due to their ectothermic nature, are notably more sensitive to alterations in climatic conditions than endothermic species (Aragón *et al.,* 2010; Kingsolver *et al.,* 2013; Gibson-Reinemer *et al.,* 2015). Within a planet characterised by a changing climate, this phenomenon highlights the necessity to investigate the status and

condition of the world's reptile populations in order to identify species of conservation concern and thus establish effective conservation measures.

Among reptiles, and in particular within snakes (suborder Serpentes), the members of the family Viperidae represent a significant conservation concern (Maritz *et al.*, 2016). Vipers are in fact characterised by slow life histories, low vagility, high philopatry and, due to their venomous nature, these snakes are also highly subjected to anthropogenic persecution (Phelps, 2004; Pleguezuelos *et al.*, 2007; Barbanera *et al.*, 2009; Toth *et al.*, 2010).

1.1 The adder: an overview

The adder (*Vipera berus*, Linneus 1758) is a small-to-medium-size viper characterised by an extremely wide geographic range that includes most of the Eurasian continent, ranging from western France to Korea, and from northern Norway to northern Greece (Mallow *et al.*, 2003; Phelps, 2010) (Figure 1). Due to the extent of its geographic range, the adder is recognised as the most widely distributed terrestrial snake on the planet (Saint Girons, 1980).

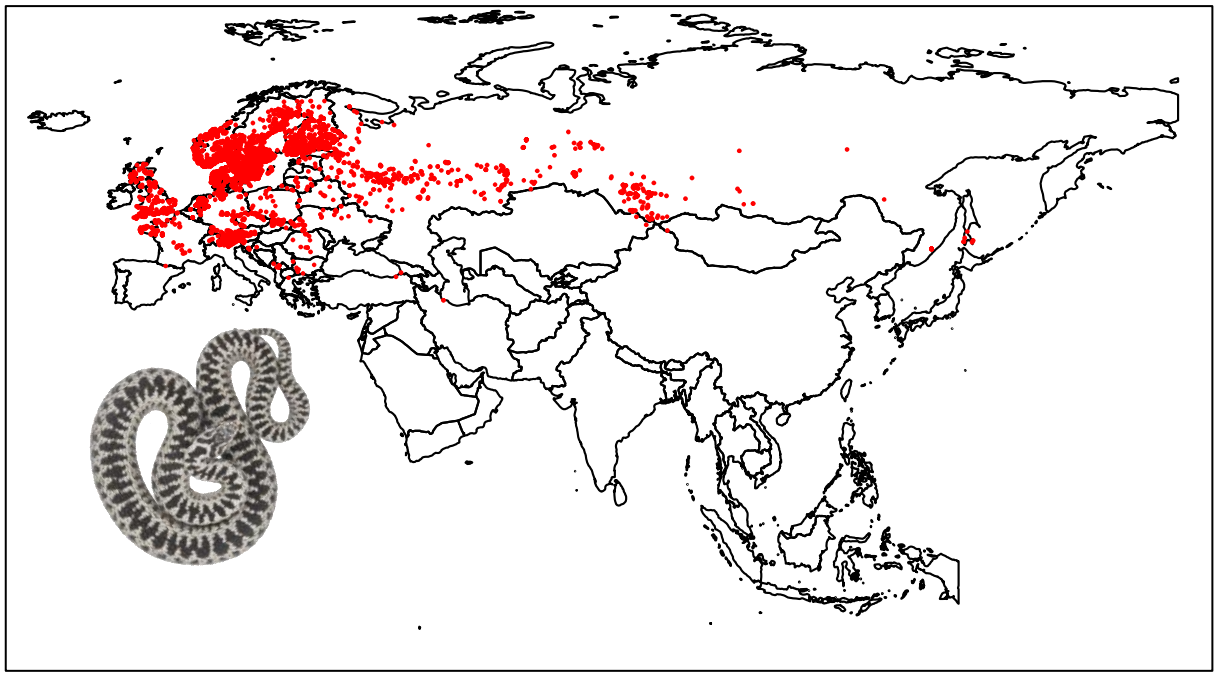

Figure 1. Approximate global distribution of the adder based on over 10000 publicly available presence data (www.gbif.org). Adder picture credit: Thor Håkonsen.

Currently, this species is subdivided into two subspecies characterised by distinct geographic ranges: *V. b. bosniensis* (Boettger, 1889) distributed in the Balkan peninsula, and the nominal subspecies which occupies the rest of the distributional range (Ursenbacher *et al.* 2009b).

Nevertheless, the systematics of the adder are still debated. The validity of *V. b. sachalinensis* (Zareveskij, 1917) endemic to Eastern Russia, North Korea and China has been questioned (Ursenbacher *et al.* 2009b; Cui *et al.* 2016). A recent research effort, involving the employment of an integrative taxonomic approach, suggested that *V. berus* should also include, at a subspecies level, *V. barani* (North-west Turkey) and *V. nikolskii* (Ukraine, southern Russia, Romania, and Moldova) (Freitas *et al.*, 2020). Further subspecies such as *V. b. marasso* (north-western Italy and southern Switzerland) and *V. b. walser* (north-eastern Italy) have been proposed by Schmidtler and Hansbauer (2020), Speybroeck *et al.*, (2020), Sindaco and Razzetti (2021). Despite that, due to the lack of strong supporting data, more work is needed to confirm the taxonomic validity of the latter two taxa.

Along its range, the adder inhabits a wide variety of heterogeneous habitats that span from sea-level heathlands and sand dune systems in the northern part of its distribution, to alpine prairies up to 3000 m asl in the southern part of its range (Grano *et al.*, 2017; Otte *et al.*, 2020).

Due to its vast geographic range, the adder is currently classified as Least Concern by the IUCN Red List (Munkhbayar *et al.*, 2021). Despite that, the same assessment highlights a worrying global population decline. In fact, significant local population reduction and extirpation events have been documented across most of its geographic range (e.g., Reading *et al.*, 1996; Ursenbacher *et al.*, 2009b; Madsen and Ujvari, 2011; de Massary *et al.* 2015; Podloucky *et al.* 2020). As an example, an ecologically unique and potentially distinct adder subspecies, which inhabited the lowland plain of the Italian Po River, went extinct during the second half of the 20th century (Gentilli *et al.* 2006).

The major causes of the global decline of the adder must be identified within direct and indirect anthropogenic pressures such as habitat loss and fragmentation, persecution, and climate change (Julian and Hodges, 2019; Francois *et al.*, 2021).

1.2 The adder in the UK

The UK, due to its biogeographic history and high latitude, is characterised by a poorly diverse herpetofauna that includes just three native species of terrestrial snakes (Speybroeck *et al.*, 2016). Among these, the adder is the sole representative of the family Viperidae in the country and the only venomous snake species. This reptile is geographically widespread within the UK, and it is present within all the country members apart from Ireland (Phelps, 2010) (Figure 2).

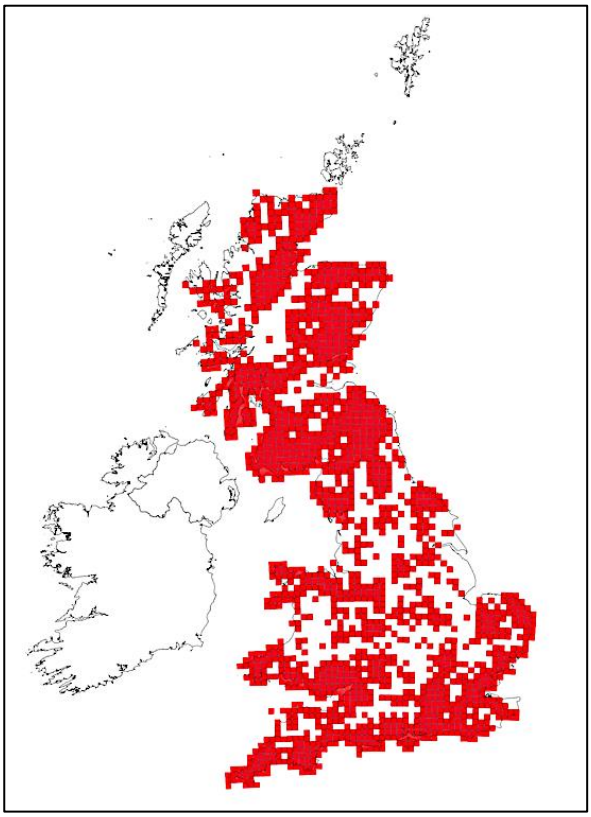

Figure 2. Current geographic distribution of the adder in the UK based on UTM 10x10 km grid cells. Data and map obtained via NBNatlas (https://nbnatlas.org/).

The adder has been considered historically common and abundant in the UK, to the extent that its presence was erroneously considered a potential public health issue (Leighton, 1901). Despite that, this phenomenon didn't lead to the institution of a government-promoted bounty system as happened in other parts of the range of *V. berus* (e.g., Toth *et al.*, 2010). Systematic persecution has been identified as the major cause of local extirpation of other viper species such as the timber rattlesnake (*Crotalus horridus*) in part of the USA and Canada (Brown, 1993; Cook, 2004) and the Hungarian meadow viper (*Vipera ursinii rakosiensis*) in Austria (Edgar and Bird, 2005).

Despite being historically widespread, the adder has gained legal protection from intentional persecution with the inclusion of the species within the Wildlife and Countryside Act back in 1981. Furthermore, this species has been recognised as a species of conservation concern and thus incorporated into the priority species list of the Biodiversity Action Plan in 2007 (BRIG, 2007). More recently, in the light of the current decline in population size and distributional range (Baker *et al.*, 2002; McInerny, 2018; Gardner *et al.*, 2019), the adder has been reclassified under the IUCN Red List criteria as vulnerable to extinction in England and as near threatened within the rest of its UK range (Foster *et al.*, 2021). At the moment, with the

quinquennial review of the Wildlife and Countryside Act's appendix, the legal conservation status of the adder in the UK is again under debate and it's possible that the species, due to conservation concerns, will likely gain complete legal protection (Amphibian and Reptile Conservation Trust, pers. comm.).

The causes behind the decline of the adder in the UK seem to be multiple and concatenated, nevertheless, the loss of suitable habitat and anthropogenic disturbance seem to represent the major sources of such a significant and widespread population decline (Gardner *et al.*, 2019; Julian and Hodges, 2019; Foster *et al.*, 2021). It has in fact been estimated that during the last few decades, solely within England, the adder's distributional range has been reduced by 40% (Gleed-Owen and Langham, 2012). The fragmentation of the suitable habitat has led to the formation of small and isolated populations. The results of an 11-year-long survey conducted at a UK-wide geographic level highlighted that out of 130 surveyed sites, 90% of them housed small (less than 10 adult specimens) adder populations, with large populations (more than 10 adult specimens) occurring only within 10% of the study sites (Gardner *et al.*, 2019). Moreover, the same research further reported a significant decline in population size across all the surveyed small populations, predicting the extirpation of the latter within the next two decades. According to the authors, by 2032 the adder in the UK will be restricted to a few large populations, which are currently undergoing a slight population increase.

Events of local extinction have been already reported within the UK (e.g., Foster *et al.*, 2021) and these latter may have been caused by a decline in population fitness due to the lack of gene flow, this latter ultimately caused by habitat loss and fragmentation.

A textbook example of this phenomenon is represented by an isolated Swedish adder population intensively studied by Madsen and colleagues (1996; 2011). At the beginning of 1990, the population consisted of only nine individuals and the authors reported a high percentage of stillborn and deformed offspring, thus highlighting a low level of population recruitment (Madsen *et al.*, 1996). This phenomenon was then correlated with low levels of genetic diversity, significant inbreeding depression and small effective population size, which potentially have contributed to the fixation of deleterious alleles leading in this way to a reduction in offspring survival (Madsen *et al.*, 1996). Correlation between low levels of genetic diversity, phenotypic abnormalities, and reduced offspring survival have been noticed in other European viper species (e.g., *Vipera ursinii rakosiensis*: Újvári *et al.*, 2002; Tóth *et al.*, 2005). In the Swedish adder population, the lack of gene flow coupled with the low effective population size and the high level of inbreeding depression pushed the population to the brink of extinction (Madsen and Ujvari, 2011).

Similarly, due to the persistence of most UK adders within small and isolated populations, it is highly likely that the lack of gene flow between populations, inbreeding depression and small effective population size, might represent significant threats to the persistence of this reptile in the UK. Unfortunately, data regarding the genetic status of the adder in Britain are extremely scarce.

The sole genetic study conducted so far on UK adders highlighted significant levels of intrapopulation relatedness among individuals from various populations in southern Britain (Ball *et al.,* 2020). Despite that, the same study didn't underline any evidence of loss in heterozygosity or inbreeding depression among the assessed populations. Nevertheless, as the same authors suggested, this latter phenomenon may be due to flaws in the applied methodology which, relying on the employment of mitochondrial DNA data and microsatellites (these latter represented by pre-existing microsatellites for *V. berus* and *V. ursinii*), may be inadequate to capture the real extent of population structure and heterozygosity loss (see below).

A promising conservation approach that would potentially help to assure the persistence of the adder in the UK is the implementation of genetic rescue (Madsen *et al.,* 2004; Whiteley *et al.,* 2015; Madsen *et al.,* 2020). This aims to increase genetic diversity via the introduction, within an inbred population, of foreign alleles through the temporal or permanent human-mediated migration of individuals from a source population (Ingvarsson, 2001). This strategy has been applied successfully in different occasions involving various and diverse taxa (e.g. Hogg *et al.,* 2006; Benson *et al.,* 2011; Åkesson *et al.,* 2016). For what concerns the adder, the implementation of genetic rescue has played a vital role in guaranteeing the survival of the previously mentioned highly inbred Swedish population. In fact, following the temporal introduction of 20 males (foreign individuals were removed after four breeding seasons [Madsen *et al.,* 1999]) from a neighbouring source population, the focal population has undergone a drastic improvement represented by a significant increase in genetic diversity (H_o prior to genetic rescue (1991-1992) = 0.181, H_o subsequent to genetic rescue (2017) = 0.243) and population recruitment (number of recruits in 1991-1992 < 5, number of recruits in 2017 = 40 circa) (Madsen *et al.,* 2020). Notably, the authors highlighted the long-term genetic benefits on the recipient population, whose genetic diversity kept increasing even after a bottleneck event caused by further habitat fragmentation and a consequent reduction in population size by 80% between 2009 and 2010.

Unfortunately, genetic rescue has been scarcely implemented as a conservation strategy due to the risk of outbreeding depression (Tallmon *et al.,* 2004; Frankham, 2015; Waller, 2015).

This phenomenon usually takes place when two or more distantly related populations, which may be characterised by fixed chromosomal differences, historical lack of gene flow, adaptive genetic differences, and coadapted-gene complexes, come back into contact leading in this way to a significant loss of fitness, reducing in this way the beneficial effects of genetic rescue (Frankham *et al.,* 2011). Outbreeding depression following genetic rescue has been observed in different cases covering phylogenetically diverse taxa (for review, Edmands, 2007). Furthermore, the translocation of individuals from one population to another can also lead to the introduction of cryptic and unwanted deleterious alleles, which may have a drastic impact on the recipient population (Kyriazis *et al.,* 2021). For these reasons, in order to avoid the potential negative side effects of genetic rescue, it is essential to obtain reliable preliminary data regarding the genetic status of all the populations involved. This allows the potential efficacy of implementing such conservation strategy to be assessed, while also identifying the most appropriate source population (Kyriazis *et al.,* 2021).

1.3 Conservation in the genomic era

During the past decades, various genetic tools have been exploited to assess and monitor the genetic status of wild populations (DeWoody *et al.,* 2021). From the use of allozymes and mitochondrial markers, in more recent years, microsatellites have represented the most used molecular tool within the fields of population and conservation genetics (Selkoe and Toonen, 2006; Vieria *et al.,* 2016). Microsatellites are co-dominant nuclear markers made up of repetitive tandem short sequences (between 1-10 nucleotides) characterised by a high, even if variable across the genome, mutation rate (Jarne and Lagoda, 1996; Fischer *et al.,* 2017). Microsatellites have been widely implemented within population genetics studies mainly because of their statistical power and allelic richness (Guichoux *et al.,* 2011; Abdul-Muneer, 2014; Vieria *et al.,* 2016). Furthermore, the polymorphic nature of these markers allows them to capture recent evolutionary events while maintaining a low cost of development (Tsitrone *et al.,* 2001; Sun *et al.,* 2009). On the other hand, microsatellites are affected by various biases and limitations. Firstly, the high rate of null alleles during microsatellites amplification may be misleading in capturing the genuine genetic diversity of a population, leading to underestimations of the latter parameter (Hedgecock *et al.,* 2004; Chapuis and Estoup, 2007). Furthermore, homoplasy commonly affects microsatellites causing significant biases in the estimation of intrapopulation genetic diversity and interpopulation structuring (Angers *et al.,* 2000; Estoup *et al.,* 2002). Moreover, microsatellites are population/species-specific, making them poorly transferable across taxa, causing ascertainment bias, and thus generating

misleading outcomes in the case of cross-species application (Barbara *et al.*, 2007; Zhan *et al.*, 2008; Aiello *et al.*, 2020). In addition, microsatellites made up a small percentage of the entire genome (e.g., 3-5% mammalian genome), capturing in this way just an extremely small proportion of the whole genomic diversity (Warren *et al.*, 2008). Finally, the outcomes produced by microsatellites analysis are highly influenced by the sample size, demanding a large number of sampled individuals in order to obtain precise genetic measurements (Coltman and Slate, 2003; Hale *et al.*, 2012; Sunde *et al.*, 2020). However, some authors still advocate for the application of microsatellites in population and conservation genetics, particularly within contexts characterised by scarce financial availabilities (Hauser *et al.*, 2021).

With the advent of Next-Generation Sequencing technologies, microsatellites have been largely replaced by the use of Reduced Representation Sequencing approaches (Flanagan *et al.*, 2019; Roques *et al.*, 2019; Sunde *et al.*, 2020). Among these, methods based on the employment of restriction enzymes, such as RAD sequencing and ddRAD sequencing, appear to be the most used within the fields of population and conservation genetics (Davey and Blaxter, 2010; Peterson *et al.*, 2012; Andrews *et al.*, 2016). These approaches allow the recovery of genetic markers at a higher number and density compared to microsatellites, providing more reliable and accurate results of genome-wide diversity compared to the latter markers. (Vendrami *et al.*, 2017; Thrasher *et al.*, 2018; Lemopoulos *et al.*, 2019; Zimmerman *et al.*, 2020). For these reasons, Reduced Representation Sequencing approaches allow assessing various population parameters with a smaller sample size compared to microsatellites (Jeffries *et al.*, 2016). Furthermore, due to the increasing application of Reduced Representation Sequencing methods and the genome-wide nature of the markers recovered through them, some authors sustain the existence of a conceptual shift from the fields of population and conservation genetics to the fields of population and conservation genomics (Stahlke *et al.*, 2020). Despite that, the genetic variation assessed through Reduced Representation Sequencing approaches is restricted to the variable sites located in the proximity of the cut sites, capturing in this way just a limited proportion of the real genomic variation (Fuentes-Pardo and Ruzzante, 2017). Additionally, these approaches require high-quality genetic samples and are particularly affected by biases during library preparation and sequencing (Arnold *et al.*, 2013; Shafer *et al.*, 2017; Flanagan and Jones, 2018).

While the above-mentioned approaches, due to their limitations and biases, may turn out to be insufficient to reliably obtain the population genetic data needed for the implementation of conservation actions, such as genetic rescue, whole-genome sequencing allows those

constraints to be overcome, thus providing a clearer picture of the genetic status and health of the focal populations (Fuentes-Pardo and Ruzzante, 2017; Saremi *et al.,* 2019). The approach provides the highest number and density of genetic markers out of all the previously mentioned methods (Lou *et al.,* 2021). This allows the real extent of genome-wide heterozygosity to be reliably measured, which is currently considered one of the fundamental parameters in conservation and population genomics (Kardos *et al.,* 2021; DeWoody *et al.,* 2021). Moreover, whole-genome sequencing permits to investigate a wide array of population and demographic parameters while requiring a limited number of sampled individuals, which makes the latter approach ideal to investigate the genetic status of species occurring in small populations (Li and Durbin, 2011; Westbury *et al.,* 2018; Paijmans *et al.,* 2021; Foote *et al.,* 2021). The major drawback associated with the application of whole-genome sequencing in population and conservation genomics is represented by the sequencing cost associated with this approach, especially at high sequencing depths, as well as the cost associated with the required bioinformatic expertise (Hendricks *et al.* 2018; Schweizer *et al.* 2021; Hogg *et al.* 2022). This economic factor constitutes a major obstacle for most of the population and conservation projects, the latter usually characterised by extremely restricted financial and computational resources.

1.4 The application of low coverage whole-genome sequencing in population and conservation genomics

A promising and cost-effective alternative to reduced-representation sequencing and whole-genome sequencing approaches in conservation genomics may be represented by the exploitation of low-coverage whole-genome sequencing (Fuentes-Pardo and Ruzzante, 2017). This latter allows genome-wide data to be recovered while limiting the sequencing effort at low-to-medium coverage depths (0.5-10x). (Lou *et al.,* 2021). The most evident benefit of this approach compared to high-depth whole-genome sequencing is represented by the significant difference in sequencing costs, due to the lower coverage depth of the former approach (Rustagi *et al.,* 2017; Martin *et al.,* 2021). This phenomenon, summed with the exponential drop during the past two decades in the per-Mb sequencing costs, often allows the employment of low-coverage whole-genome sequencing at a comparable price-point to reduced representation sequencing (Preston *et al.,* 2021; Lou *et al.,* 2021). In this way, taking into consideration the often limited budget of conservation studies, it's thus possible to cost-effectively sequencing a considerable number of individuals, covering in this way a wide geographic scenario (Fumagalli, 2013). In regard to reduced-representation sequencing

approaches, another major benefit of low-coverage whole-genome sequencing consists in the wider breadth of genomic coverage of this latter approach. In fact, while the former approaches, such as RAD-seq, provide a limited and random window on the study subject's genome (Fuentes-Pardo and Ruzzante, 2017), low-coverage whole-genome sequencing is able to retrieve an impressive high density of genetic markers from the entire genome of the study subject (Bizon *et al.* 2014). Furthermore, this latter approach is not limited, like reduced-representation sequencing, to the sole detection of changes in nucleotide bases, but it additionally allows structural differences to be recovered and mutations within regulatory elements to be detected (Mathelier *et al.*, 2015). The wider sequencing breadth of the latter approach compared to more traditional sequencing techniques permits to investigate intra and inter-population parameters at a higher accuracy while requiring a smaller sample size (Wright *et al.*, 2019; Lou *et al.*, 2021). This latter feature of low-coverage whole-genome sequencing appears to be extremely important when dealing with rare, threatened, and declining species, which usually occur at low densities, and on which is fundamental to strongly limit the sampling pressure (Barbosa *et al.*, 2020; Hohenlohe *et al.*, 2021).
This is the case of the adder in the UK, where the species mainly occurs in small, isolated, and declining populations that wouldn't be suitable for the employment of traditional sequencing approaches in terms of sample number and sampling pressure. On the other hand, due to the above-mentioned benefits, the employment of low-coverage whole-genome sequencing seems to represent a valid option to investigate the genetics and demographic history of this declining taxon.
Unfortunately, mainly due to its low sequencing depth, this method is characterised by a series of constraints and limitations. Firstly, due to the short nature of the reads obtained during the sequencing process, low-coverage whole-genome sequencing requires mapping the reads to a pre-assembled reference genome of the species concerned (Supple and Shapiro, 2018). This represents a significant limitation for conservation studies as just a small percentage of Earth's biodiversity is currently provided with a reference genome (Lewin *et al.*, 2018; Brandies *et al.*, 2019). In order to overcome this issue, reference genomes from closely related species can be used, even if the relative outcomes must be interpreted with caution (Galla *et al.*, 2018; Bohling, 2020). Moreover, various projects are aiming to sequence all the currently known eukaryotic species within the next decade (Blaxter *et al.*, 2022; Lewin *et al.*, 2022).
Furthermore, the low sequencing depth that characterises this method does not allow individual genotypes to be confidently inferred (Nielsen *et al.*, 2012; Benjelloun *et al.*, 2019).

Luckily, this latter limitation can be overcome with the employment of probabilistic methods such as genotype likelihood (GLs) (Fumagalli *et al.*, 2014; Hui *et al.*, 2020; Chat *et al.*, 2021). Finally, due to the vast amount of genetic data produced by low-coverage whole-genome sequencing, this approach can be extremely demanding in terms of informatic resources, requiring the use of computer clusters and a significant amount of bioinformatic skills (Sboner *et al.*, 2011; Hendricks *et al.*, 2018; Luikart *et al.*, 2018).

Here, a low-coverage whole-genome sequencing approach was employed for 16 adder specimens from four English and Welsh populations (four adders per each population) characterised by different population size and biogeographic histories. The presence of an adder reference genome was exploited to measure various population parameters, assessing in this way the status of the sampled population while limiting the number of sampled individuals and thus the impact on the whole population. Moreover, the presence of *Ophidiomyces ophidiicola* was investigated among the sequenced adder genomes. The latter represents the causative agent of the snake fungal disease (SFD), a potentially lethal and globally widespread disease, already recorded within the UK (for review, Di Nicola *et al.* 2022). Levels of heterozygosity from the adder populations were then compared with those from other vertebrate taxa and the ability of genome-wide heterozygosity in capturing the real extent of genetic diversity was investigated.
This study aimed to investigate the genetic and population status of various UK adder populations, assess the presence of historic or current decline, and provide preliminary evidence potentially useful for conservation managers. To the authors' knowledge, this pilot study represents the first attempt to explore the population status and genetic health of the adder through the application of a whole-genome sequencing approach.

2. AIMS AND OBJECTIVES

2.1 Primary aims

I. To assess population genetic patterns and genome-wide heterozygosity within adder populations.
II. To compare population structure and genome-wide heterozygosity between adder populations.

2.2 Secondary aims

III. To test the effectiveness of non-invasive sampling methods for population genomic analysis.

IV. To assess for the genomic presence of *Ophidiomyces ophidiicola* in the analysed adders.

3. MATERIALS AND METHODS

3.1 Sampling and data generation

3.1.1 Populations details

Adder samples were collected under the Bangor University ethical approval for sample collection: COESE2023WW01A.

These originated from three populations in North Wales (Anglesey 1, Anglesey 2, Gwynedd) and one population in England (Staffordshire) (Figure 3). Population were divided into small and large on the bases of the number of reproductive specimens (10 individuals threshold) (Gardner *et al.,* 2019).

Brief descriptions of these populations are herein reported:

- Anglesey 1: This population is situated in an urbanised area on the eastern side of Anglesey Island. The area's overall extension is under 100 hectares. The latter is defined by a high density of heather (*Calluna vulgaris*) and gorse shrubs (*Ulex europaeus*). The population is considered to be small with fewer than 10 reproductive individuals.
- Anglesey 2: This population is situated on the western coast of Anglesey Island. The area's overall extension is greater than 100 hectares. The area is characterised by sand dunes covered by marram grass (*Ammophila sp.*). This population is considered to be large with more than 10 reproductive individuals.
- Gwynedd: This population is situated within an area surrounded by farmland in Gwynedd County, North Wales. The area's overall extension is under 100 hectares. The latter is defined by a high density of heather (*Calluna vulgaris*) and gorse shrubs (*Ulex europaeus*). The population is considered to be small with fewer than 10 reproductive individuals.
- Staffordshire: This population is situated in the County of Staffordshire, England. The area's overall extension is greater than 100 hectares. The latter is characterised by

heathland bordered by coniferous forest. The population is considered to be large, with more than 10 reproductive individuals.

The Euclidean distance between Anglesey 1 and Anglesey 2 populations is around 20km. The Gwynedd population is located 45 km circa from the two Anglesey populations and around 150 km from the Staffordshire one.

Geological evidence show that during the last five thousand years, Anglesey Island has been completely isolated from the mainland (Roberts *et al.* 2011). The island is separated from mainland Wales by the Menai Strait, which measures 250m at its narrowest point.

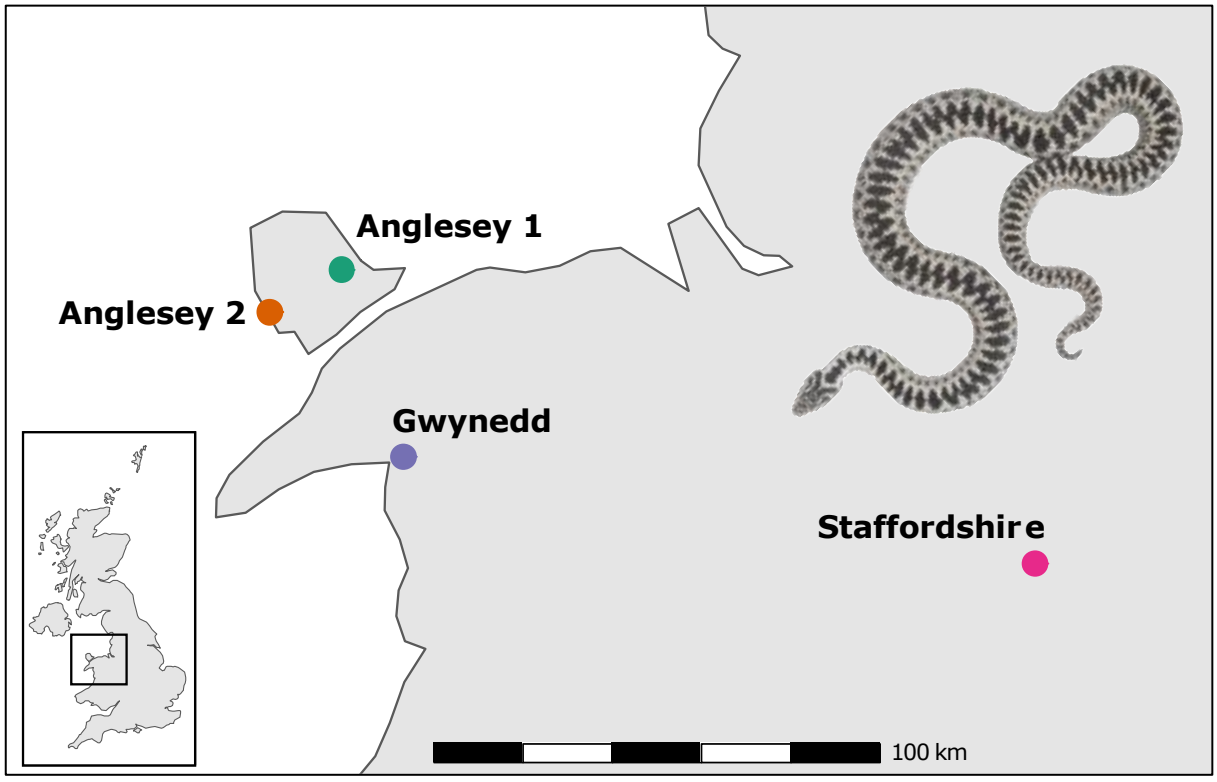

Figure 3. Sampling location of the four adder populations. Adder picture credit: Thor Håkonsen

3.1.2 Samples

A total of sixteen samples, four samples from each one of the four populations, were used during this project. These consisted of scale tissue stored in 75% ethanol. In order to test the efficacy of non-invasive samples in providing enough genetic material for population genomic analysis, a sample from Anglesey 2 (ww5395) consisted of a freshly collected shed skin, subsequentially dried and stored within a paper envelope.

In the context of phylogenetic analysis, publicly available low-coverage whole-genome sequences from two Danish individuals of *V. berus* were retrieved from NCBI database: ZMUCR90374 (NCBI accession number: SAMN14333252) and ZMUCR90375 (NCBI

accession number: SAMN14333253). The currently available adder reference genome (NBCI accession number: SAMN02780930) is characterised by a scaffold assembly level, a total sequence length of 1.5 Gb, a scaffold count of 28883 and scaffold N50 of 126452.

Table 1. List of UK adder samples. The table contains data regarding the sample location and the nature of the collected sample.

Specimen ID	Location	Sample type
ww5272	Anglesey 1	Scale clip
ww5273	Anglesey 1	Scale clip
ww5274	Gwynedd	Scale clip
ww5275	Gwynedd	Scale clip
ww5280	Anglesey 1	Scale clip
ww5281	Anglesey 1	Scale clip
ww5287	Staffordshire	Scale clip
ww5288	Staffordshire	Scale clip
ww5289	Staffordshire	Scale clip
ww5290	Staffordshire	Scale clip
ww5300	Anglesey 2	Scale clip
ww5301	Anglesey 2	Scale clip
ww5302	Anglesey 2	Scale clip
ww5305	Anglesey 2	Shed skin
ww5395	Gwynedd	Scale clip
ww5396	Gwynedd	Scale clip

3.1.3 Laboratory procedures

DNA from both types of samples was extracted through the use of commercially available DNA extraction kits (DNeasy Blood & Tissue Kit, QIAGEN) following the manufacturer's protocol (Appendix I). Extracted DNA was quantified using a Qubit 3 fluorometer and a dsDNA HS assay kit, following the manufacturer's instructions (Appendix II).
Library preparation, quality control, and whole-genome sequencing were carried out by an external commercial facility (Novagene Ltd., Cambridge), using a NovaSeq platform producing paired-end 150bp sequencing reads, aiming for 8x coverage of the genome of each adder.

3.1.4 Data processing

The BEARCAVE environment was used for data curation and processing (https://github.com/nikolasbasler/BEARCAVE).

Initially, Cutadap V 1.1.8 (Martin, 2011) was used to trim adapters and exclude trimmed reads shorter than 30 bp from the raw genetic sequences with a minimum overlapping threshold of one base between read and adapter. Afterwards, FLASH v. 1.2.11 (Magoč and Salzberg, 2011) with default settings was employed to merge overlapping pair-end reads. Subsequentially, reads were mapped to the adder reference genome (Swedish origin) using the BWA v. 0.7.17 mem algorithm (Li *et al.* 2009a). Finally, Samtools v 1.3.1 (Li *et al.* 2009b; Danecek *et al.* 2021) was used to remove reads that didn't map to the reference genome (-F 4), secondary alignments (-F 256), reads characterised by low mapping score (-q 30), and products of PCR duplication (- rmdup).

The same protocol was then used to map individuals' genome sequences to the *Ophidiomyces ophiodiicola* reference genome (NBCI accession number: MWKM00000000), in order to assess for the presence of the keratinophilic fungus within the assessed adder samples. The latter represents the causative agent of the Snake Fungal Disease (SFD), a widespread condition of conservation concern (Di Nicola *et al.* 2022, for review).

3.2 Population structure

3.2.1 Principal component analysis

Patterns of population structure were investigated among the UK adder samples via principal component analysis (PCA). This latter multivariate analysis is one of the most widely employed dimension reduction methods for the assessment of population structure in genomic datasets (Alhusain and Hafez, 2018) (but see Elhaik, 2022 for a critical view). ANGSD v. 0925 (Korneliussen *et al.* 2014) was used to calculate a covariance matrix by sampling random single reads (-doIBS 1). During the process, only genome scaffolds larger than 100 kb were analysed (-rf), singletons were removed by setting the minimum minor allele frequency at 0.06 (-minFreq 0.06), the minimum base quality was set at 30 bp (-minQ 30), the minimum read mapping quality was set at 30 bp (-minMapQ 30), and finally, sites with missing data were discarded setting the minimum individual filter to include all the individuals (-minInd 16). Subsequentially, PCA was performed in R using the "eigen" function and the obtained covariance matrix (R Core Team, 2021).

3.2.2 Neighbour-joining tree

In order to investigate the relationship among clusters, a Neighbour-joining (NJ) tree approach was employed.

Initially, ANGSD v. 0925 was used to retrieve a distance matrix via random sampling of reads (-doIBS 1) from the 16 UK adders and using the 2 Danish adders as an outgroup. The distance matrix retrieval process was performed applying the following filters: minimum minor allele frequency (-minFreq 0.06), minimum base quality (-minQ 30), minimum read mapping quality (-minMapQ 30), minimum individual (-minInd 18).

Subsequentially, the resulting distance matrix was used to produce the relative NJ tree in R studio employing the APE v. 5.6.1 library (Paradis and Schliep, 2019). During the latter process, the two Danish samples were used to root the NJ tree.

3.2.3 Ancestry analyses

In order to investigate potential patterns of gene flow among the UK populations, ancestry analyses were performed in NGSadmix v. 32 (Skotte *et al.* 2013). This approach allows individual's ancestry to be reliably investigated while accounting for the uncertainty correlated with the use of low and medium coverage whole-genome sequencing data thanks to the employment of genotype likelihoods.

In the first place, ANGSD v. 0925 was used to generate a BEAGLE file containing the UK adders' genotype likelihoods data. To avoid the introduction of computational biases, the analyses were focused on genomic scaffolds larger than 100 Kb (-rf) and a SAMtools model for genotype likelihood estimation was employed (-GL 1). SNPs filtering threshold was set at 1e-6 (-SNP_pval). Minimum base quality was set at 30 bp (-minQ 30), and minimum read mapping quality was set at 30 bp (-minMapQ 30). Minimum and maximum individual sequencing depth filters corresponded to the half and the double of the average sequencing coverages' value respectively (-setMinDepthInd 6) (-setMaxDepthInd 24). Additionally, in order to remove singletons, a minor allele frequency filter was set at 0.1 (-minMaf). Ancestry analyses were performed for three different values of K, corresponding to the number of potential ancestral populations (K=3, K=4, and K=5). The choice of the K values was based on a priori geographic assumption of 4 populations, further tested for 1 population above and below this assumption.

The robustness of each K value was tested via four independent runs, each one characterised by unique seed values (-seed).

Resulting estimated admixture proportion files were then processed in R and displayed as colour-coded bar plots.

3.3 Genetic diversity

3.3.1 Heterozygosity estimation

Average genome-wide heterozygosity was used as a proxy to assess the genetic health of the UK adders. This genetic parameter is highly used in population and conservation genetics and allows to account for neutral and adaptative variations (Schmidt *et al.* 2021).

ANGSD v 0925 was used to retrieve individual average genome-wide estimations on the bases of maximum likelihood site frequency spectrum (SFS) calculations (Nielsen *et al* 2012). Initially, site allele frequency likelihoods (-doSaf 1) were generated for each individual UK adder: analyses were restricted to genomic scaffolds larger than 100 Kb (-rf), minimum base quality was set at 30 bp (-minQ 30), the minimum read mapping quality was set at 30 bp (-minMapQ 30), while minimum and maximum sequencing depth filters corresponded to the half and to the double of the average sequencing coverages' value respectively (-setMinDepth 6) (-setMaxDepth 24), the reference genome assembly of the adder was used as the ancestral state (-anc), and a SAMtools model for genotype likelihood estimation was employed (-GL 1).

Subsequentially, individual site allele frequency likelihoods were employed for Maximum Likelihood estimation of the SFS (Site Frequency Spectrum), using the "realSFS" function, a maximum number of iterations in the EM algorithm of 100 (-maxIter 100), and limiting the optimisation to 10000 sites (-nSites 100000).

Average population genome-wide heterozygosity was calculated for each of the four adder populations (mean of individual's heterozygosity values) and then compared with heterozygosity values from other vertebrate taxa obtained from Westbury *et al.* 2018. The latter study employed methods for heterozygosity estimation that allow for comparison with estimates from this study.

Finally, the distribution of genome-wide heterozygosity along 100 kb non-overlapping windows within the individual adder genomes was graphically explored through the use of histogram plots in R.

3.3.2 Nucleotide diversity

The rate of allele fixation within each adder population was investigated via the estimation of nucleotide diversity (π) along 100 Kb genomic windows.

In ANGDS v. 0925, population-wide SFS estimations were obtained for each of the four adder populations (see above for filters details). Afterwards, population-wide SFS were employed to obtain per-site nucleotide diversity (in ANGSD denominated as pairwise-theta, "Pt") estimation (-saf2theta). Finally, a sliding-window approach was employed to collect nucleotide diversity estimation along 100 Kb windows (thetaStat) (do_stat).

Resulting nucleotide diversity estimations were processed and visualised in R.

3.3.3 Fst

Genomic fixation between populations pairs was investigated via Fixation index (Fst) estimation.

In ANGSD v. 0925, population-wide SFS estimations (-doSaf 1) were carried out for each one of the four adder populations (see above for filtering details). Subsequently, estimation of 2d SFS was performed for each population pair. Afterwards, these were employed to obtain weighted global Fst estimations (fst stat). Finally, a sliding window approach was employed in order to obtain Fst estimation along 100 Kb genomic windows (fst stat2).

Resulting sliding-windows Fst estimations were visualised via R.

4. RESULTS

4.1 Resulting data

During the trimming and merging processes, an average of 37% of the reads form UK adder sequences were characterised by sequencing adapters and an average of 28.37 % of UK adder sequences contained combined reads (Appendix IV).

Sequences from all the 16 UK adder samples, including the non-invasive shed skin sample, were employed for population and genetic analyses due to the high mapping coverage. The latter were in fact characterised by a mapping coverage of over 11 Gb (total amount of mapped data). On the other hand, due to the low mapping coverage of the two Danish adder sequences (2.16 Gb and 4.83 Gb, total amount of mapped data), these were employed exclusively during phylogenetic analyses and excluded from further analyses.

The UK adder sequences were characterised by a mean read depth that spanned from 8.5x to 12.2x. Consistent with the low mapping coverage, the two Danish adder sequences were characterised by a read depth of 2.1x and 3.9x (Table 2).
The low average breath of coverage (0.34 %) and the high average read depth (75x) highlighted the absence of *O. ophiodiicola* in the investigated UK adder sequences (Appendix V).

Table 2. Mapping coverage (Gb) and read depth for individual adder sequences mapped to the adder reference genome post-filtering. Results relative to the non-invasive sample (shed skin) are highlighted with a bold font. Further details can be found in the Appendix IV.

Sample ID	**Mapping coverage (Gb)**	**Read depth**
WW5272	11.61	8.9
WW5273	14.56	11.2
WW5274	11.43	8.8
WW5275	11.24	8.7
WW5280	11.33	8.8
WW5281	13.00	10.0
WW5287	11.49	8.9
WW5288	15.83	12.2
WW5289	14.53	11.2
WW5290	14.01	10.8
WW5300	14.17	10.9
WW5301	13.84	10.7
WW5302	11.02	8.5
WW5305	**12.99**	**10.0**
WW5395	14.38	11.1
WW5396	12.51	9.67
ZMUCR90374	2.16	2.1
ZMUCR90375	4.83	3.9

4.2 Population structure

4.2.1 Principal component analysis

After the filtering process, a total number of around 7.6 million informative sites were employed for further analysis. The results of the PCA analysis highlighted the presence of

four distinct population clusters, where each specimen clustered according to its geographic origin (Figure 4).

An east-to-west segregation pattern was observed along the PC1, the latter explaining 11.4% of the total variation. On the other hand, a subdivision between English (Staffordshire) and Welsh (Anglesey 1, Anglesey 2, and Gwynedd) populations was observed along the PC2, the latter accounting for 10.1 % of the total data variation. A slight overlap between adders from Anglesey 1 and Anglesey 2 populations was highlighted by the analysis, but exploration of further components didn't resolve the latter pattern.

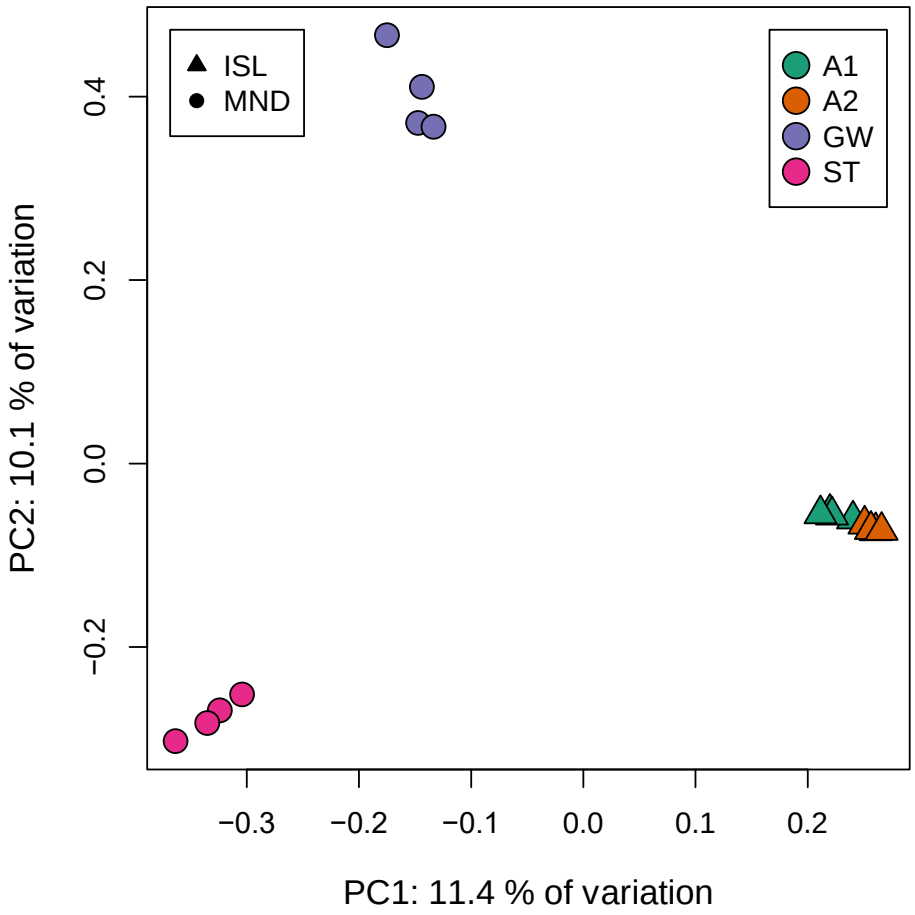

Figure 4. Principal Component Analysis (PCA) performed on 7.6 million variable sites. Principal Component 1 (PC1) accounts for 11.4% of the total variation, while Principal Component 2 (PC2) accounts for 10,1% of the total variation of the data.

The four colours correspond to the four sampled populations: green for the Anglesey 1 (A1) population, orange for the Anglesey 2 (A2) population, purple for the Gwynedd (GW) population, and fuchsia for the Staffordshire (ST) population. The different shapes represent the different biographic nature of the populations: a triangle for the insular (ISL) populations and a circle for the mainland (MND) populations.

4.2.2 Neighbour-joining tree

After the filtering process, a total number of 6.1 million informative sites were employed for further analysis. The results of the NJ tree analysis highlighted the existence of two main clades: an English one, containing all specimens from the Staffordshire populations; and a Welsh clade, containing all the specimens from Anglesey 1, Anglesey 2, and Gwynedd populations (Figure 5).

Within this latter cluster, further sub-clustering revealed the presence of a mainland clade, containing all the specimens from the Gwynedd population; and an insular clade, containing all the specimens from Anglesey 1 and Anglesey 2 populations, the latter subdivided into two distinct and non-overlapping clades corresponding to the two sampled populations. Notably, the branch length separating Anglesey 1 and Anglesey 2 populations is shorter that occurs between the other geographic populations.

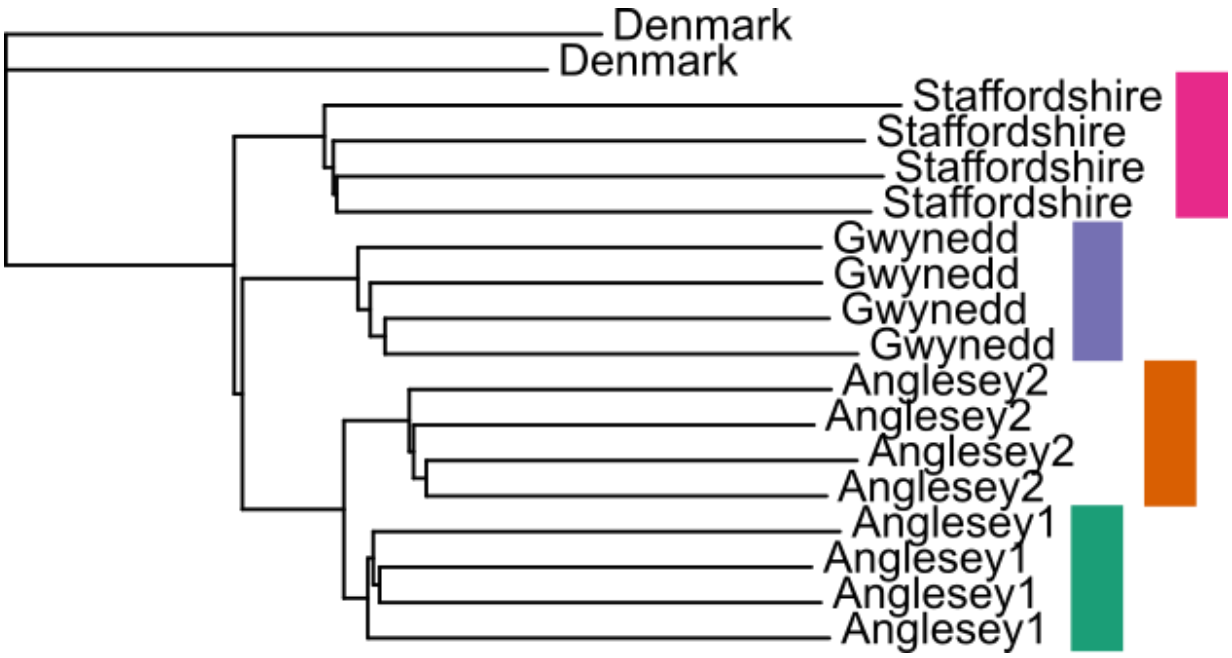

Figure 5. Whole genome phylogeny of the assessed adder populations via Neighbour-joining tree**.**

4.2.3 Ancestry analyses

The ancestry analysis performed for K=3 highlighted the presence of three different clusters: an English cluster containing all the specimens from the Staffordshire population, a mainland Welsh cluster containing all the specimens from the Gwynedd population, and a final insular Welsh cluster containing all the specimens from the Anglesey 1 and the Anglesey 2 populations. This pattern was consistently recorded during all four runs (Appendix VI).

The ancestry analysis performed for K=4 highlighted the presence of four distinct population clusters corresponding to the four geographic populations. In fact, the analysis underlined the presence of an English cluster containing all the specimens from the Staffordshire population, a mainland Welsh cluster containing all the specimens from the Gwynedd population, two distinct insular Welsh clusters, one containing all the specimens from Anglesey 1 and the other containing all the specimens from Anglesey 2. This pattern was consistently recorded during all four runs (Figure 6).

The ancestry analysis performed for K=5 highlighted the presence of a fifth population cluster with no consistency among all four runs (Appendix VII). In fact, the analysis underlined the presence of specimens belonging to a fifth population cluster which appeared to be distributed randomly within the four geographic populations. Specimens characterised by the fifth genetic ancestry were present within the Anglesey 1 population during the first run, the Staffordshire population during the second and the third runs, and the Gwynedd population during the fourth run.

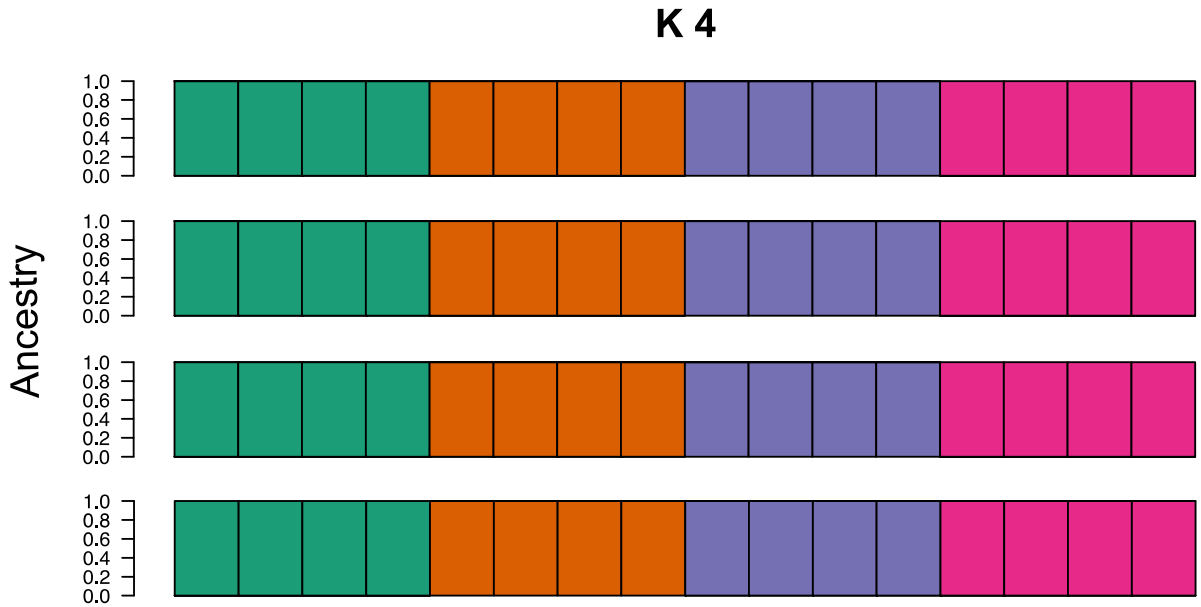

Figure 6. Ancestry analysis for K=4. Each bar of the plot represents a different individual adder. Each line represents a different run performed using a unique seed value. The colour of each bar represents the proportion of ancestry of each sampled genome to K=4 clusters. The four colours correspond to the four sampled populations: green for the Anglesey 1 (A1) population, orange for the Anglesey 2 (A2) population, purple for the Gwynedd (GW) population, and fuchsia for the Staffordshire (ST) population.

4.3 Genetic diversity

4.3.1 Heterozygosity estimation

The analyses highlighted remarkably high levels of heterozygosity among all the 16 UK adders, with values ranging from 1.3 het. sites per kb to 2.5 het. sites per kb (Table 3). The Staffordshire population was characterised by the highest genetic diversity (average population genome-wide heterozygosity = 2.32 het. sites per kb), followed by the Anglesey 1 population (average population genome-wide heterozygosity = 1.75 het. sites per kb), the Anglesey 2 population (average population genome-wide heterozygosity = 1.70 het. sites per kb), and finally the Gwynedd population (average population genome-wide heterozygosity = 1.65 het. sites per kb).

Table 3. Individual average-genome wide heterozygosity values (heterozygous sites per kb).

Sample ID	Population	Heterozygosity (het. sites per kb)
WW5272	Anglesey 1	1.8
WW5273	Anglesey 1	1.8
WW5274	Gwynedd	1.8
WW5275	Gwynedd	1.3
WW5280	Anglesey 1	1.7
WW5281	Anglesey 1	1.7
WW5287	Staffordshire	2.3
WW5288	Staffordshire	2.2
WW5289	Staffordshire	2.5
WW5290	Staffordshire	2.3
WW5300	Anglesey 2	1.7
WW5301	Anglesey 2	1.7
WW5302	Anglesey 2	1.6
WW5305	Anglesey 2	1.8
WW5395	Gwynedd	1.8
WW5396	Gwynedd	1.7

When compared with other vertebrate taxa, the focal adder populations were characterised by levels of genetic diversity higher or comparable to those from highly diverse vertebrates, such as the yellow baboon (genome-wide average heterozygosity = 1.68 het. sites per kb) and the

chimpanzee (genome-wide average heterozygosity = 1.08 het. sites per kb) (Figure 7) (Appendix VIII).

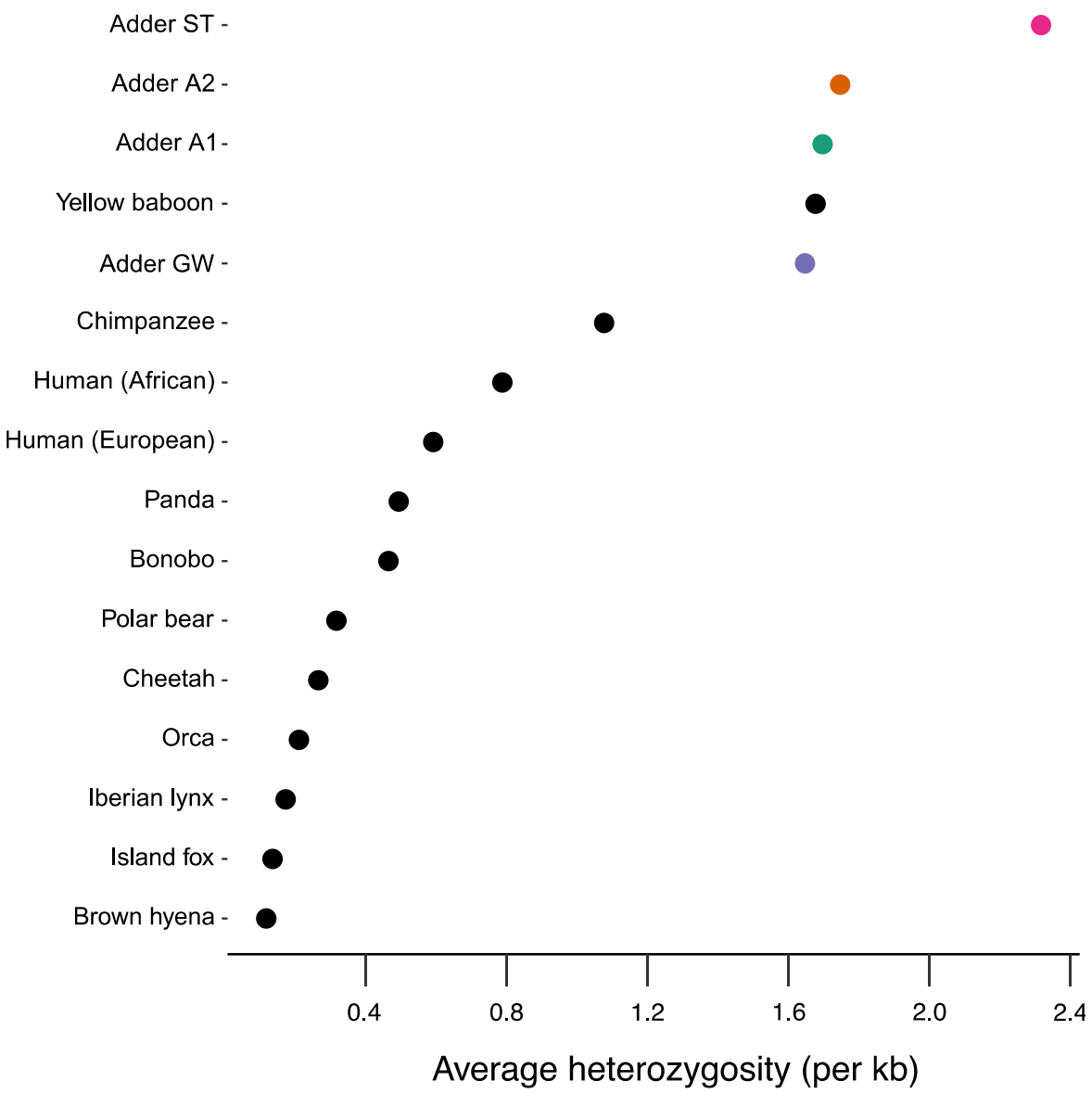

Figure 7. Between-species comparison of average genome-wide heterozygosity values. The average genome-wide heterozygosity on the Y axis is recorded as "heterozygous sites per kb". Population values for the four UK adder population were calculated as the mean of relative individual's heterozygosity values. The average genome-wide heterozygosity values for the other vertebrate taxa were obtained from Westbury *et al.* 2018. The four colours correspond to the four sampled populations: green for the Anglesey 1 population, orange for the Anglesey 2 population, purple for the Gwynedd population, and fuchsia for the Staffordshire population.

The distribution of genome-wide average heterozygosity within the sequenced adder genomes showcased an unexpected distributional pattern (Figure 8). Within all the adder

genomes, the distribution of heterozygosity presented an asymmetric, negative-exponential pattern, highlighting the presence of a high number of genomic regions characterised by heterozygosity values close to zero. This distributional pattern was less accentuated among the adders from the Staffordshire population (Figure 8).

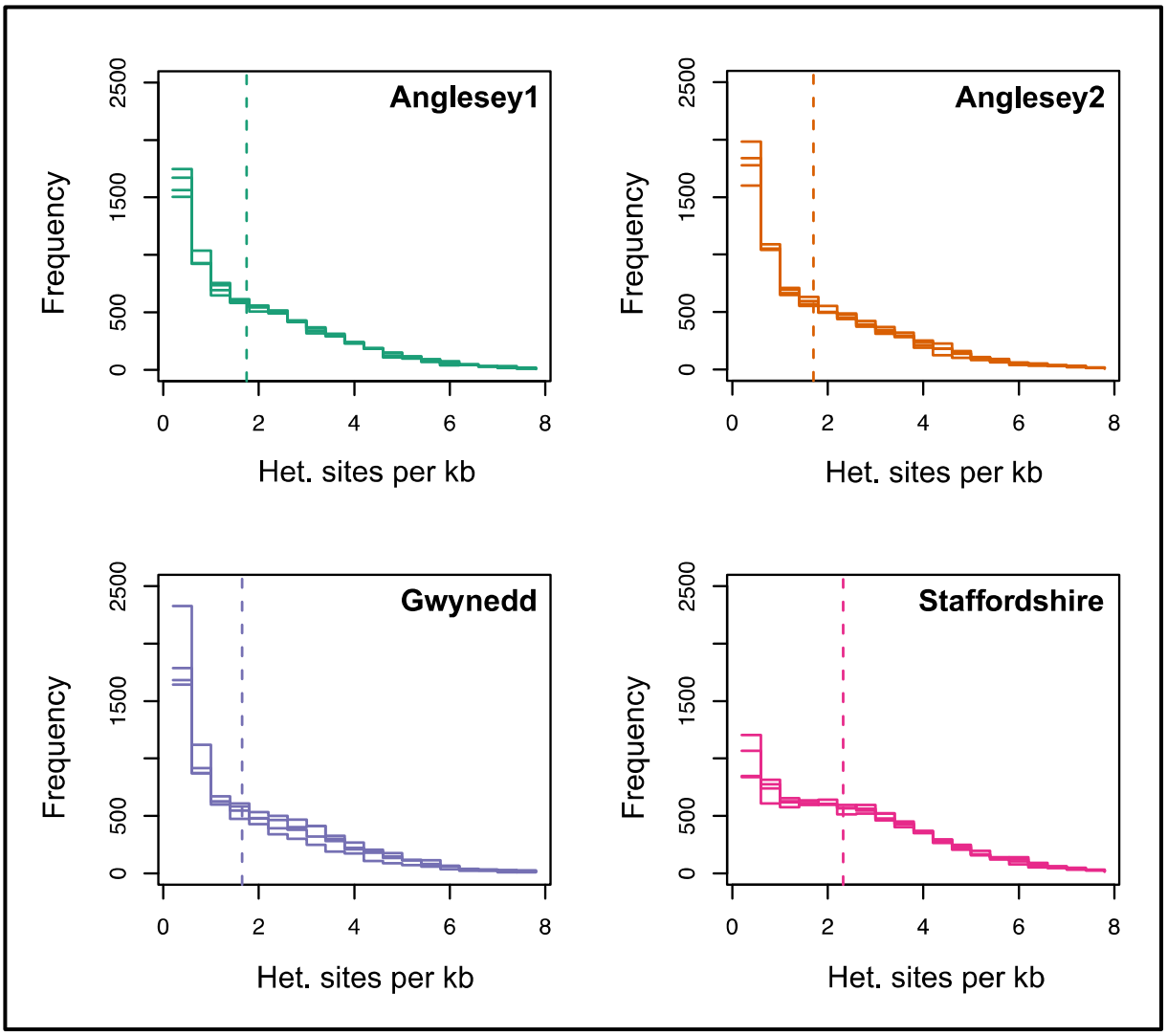

Figure 8. Distribution of heterozygosity along 100 kb widows for the four adder populations. The values on the Y axis are recorded as "heterozygous sites per kb". Each plot represents one of the four sampled adder populations. Individual continuous and overlaid lines represent different adder specimens. Dashed lines represent the relative population average genome-wide heterozygosity value.

4.3.2 Nucleotide diversity

The analysis highlighted a noticeable difference in terms of intra-population nucleotide diversity between the English population (Staffordshire) and the three Welsh populations (Anglesey 1, Anglesey 2, and Gwynedd) (Figure 9). Individuals from the Staffordshire

population seems in fact to present lower patterns of allelic fixation compared to the Welsh populations.

Average nucleotide diversity was higher in the Staffordshire population ($\pi = 2.25$ substitutions per kb) followed by the Anglesey 1 population ($\pi = 1.81$ substitutions per kb), the Anglesey 2 population ($\pi = 1.79$ substitutions per kb), and the Gwynedd population ($\pi = 1.69$ substitutions per kb).

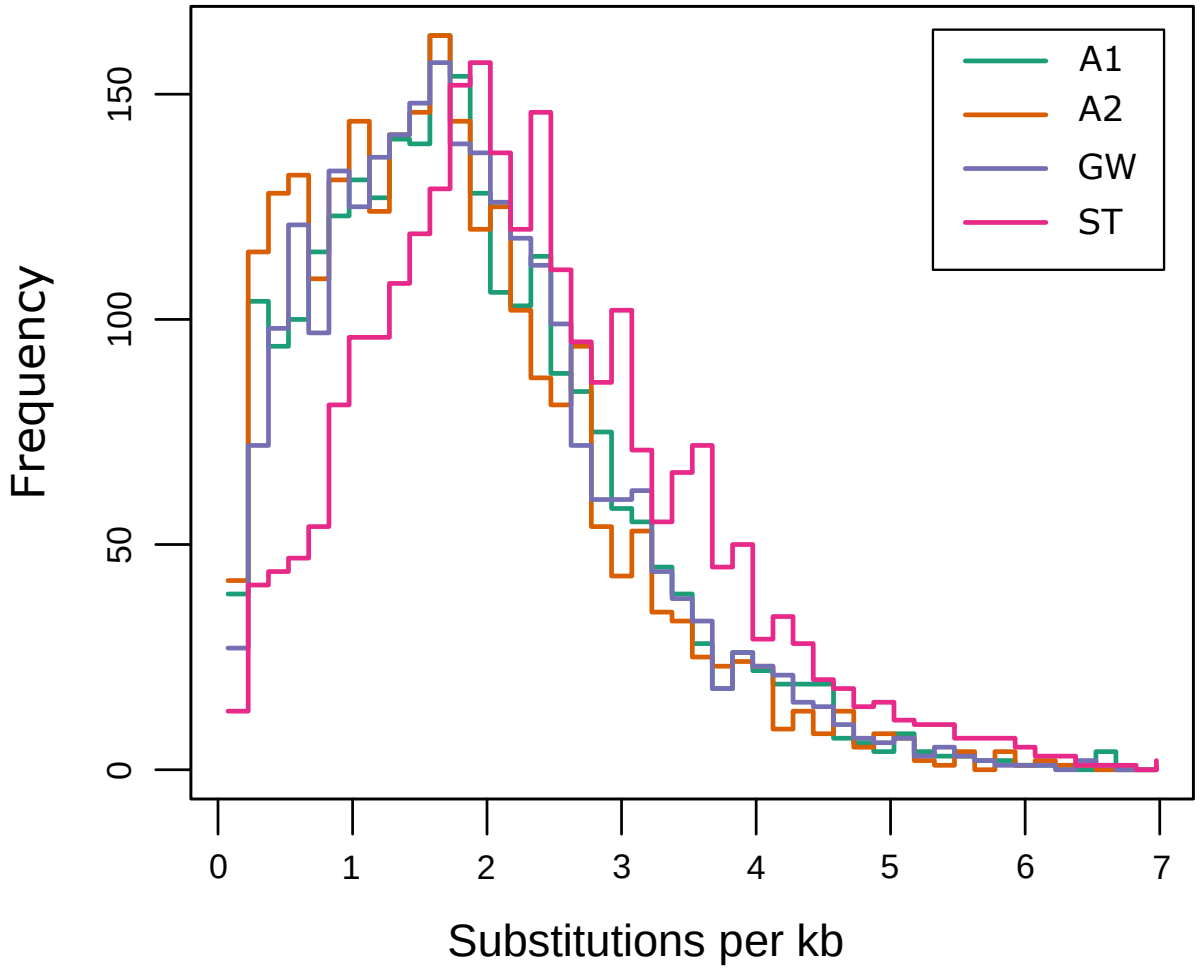

Figure 9. Distribution of nucleotide diversity within UK adder populations along 100 kb windows. The latter is recorded on the Y axis as "substation per kb", while its frequency is recorded on the X axis. Each line represents a different one of the four sampled populations. The four colours correspond to the four sampled populations: green for the Anglesey 1 (A1) population, orange for the Anglesey 2 (A2) population, purple for the Gwynedd (GW) population, and fuchsia for the Staffordshire (ST) population.

4.3.3 Fst

The sliding windows Fst analysis highlighted discrete levels of genetic differentiation between population-pair, which were largely characterised by modest proportions of low-Fst

genetic windows (Figure 10). Exceptional was the case of the two insular Welsh population, Anglesey 1 and Anglesey 2, the latter marked by a remarkably higher proportion low-Fst genetic windows.

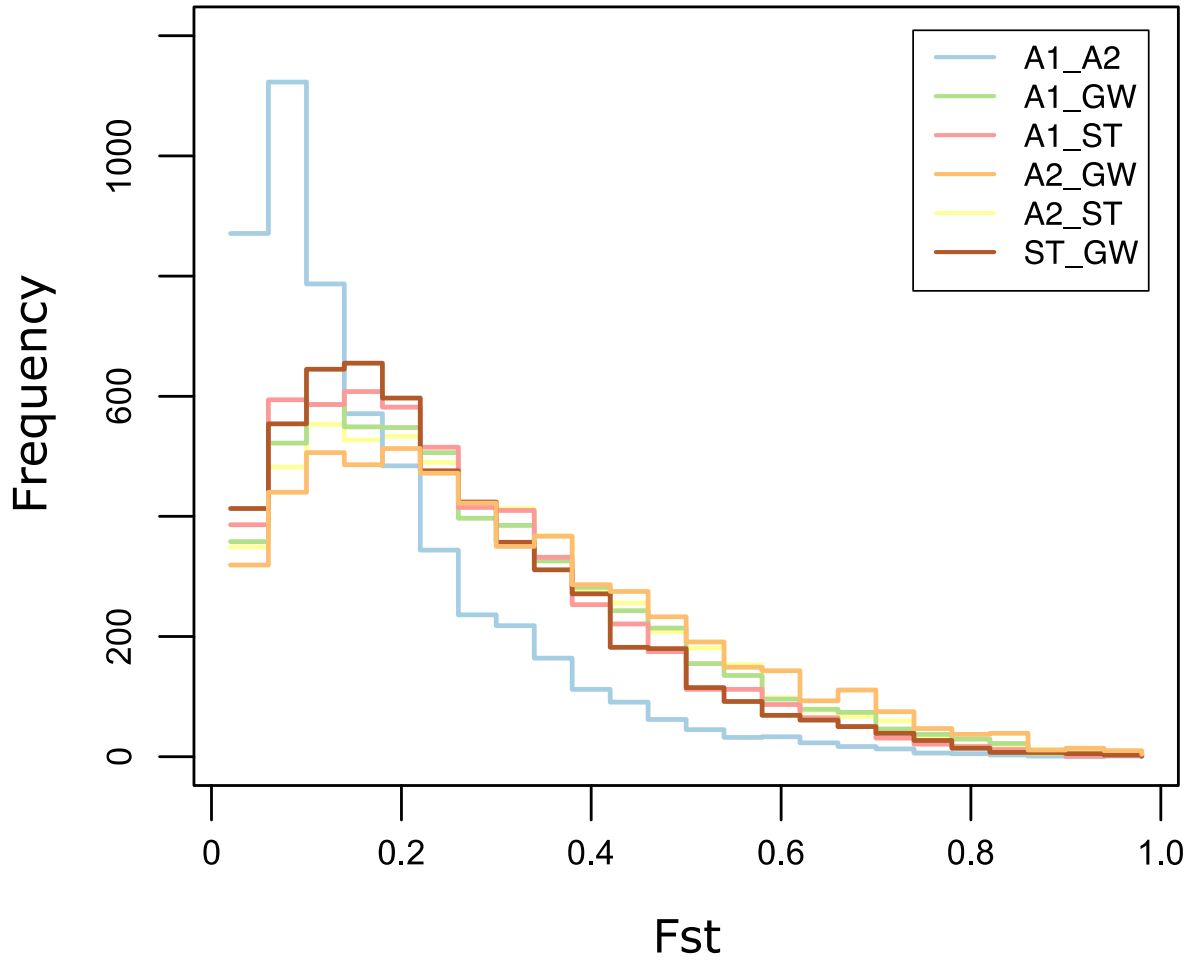

Figure 10. Distribution of pairwise weighted Fst along 100 kb windows. Each line represents a different population pair. Each of the six colours corresponds to a different population pair: light blue for the Anglesey 1 and Anglesey 2 populations pair (A1_A2), light green for the Anglesey 1 and Gwynedd populations pair (A1_GW), pink for the Anglesey 1 and Staffordshire populations pair (A1_ST), salmon for the Anglesey 2 and Gwynedd populations pair (A2_GW), yellow for the Anglesey 2 and Staffordshire populations pair (A2_ST), and brown for the Staffordshire and Gwynedd populations pair (ST_GW).

The global Fst estimates painted a similar picture (Table 4). The lowest values were observed between the Anglesey 1 and the Anglesey 2 populations (global Fst = 0.16), while the highest global Fst estimate, double the one recorded between the two insular Welsh populations, was found between the Anglesey 2 and the Gwynedd populations (global Fst = 0.32).

Table 4. Pairwise global weighted Fst values for UK adder population pairs.

Population pair	Global weighted Fst
Anglesey 1 - Anglesey 2	0.16
Anglesey 1 - Gwynedd	0.290
Anglesey 1 - Staffordshire	0.255
Anglesey 2 - Gwynedd	0.320
Anglesey 2 - Staffordshire	0.283
Staffordshire - Gwynedd	0.251

5. DISCUSSION

5.1 High genetic diversity despite the lack of gene flow

Habitat fragmentation can lead to the disruption of gene flow between populations and patterns of strong population structuring (Keyghobadi, 2007; Wang *et al.* 2017; Schlaepfer *et al.* 2018). During the last few decades, the UK has undergone a drastic process of industrial agriculture intensification and urban expansion, drastically transforming land use and reducing the suitable habitat for a wide variety of taxa (Rushton *et al.* 2001; Rounsevell and Reay, 2009; Cole *et al.* 2022). Solely in England, the adder has lost almost 40% of its historic range (pre-2006), leading to the formation of isolated and potentially highly structured populations (Gleed-Owen and Langham, 2012; Gardner *et al.* 2018).

Among the assessed adder populations, high levels of population structuring and lack of gene flow between populations were recovered using whole-genome sequencing data as shown by lack of detectable admixture in NGSadmix analysis and the recovered Fst value between populations.

On a finer scale, intrapopulation structuring was not observed, as highlighted by the lack of signal recovered from ancestry analysis for k=5 (Appendix VII). In adder, population structuring has been correlated with habitat degradation (François *et al.* 2021). In particular, alteration of fine-scale vegetation structure (e.g. hedgerows) due to the intensification of agricultural activities and bad habitat management practises (Ball *et al.* 2020), is likely to play a role in population structure due to the increase in predation rate and the reduction of connectivity between suitable patches of habitat (Davies and Pullin, 2007; Worthington-Hill and Gill, 2019; Duchesne *et al.* 2022). This phenomenon is likely to have a stronger impact on juvenile adders which, due to their reduced size, represent a potential prey item for a wide

variety of avian and terrestrial predators (Herczeg *et al.* 2007). Ecological studies have in fact highlighted the crucial role of juvenile adders as the primary source of population emigration during the natal displacement process (Ursenbacher *et al.* 2009a; Hodges and Seabrook, 2019). As whole-genome sequencing data allow for the recovery of population structure at fine scale, even with limited samples (Ekblom and Wolf, 2014; Iannucci *et al.* 2021) the absence of evident structing within the assessed adder population seems to highlight how the abovementioned factors may not occur within the focal population, or their effects may still have to affect intrapopulation structure (but see Tolley *et al.* 2022).

On a wider scale, the lack of evidence for recent gene flow observed between the focal adder populations (Figure 6) is likely to be the product of rapid and extensive events of anthropogenic-led habitat loss and fragmentation (Gardner *et al.* 2019; Ball *et al.* 2020; Paijmans *et al.* 2021). Accordingly, the recovered Fst values between adder populations (Table 4) (Figure 10) seem to support the recent and drastic habitat fragmentation hypothesis due to the rapid response of genetic structure to habitat alteration (Tolley *et al.* 2022). Interestingly, Ursenbacher and colleagues (2009b) highlighted a lack of gene flow between Swiss adder populations even where habitat characteristics did not seem to limit individuals' dispersal abilities. Therefore, besides habitat fragmentation, the interplay of adder's high philopatry, reproductive ecology (sex-biased dispersal) and trophic ecology (ambush strategy), is likely to contribute to the herein reported patterns of population structuring and the observed lack of recent gene flow (Madsen and Shine 1992; Ursenbacher *et al.* 2009a; De Fraga *et al.* 2017).

Adders in the UK are currently restricted to extremely small and isolated populations (Gardner *et al.* 2019; Ball *et al.* 2020). According to the current theoretical framework, low genetic diversity may represent a threat to these populations (Ellstrand and Elam, 1993; Frankham *et al.* 2019).

Surprisingly, this seems not to be the case for the investigated UK adder populations. Instead, extremely high levels of average genome-wide heterozygosity were recovered among the assessed adder individuals and populations (Table 3). Even if on a different scale, these outcomes agree with the insights showcased by Ball and colleagues (2020), where high levels of heterozygosity, comparable to those from mainland European populations, were recovered in various UK adder populations. Similarly, high levels of heterozygosity despite significant population structure and decline were recovered within other Palearctic vipers. For example, Adavodi *et al.* (2019) found high levels of genetic diversity within small and genetically isolated populations of Caucasian pit vipers (*Gloydius caucasicus*). Recently, high levels of

heterozygosity were recovered among various small and isolated populations of two highly endangered vipers, the Hungarian meadow viper (*Vipera ursinii rakosiensis*) and the Moldavian meadow viper (*Vipera ursinii moldavica*) (Vörös *et al.* 2022). Nevertheless, it is important to highlight that the latter three studies employed traditional genetic markers (microsatellites) which tend to overestimate the real extent of genetic diversity due to the variable nature of these markers and via indirect estimations (Smeds and Ellegren, 2023). Nonetheless, the recovered average genome-wide heterozygosity values of the assessed adder populations were even higher or comparable to the heterozygosity values from remarkably highly diverse vertebrates (Figure 7) (Appendix VIII). For example, the vast majority of the focal adder populations were characterised by heterozygosity values higher than those from the yellow baboon (Heterozygosity = 1.68) and the chimpanzee (Heterozygosity = 1.08). Even if between species comparison of heterozygosity may be sometimes biased by differences in the applied bioinformatics pipelines (Ekblom *et al.* 2018), the herein reported comparison still provides an immediate picture of the extremely high levels of heterozygosity recovered among the assessed UK adder populations. The latter comparison further opens a question regarding the mechanisms underlying the highlighted high levels of genetic diversity. In fact, in addition to the relationship between gentic drift and population size (Kimura and Ohta, 1969), the high levels of heterozygosity that characterise a good proportion of the vertebrate taxa reported by Westbury *et al.* 2018 can be correlated with the presence of social structure (e.g. presence of individuals in a non-reproductive status) (Parreira and Chikhi, 2015; Parreira *et al.* 2020; Guevara *et al.* 2021). This correlation between genetic diversity and social structure has been highlighted in a wide variety of mammalian taxa (Winterrowd *et al.* 2009; Huchard *et al.* 2010; Parreira *et al.* 2020; Guevara *et al.* 2021). Nevertheless, due to the absence of social structure in adders, other more cryptic mechanisms must be involved in the maintenance of high genetic diversity. Madsen and colleagues (2023) correlated patterns of negative assortative mating with the high genetic diversity recovered within a small and isolated adder population in Sweden. Simulations and empirical evidence showcased how polyandry and non-random mating seems to promote heterozygosity within small and isolated adder populations.

Besides, effective ancestral population size and recent patterns of population decline may be further associated with the herein recovered extremely high levels of genetic diversity (Westbury *et al.* 2019; Iannucci *et al.* 2021; Xie *et al.* 2022). Long-term small effective population size has been correlated to current low levels of heterozygosity in various taxa (Westbury *et al.* 2018; Liu *et al.* 2021; Robinson *et al.* 2022; Mooney *et al.* 2023; Foote *et al.*

2023). On the other hand, the high levels of genetic diversity recovered among the focal adder populations may reflect the presence of large and interconnected ancestral populations characterised by high levels of heterozygosity (Armstrong *et al.* 2021; Von Seth *et al.* 2021). Similarly, remarkable levels of genetic diversity found in the endangered Armenian viper (*Montivipera raddei*) were correlated to historically large effective population and recent patterns of anthropogenic-led population decline (Ettling and Parker, 2017). Empirical and theoretical evidence highlighted how extreme and rapid bottleneck events are likely to only moderately reduce heterozygosity in the short term (Ralls *et al.* 1983; Beichman *et al.* 2023), as loss of genetic diversity may tend to "lag behind" population depletion (Lukoschek, 2018; Bradke *et al.* 2021; Salado *et al.* 2022; Tolley *et al.* 2022). Therefore, is plausible to assume that the herein recovered high levels of genetic diversity could be correlated to large ancestral effective population size and recent and extreme human-induced bottleneck events (Gardner *et al.* 2019; Ball *et al.* 2020). Consequently, the assessed adder populations may still have to face the genetic effects of this drastic population decline and their current population size (Sovic *et al.* 2019).

5.2 Hidden patterns of genomic erosion

According to the current theoretical framework, due to the extremely high levels of genetic diversity recovered among the assessed adder populations, the latter should not be considered a conservation concern, at least from the genetic point of view (Frankham, 2005; Market *et al.* 2010; García-Dorado and Caballero, 2021; Schmidt el al. 2023).

However, analyses aimed to assess the distribution of heterozygosity within the focal genomes revealed the presence of hidden patterns of genomic erosion (Figure 8). Specifically, within the investigated genomes, a high density of genomic windows of 100Kb characterised by heterozygosity values close to zero were recovered. These outcomes are likely to have profound consequences on the conservation of the investigated adder populations.

Firstly, the highlighted distribution of heterozygosity within the analysed genomes shows how genome-wide heterozygosity values may lead to misinterpretation and overestimation of the real genetic diversity. This may be correlated to the recovered skewed distribution of heterozygosity within the assessed adder genomes (Figure 8). Average genome-wide heterozygosity is widely used as a proxy for populations' genetic health (Schmidt *et al.* 2021), even if its relationship with populations' conservation status is not straightforward (Schmidt *et al.* 2023). Nonetheless, the herein reported results show that this widely

employed parameter may lead to overestimating the real extent of genetic diversity while masking extensive patterns of genomic erosion. Therefore, caution must be used when average genome-wide heterozygosity is employed as the only metric to assess populations' genetic health.

Secondly, the highlighted pattern of genomic erosion and the known adder population history in the UK indirectly showcase the likely presence of Runs of Homozygosity (ROH) at high frequency within the assessed genomes (van Oosterhout *et al.* 2022). These represent contiguous homozygous genomic regions identically inherited from both parents (Gibson *et al.* 2006). The presence of the latter provides a window into the demographic history and future persistence of the assessed adder populations (Ceballos *et al.* 2018; Supple and Shapiro, 2018; Brüniche-Olsen *et al.* 2018). ROHs' distribution, length and abundance are in fact largely influenced by population demographic patterns (Curik *et al.* 2014; Ceballos *et al.* 2018; Dixit *et al.* 2020; Hewett *et al.* 2023). Therefore, contiguity of ROHs has been shown to be highly affected by haplotype recombination patterns, with shorter ROHs (< 1Mb) likely representing the product of historic population contractions and longer ROHs (>1 Mb) likely arising from recent bottleneck events (Jensen *et al.* 2021; Wotton *et al.* 2022; Martin *et al.* 2023; Mooney *et al.* 2023).

The scaffolds' length of the employed adder's reference genome does not allow for the length of the recovered ROH to be precisely estimated, therefore it is not possible to assess the relative proportion of long and short RHO within the assessed genomes. Nevertheless, it is possible to infer, with a discrete degree of certainty, the length of a part of the recovered RHOs on the basis of the known species' demographic patterns and the highlighted levels of heterozygosity. Empirical evidence have shown a devastating and rapid country-wide population collapse of the adder during the last few decades (Baker *et al.* 2002; Gleed-Owen and Langham, 2012; Gardner *et al.* 2019; Ball *et al.* 2020). Thus, it is possible to assume that a good proportion of the recovered ROHs may represent long stretches of ROHs arose during the recent population decline (Wilder *et al.* 2022). Moreover, the extremely high levels of genome-wide heterozygosity highlighted by the analyses, despite the small population size of the assessed populations the lack of gene flow among populations, and the herein reported pattern of genomic erosion, argue in favour of a recent and exceptionally rapid population collapse. This contrasting pattern has been highlighted within two canid taxa characterised by recent population collapse histories, the Mexican wolf (*Canis lupus baileyi*) and the grey wolf from Isle Royale wolf (*Canis lupus*) (Robinson *et al.* 2019). Similarly, high heterozygosity

despite the presence of long ROHs has been correlated to human-mediated population decline in Indian tigers (*Panthera tigris*) (Armstrong *et al.* 2021).

Consequently, it is possible to assume that a discrete proportion of the recovered ROHs within the assessed adder genomes are likely to represent long ROHs arose due to recent and extremely rapid population decline. Therefore, this outcome may highlight recent inbreeding patterns within the assessed adder populations. Inbreeding patterns were not recovered within previous population genetic studies on UK adders (Ball *et al.* 2020). As even low levels of inbreeding are known to have drastic consequences on species' fitness and reproductive abilities (Huisman *et al.* 2016), further investigations are required. Moreover, these outcomes showcase the higher ability of the whole-genome sequencing approach in recovering fine demographic patterns compared to more traditional genetic methods (Iannucci *et al.* 2021). However, it is possible to argue that the presence of ROH in the genomes of the focal adders may be correlated with the presence of mechanisms of non-random mating (Li *et al.* 2006). Despite that, ROHs arising as a product of this phenomenon are expected to be present within the genome at low frequency (Martin *et al.* 2023), which appears to be in contrast with the high-frequency levels herein reported.

Taking into consideration the known demographic history of the adder in the UK, the current population status of the species, and the herein reported contrasting genetic patterns, a concerning scenario arises. In fact, the assessed adder populations are likely to have undergone a drastic and rapid population collapse and fragmentation starting from historically large and well-interconnected ancestral populations (see section 5.1). This phenomenon may have had a negative impact on the presence and frequency of deleterious alleles within adders' genomes.

While individuals from small populations characterised by stable demographic histories and small ancestral population sizes seem to harbour a lower number of extremely deleterious alleles within their genomes thanks to the effect of genetic purging (García-Dorado, 2012; Xie *et al.* 2022; Kleinman-Ruiz *et al.* 2022; Wotton *et al.* 2023; Stanhope *et al.* 2023; Mooney *et al.* 2023). On the other hand, the genomes of individuals from small and fragmented populations characterised by large ancestral population size which have undergone recent population contractions seem to be characterised by a significantly higher number of extremely deleterious alleles (Hu *et al.* 2020; Kyriazis *et al.* 2021; Tian *et al.* 2022; Zhang *et al.* 2022; Smeds and Ellegren, 2023; Quinn *et al.* 2023). Although between-species comparison regarding the effect of demographic patterns on the frequency of deleterious alleles and relative fitness consequences can be hard to make (Dussex *et al.*

2021), the current theoretical framework suggests an increase of deleterious alleles within the focal adder populations due to recent bottleneck events, a hypothesis likely supported by the herein recovered patterns of genomic erosion. Even if the assessed UK adder populations did not yet present worrying signs of fitness decrease, such as those found within the highly inbred Swedish adder population of Smygehuk (Madsen *et al.* 1996, 2011), it is likely that these populations have still to showcase the fitness consequences correlated with the observed patterns of genomic erosion (von Oosterhout *et al.* 2022).

In the near future, access to a higher-quality reference genome for the adder will allow the length and the origin of the here identified ROHs to be precisely estimated and the presence of extremely deleterious alleles and their future effects on the adders' fitness to be quantified.

5.3 The potential for genetic rescue

Despite the extremely high level of heterozygosity recovered within the assessed adder populations, genomic erosion coupled with the current small population size is likely to lead to a future decrease in heterozygosity, an increase of ROH regions, enhancement of mutational load, and eventually local extinction (Bijlsma *et al.* 2012; von Seth *et al.* 2021; Sánchez-Barreiro *et al.* 2021; van Oosterhout *et al.* 2022). While cryptic mechanisms (polyandry and non-random mating) may have played a role in maintaining the recovered high levels of heterozygosity despite recent population declines and the lack of interpopulation gene flow patterns, under future scenarios of genetic diversity and population collapse, these mechanisms are unlikely to be sufficient in assuring the persistence of the populations (Wood *et al.* 2020; Madsen *et al.* 2023). For these reasons, genetic conservation measures such as genetic rescue, may be needed in the near future in order to assure the persistence of the assessed populations (Ingvarsson, 2001; Frankham, 2015). However, genetic rescue measures require preliminary genetic data from both donor and recipient populations to maximise the conservation benefits and avoid outbreeding depression (Whiteley *et al.* 2015; Bell *et al.* 2019; Frankham *et al.* 2019). Thus, taking into consideration the positive results obtained via genetic rescue in adders (Madsen *et al.* 2020) and in a wide variety of other vertebrate taxa (e.g. Hogg *et al.* 2006; Pimm *et al.* 2006; Åkesson *et al.* 2016), the potential of genetic rescue within the assessed adder populations was therefore explored.

Fixation of the whole population for a single allele represents the dead-end of the inbreeding process, causing the complete loss of adaptation potential (Otto and Whitlock, 1997; Barret and Schluter, 2008). Therefore, nucleotide diversity can be exploited to assess this

intrapopulation degree of genomic fixation and compare results between populations. The analyses highlighted moderate levels of genomic fixation (measured via nucleotide diversity) between individuals from the same population (Figure 9). In some cases, strong habitat fragmentation within a single population has been shown to lead to the formation of fragmented and isolated subpopulations characterised by compatible fixed genomic regions, favouring in this way the population's overall genetic diversity (e.g. Löytynoja *et al.* 2023). The recovered levels of intrapopulation genomic fixation highlighted by the analyses argue against this possibility. Instead, it is likely that within population genetic exchange, due to the incompatibility between the fixed genomic regions, will lead to a decrease in the overall populations' genetic diversity (Frankham, 2003, 2005; Frankham *et al.* 2019). Thus, without human intervention (e.g. genetic rescue, creation of habitat corridors, etc..) the assessed populations, even if at different rates, are likely to lose genetic diversity and suffer the relative consequences. Therefore, the potential for anthropogenic-mediated migration of individuals between the assessed adder populations was investigated.

A lack of shared fixed genomic regions (high Fst values) was recovered between most of the adder populations (Figure 10) (Table 4). These results highlighted between population discrete levels of genetic compatibility in terms of genomic window fixation, arguing in favour of potential conservation benefits of future genetic rescue initiatives (Saremi *et al.* 2019). Nevertheless, extensive genomic fixation (low Fst value) was recovered between the two Anglesey populations (Fst = 0.16). This outcome argues against the translocation of individuals between the latter populations, as such intervention is likely to provide little if no conservation benefits (Fitzpatrick and Funk, 2019; Byrne *et al.* 2021; Melis *et al.* 2022). Interestingly enough, the current theoretical framework portrays geographic proximity as one of the main factors to take into consideration during the choice of both donor and recipient populations in the context of genetic rescue actions (Frankham *et al.* 2011,2019). Geographically close populations are in fact more likely to be adapted to similar environmental conditions, lowering in this way the possibility of diluting genetically adapted variations with the introduction of foreign alleles (Tallmon *et al.* 2004). The quality of the currently available adder reference genome did not allow for the genes within the recovered fixed genomic windows to be identified. For this reason, it is not possible to completely exclude that the highlighted fixation pattern may be correlated with environmentally adapted variations shared between the two Anglesey populations. In spite of that, taking into consideration the contrasting environments inhabited by the two adder populations, it is more likely that the recovered fixation pattern may be correlated with the shared demographic

history of the two populations (Kitada *et al.* 2021). Consequently, these results seem to suggest that higher conservation benefits may be achieved with the introduction of individuals adders from the geographically distant mainland populations (Gwynedd and Staffordshire) within the two Anglesey populations.

However, caution must be used during the translocation of individuals coming from populations characterised by different population sizes. In fact, populations characterised by larger effective population size may harbour a higher proportion of deleterious heterozygous alleles (hidden mutational load) due to the lower influence of purging (Khan *et al.* 2021; Pérez-Pereira *et al.* 2022). Thus, the introduction of foreign deleterious alleles within populations characterised by small effective population sizes can lead to a critical loss in fitness via the expression of those alleles, which are not purged out of the gene pool due to population-size constraints (Kyriazis *et al.* 2021; Ochoa *et al.* 2022). Therefore, the herein reported results represent only a preliminary investigation and further genome scanning and quantification of individuals 'mutational loads are required before taking into consideration any genetic rescue actions (Wotton *et al.* 2022).

6. CONCLUSION

6.1 Conclusions and future perspectives

A growing body of empirical evidence has highlighted the dramatic population decline affecting the UK adders, pointing the attention towards necessary conservation measures. Using a pioneering approach based on whole-genome sequencing data, this study provided detailed data regarding the genetic health of four UK adder populations characterised by different population sizes and biogeographic histories. Surprisingly, extremely high levels of genome-wide genetic diversity were recovered within all the assessed populations despite strong population structure and the lack of gene flow between populations. Nevertheless, further analyses revealed hidden patterns of genomic erosion, shining a light on the future potential genetic collapse of the assessed populations. This contrasting pattern is likely to be the product of the historical presence of large and interconnected ancestral populations, which have recently undergone rapid and extreme population collapse.

Anthropogenic habitat loss and degradation, coupled with the further fragmentation of the adder populations are excepted to negatively impact the persistence of the species in the UK (Gardner *et al.* 2019; Julian and Hodges, 2019; Guiller *et al.* 2022; Duchesne *et al.* 2022).

Moreover, the persistence of the species seems to be further compromised by future climatic scenarios (Trumbo *et al.* 2021; Jørgensen *et al.* 2022; Dezetter *et al.* 2022; Turner and Maclean, 2022; Garcia-Costoya *et al.* 2023; Madsen *et al.* 2023). For these reasons, human-mediated actions aimed to conserve genetic adaptive potential via the preservation of high levels of genetic diversity will be likely essential in the near future.

While this study refrains from providing conservation managers with formal directions regarding the conservation of the assessed UK adder populations, the preliminary results herein reported argue for the future implementation of genetic rescue.

Despite this study representing a pilot initiative limited by the restricted sample size and the quality of the current adder reference genome, it still showcased the various benefits correlated with the use of whole-genome sequencing data in the context of population and conservation genetic studies. In particular, despite the small sample size, it was possible to recover spatially fine patterns of population structure and accurately assess the genetic health of the investigated populations. This study placed the foundations for further population genetic studies, where the implementation of whole-genome sequencing data and a higher quality (chromosome level) reference genome, may allow for the fitness impact of adders' mutation loads and the ability to adapt to future climatic scenarios to be assessed (Aguirre-Liguori *et al.* 2022; Bertorelle *et al.* 2022). Future decrease in cost of whole-genome sequencing and automation of bioinformatic pipelines may further increase the effectiveness and application of this approach in the funds-deprived field of wildlife conservation.

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APPENDIX

Appendix I – DNeasy Blood and Tissues Kit DNA extraction protocol

Approximately 2 mm^2 of tissue sample was cut into smaller fragments through the use of a sterile scalpel. Subsequentially, these were transferred into a 1.5 ml tube where 180 µl of ATL buffer were further added, followed by 20 µl of proteinase K. The tube was incubated overnight at 56°C to allow tissue digestion. Afterwards, the samples were mixed via the use of a vortex for 15 s. 200 µl of AL buffer and 200 µl of absolute ethanol were further added. Tube's content was then transferred into a spin column. Therefore, samples were placed into a centrifuge at 8000 rpm for 1 minute. Tube's content was then transferred into a new collection tube where 500 µl of AW1 buffer were subsequentially added. Therefore, samples were placed into a centrifuge at 8000 rpm for 1 minute. Following centrifugation, tube's content was placed into a new collection tube and 500 µl of AW2 buffer were added. Therefore, samples were placed into a centrifuge at 13000 rpm for 3 minutes. Following centrifugation, the column's content was placed into a 1.5 ml tube, where 100 µl of AE buffer were subsequentially added. The samples were incubated for 10 minutes at room temperature. Therefore, samples were placed into a centrifuge at 8000 rpm for 1 minute. Following centrifugation, tube's content was placed into a second elution tube where 50 µl of AE buffer were added. Samples were incubated for 5 minutes at room temperature. Finally, samples were placed into a centrifuge at 8000 rpm for 1 minute.

Appendix II – Qubit 3 DNA quantification protocol

Firstly, the lids of 0.5 ml tubes were labelled for samples and two standards. Afterwards, the Qubit working solution was prepared by mixing the Qubit dsDNA HS Reagent with the Qubit dsDNA HS Buffer with a 1:200 ratio. 190 µl of working solution was then pipetted into the standards. Subsequentially, 10 µl of Qubit standard was added. Tubes were then vortexed for 3 seconds. 199 µl of working solution was added to all the sample tubes. 1 µl of DNA for quantification was then pipetted into the sample tubes and the latter were then vortexed for 3 seconds. Tubes were then incubated at room temperature for 2 minutes.

On the Qubit 3, dsDNA high sensitivity was selected as assay type. Subsequentially the machine was calibrated via the reads of the standards. Once calibrated, the samples' DNA was quantified. Sample volume was set as 1 µl in the corresponding menu. Afterwards, samples were placed into the chamber and "Read tube" was pressed. Once the read was completed, the resulting concentration values were manually recorded. This process was then performed for all the samples.

Appendix III – DNA quantification results

Results relative to non-invasive sample (shed skin) are highlighted with a bold font.

N.B. This procedure was performed with/by John Benjamin Owens**.**

Sample ID	**DNA concentration elution 1 (ng/µl)**	**DNA concentration elution 2 (ng/µl)**
WW5272	80	80
WW5273	70	60
WW5274	140	140
WW5275	120	120
WW5280	150	120
WW5281	40	40
WW5287	70	70
WW5288	60	60
WW5289	90	90
WW5290	120	120
WW5300	120	120
WW5301	10	10
WW5302	120	120
WW5305	**150**	**150**
WW5395	96	46
WW5396	79	51

Appendix IV – Resulting data further details

The table contains results from the trimming, merging, and mapping processes. For each sample It shows the number of raw read pairs, the approximate percentage of reads (1 and 2) with adapter, the number of read pairs shorter than 30 mb, the percentage of combined pairs after short read removal, the number of mapped reads (Gb), and the read depth.

Sample ID	**Raw read pairs**	**Reads with adapter (%)**	**Read pairs < 30 mb**	**Combined pairs (%)**	**Mapping coverage (GB)**	**Read depth**
WW5272	59485998	37	2662	26.67	11.61	8.9
WW5273	76249417	37	4062	24.46	14.56	11.2
WW5274	56518134	37	3288	24.54	11.43	8.8
WW5275	55078138	37	2302	31.24	11.24	8.7
WW5280	57227177	37	2732	39.13	11.33	8.8
WW5281	66794362	37	3419	22.80	13.00	10.0
WW5287	58050421	37	2580	35.07	11.49	8.9
WW5288	83414961	37	5075	21.83	15.83	12.2
WW5289	73151161	37	4261	21.41	14.53	11.2
WW5290	74044433	37	4711	32.54	14.01	10.8
WW5300	71506150	37	3456	31.42	14.17	10.9
WW5301	73468547	37	3795	36.97	13.84	10.7
WW5302	56607401	37	2484	39.85	11.02	8.5
WW5305	66347902	37	3511	29.26	12.99	10.0
WW5395	72347587	37	2720	16.94	14.38	11.1
WW5396	61895797	37	2198	19.83	12.51	9.67
ZMUCR90374	33625072	60	9533	78.40	2.16	2.1
ZMUCR90375	15290738	59	5468	76.46	4.83	3.9

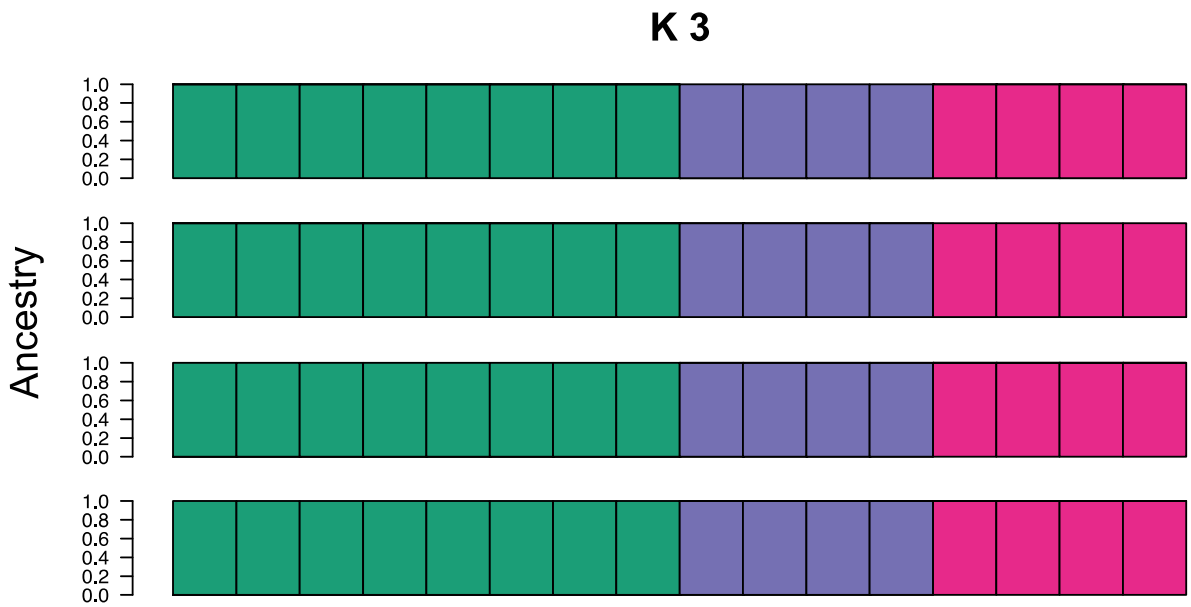
Appendix V - Mapping results *Ophidiomyces ophiodiicola*

The table shows the mapping statistics obtained via mapping of the UK adder sequences to the reference genome for *O. ophidiicola*. For each individual, the breadth of coverage (obtained via the function "depth" of Samtools and by dividing the resulting number by the size of the reference genome, which represents the percentage of the genome covered by more than one read), and the read depth are reported.

Sample ID	**Breadth of coverage (%)**	**Read depth**
WW5272	0.33	76
WW5273	0.35	91
WW5274	0.31	82
WW5275	0.30	76
WW5280	0.32	60
WW5281	0.32	79
WW5287	0.31	72
WW5288	0.36	80
WW5289	0.35	94
WW5290	0.33	93
WW5300	0.38	68
WW5301	0.38	62
WW5302	0.35	59
WW5305	0.38	64
WW5395	0.36	74
WW5396	0.34	72

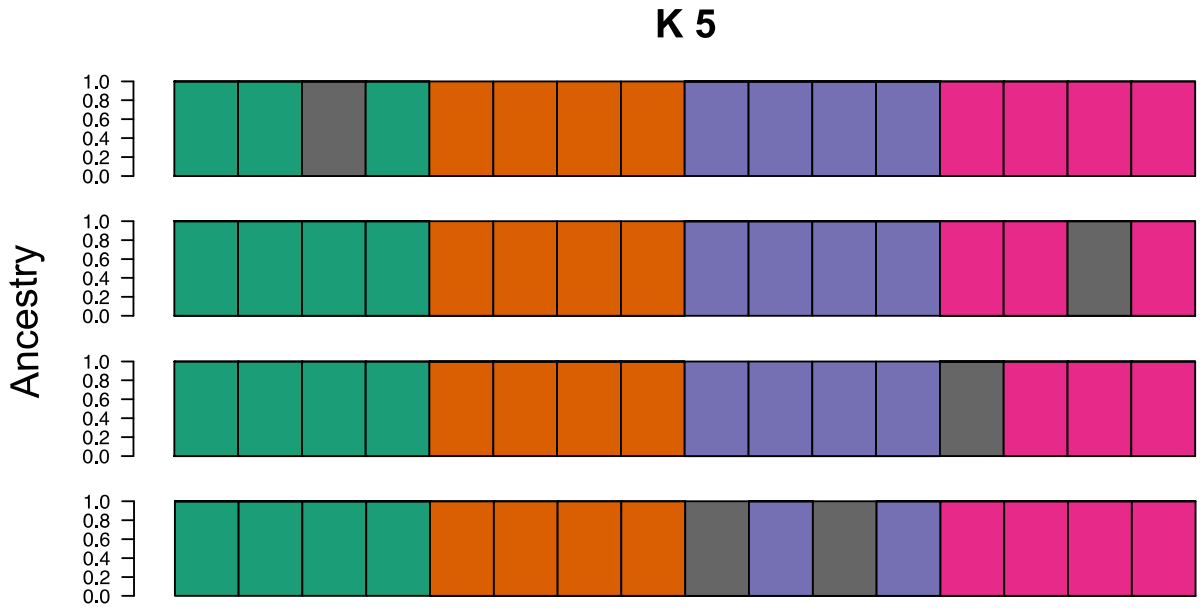
Appendix VI – Ancestry analyses for K=3

Each bar of the plot represents an individual adder. Each plot represents a different run, each was performed using a unique seed value. The three different colours represent: green for Anglesey 1 and Anglesey 2 populations, purple for the Gwynedd population, and fuchsia for the Staffordshire population.



Appendix VII – Ancestry analyses for K=5

Each bar of the plot represents an individual adder. Each plot represents a different run, each was performed using a unique seed value. The five different colours represent: green for the Anglesey 1 population, orange for the Anglesey 2 population, purple for the Gwynedd population, fuchsia for the Staffordshire population, and grey for a fifth unknown population cluster.



Appendix VIII – Between-species average genome-wide heterozygosity

The table contains the value used for the figure 7. Values for the four adder populations were obtained as the mean of the relative individual adders' average genome-wide values. Values for the other vertebrate taxa were obtained from Westbury *et al.* 2018.

Taxon	**Heterozygosity (per kb)**
Staffordshire	2.32
Anglesey 1	1.75
Anglesey 2	1.70
Gwynedd	1.65
Yellow baboon	1.68
Chimpanzee	1.08
Human (African)	0.791
Human (European)	0.595
Panda	0.497
Bonobo	0.468
Polar bear	0.320
Cheetah	0.269
Orca	0.214
Iberian lynx	0.176
Island fox	0.139
Brown hyena	0.121