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# GENETIC DIVERSITY OF EUROPEAN TREE FROGS (*HYLA ARBOREA* GROUP): A SYSTEMATIC REVIEW

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*Abstract*.
*Amphibian populations are increasingly threatened by global change and the study of their genetic diversity is a major conservation priority. Western palearctic tree frog species of the* Hyla arborea *group are commonly distributed across Europe and the Middle East and many have declining populations. We performed a systematic review based on "The Preferred Reporting Items for Systematic reviews and Meta-Analyses" (PRISMA) guidelines to gain insight into the genetic diversity of* H. arborea *group. Sixteen published studies were included in the final qualitative analysis. While the genetic diversity of* H. arborea *group species was widely variable, it could often be explained by phylogeographic history. Populations in Western and Northern Europe had lower genetic diversity linked mainly to postglacial population expansions, with some populations also affected by habitat fragmentation. However, important regions of high genetic diversity were found in the Balkan peninsula for* H. arborea *sensu stricto and around the Black Sea for* H. orientalis. *Genetic diversity of* H. molleri, H. savignyi, H. meridionalis, H. felixarabica, H. intermedia, H. sarda *has been investigated only across extensive phylogeographical studies, while data regarding their genetic diversity at the local level are missing. A database that gathers information on the studies carried out with the* Hyla arborea *species group could help the work of future ecological and genetic studies. The aim of this review is to identify knowledge gaps about the genetic diversity of the* H. arborea *group that require further investigation of and illustrate how filling these gaps might translate into future conservation efforts.*

**Key words***:* Hyla arborea *group, genetic diversity, amphibians, phylogeography, conservation, biodiversity*

## 1. Introduction

An increased number of studies report the decline of amphibian population sizes and the number of species during the last few decades with an accelerating extinction rate (Ceballos et al., 2020). Amphibians are sensitive to environmental pollution, habitat destruction and the presence of pathogens (Beebee & Griffiths, 2005; Skerratt et al., 2007), which can lead to decreased genetic diversity and variation within populations or species (Freeland, 2020). The Western palearctic tree frog species complex referred to as the *Hyla arborea* group consists of ten species from the *Hyla* genus that are distributed across Europe and the Middle East (Dufresnes et al., 2020). Six of the species (*H. arborea* sensu stricto, *H. sarda*, *H. intermedia*, *H. perrini*, *H. savignyi* and *H. meridionalis*) are included in the Red List of International Union for Conservation of Nature (IUCN, 2022) and three of them (*H. arborea* s.s., *H. sarda* and *H. meridionalis*) are also protected by the European Habitat Directive (Council Directive 92/43/EEC of 21 May 1992). All *H. arborea* group species in the IUCN Red List are listed as Least Concerned (LC) many (*H. arborea* s.s., *H. intermedia*, *H. perrini*, *H. meridionalis*) with decreasing population trends (IUCN, 2022). Many species in the *H. arborea* group have declining populations and like other amphibians are affected by habitat fragmentation (Andersen et al., 2004; IUCN, 2022).

Genetic diversity is gaining support of inclusion in species conservation assessments. Despite some criticism (Teixeira & Huber, 2021) many agree that monitoring genetic diversity of populations improves the quality of conservation measures (DeWoody et al., 2021; Laikre, 2010; Laikre et al., 2010; Phillips, 2020). It is important for a sustainable population to have a high genetic diversity as it makes them more resilient and less vulnerable to environmental changes, successfully establish new populations and distribute geographically (Forsman & Wennersten, 2016; Freeland, 2020). The loss of genetic diversity can accelerate the extinction processes of a population or even species (Allentoft & O'Brien, 2010; Frankham, 2005; Frankham et al., 2010). Even reintroduced populations are at risk of genetic diversity loss if the founder population is not genetically diverse (Freeland, 2020). Assessing ge-

**Table 1**. The inclusion and exclusion criteria for the selection of eligible studies for a systematic review on the genetic diversity of *Hyla arborea* group populations.

| Inclusion criteria | Exclusion criteria |
|---|---|
| English language | Reviews, letters, editorials, notes, comments |
| Research article | Studies on *Hyla* morphology, reproduction, song, mating, pathogens, biometry |
| No geographical restriction | |
| Publications published up to 4th April 2022 | Studies not related to any *Hyla* species |
| Studies on species in the *Hyla arborea* group | No genetic analysis |

netic diversity is an effective method to follow the viability of a population (Hamer & McDonnell, 2008). Recent advancements in molecular biology have made genetic analyses even more accessible and affordable than before. Microsatellite and single nucleotide polymorphism (SNP) analyses are nowadays particularly popular for assessing genetic diversity, in consideration of their repeatability and comparability (Beebee & Rowe, 2004; Freeland, 2020). However the use of appropriate genetic markers and cautious interpretation is strongly advised (Hoban et al., 2021; Paz-Vinas et al., 2021).

Here, we present a systematic review to summarize the evidence concerning the genetic diversity of European tree frog (*Hyla arborea* group) populations and identify the most common methods used for its assessment. The aim of this review is to identify knowledge gaps about the genetic diversity of the *H. arborea* group that require further investigation and illustrate how filling these gaps might translate into future conservation efforts. For a group of phenotypically similar species like *Hyla* frogs the knowledge of regionally available data is important for informing ecological studies.

## 2. Materials and Methods

### 2.1. Search strategy and sources

The methodology was based on "The Preferred Reporting Items for Systematic reviews and Meta-Analyses" (PRISMA) guidelines (Page et al., 2021). Four international databases were searched (Scopus, PubMed, Web of Science, ScienceDirect) for all published studies reporting the genetic diversity of *Hyla arborea* group species. Selected keywords for the databases were "hyla", "genetic" and "diversity". No limits were set on publication years or geographic regions. Only studies reported in peer-reviewed articles were considered eligible. The databases were searched for English-language publications published up to November 8th, 2022.

### 2.2. Study selection and analyses

All search results from the four databases were combined and entered in MS Excel (Microsoft Corporation, 2022). Authors (EB, GD, ADM) then independently screened the title and abstract of each article and selected eligible studies using inclusion and exclusion criteria (Table 1). Selected eligible studies from all authors were combined and if there were different results of the eligibility of a study all authors discussed it until an agreement was reached. The data representing the eligible studies for the full-text screening were merged and managed in Microsoft Excel 2021. Papers were further screened for eligibility based on the full-text by three independent reviewers (EB, GD, ADM). Exclusion criteria for the full text screening were: i) studies that did not investigate genetic diversity or variation, ii) studies that were conducted in the laboratory or relied on common-garden experiments and data simulations, iii) studies whose full text was not available.

### 2.3. Data extraction

A data extraction sheet was created in Microsoft Excel 2021. It contained the following information: article information (title, author, year), sample information (study site, species studied, age group used in study, sample type and storage, DNA sample extraction method and storage, sample tests (electrophoretic analysis, microsatellite analysis, sequencing and other tests used), other methods used in study (phylogenetic analysis, bottleneck analysis, genetic diversity analysis, population structure analysis), results (microsatellite results, enzyme results, overall genetic diversity, bottleneck effect, phylogenetic results, other results).

## 3. Results

### 3.1. Search results and eligible studies

A total of 805 studies were identified through four databases. There were 129 studies that were duplicates and 651 studies that did not meet the inclusion criteria of the screening process for either the title and abstract screening or the full-text screening. After full-text screening of the 25 articles, nine were excluded for following reasons: four studies investigated sex chromosomes with no aim of estimating population genetic diversity; four studies used genetic data from previous studies; one study did not disclose the number of populations and sites that were sampled; one study used immunological methods. A total of 16 relevant studies were included in the review after thorough screening after the inclusion and exclusion criteria (Figure 1).

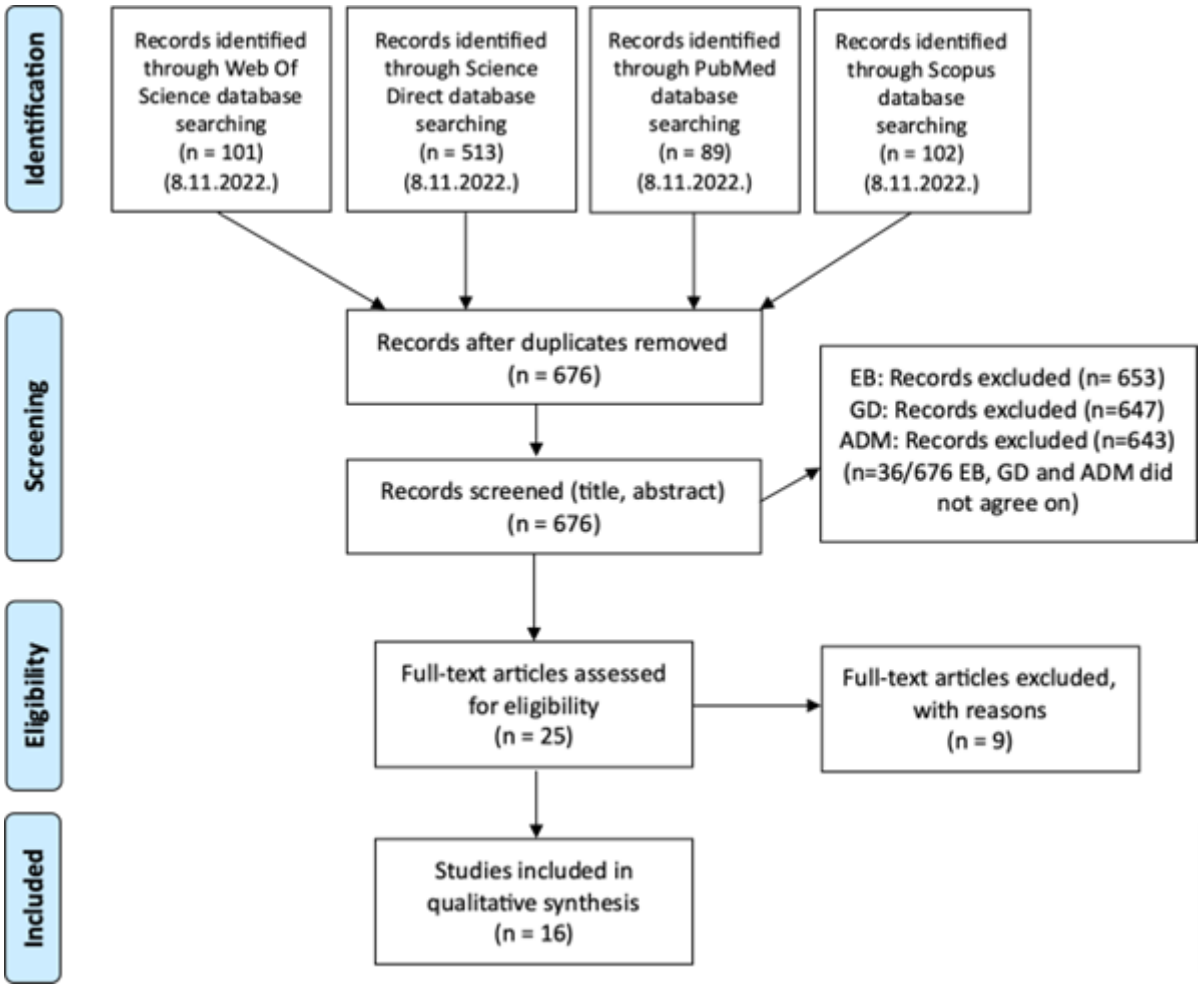


**Fig. 1.** PRISMA flow diagram of the search strategy steps for the literature review.

### 3.2. Genetic data sampling and analysis methods

The studies included in analysis originated from 44 countries in Europe, Middle East and Asia and were published between 1992 and 2022 (Table 2). The most common species studied was *Hyla arborea* s.s. (13 studies), others included *H. orientalis*, *H. molleri*, *H. intermedia*, *H. savignyi*, *H. meridionalis*, *H. japonica*, *H. felixarabica* and *H. sarda*. Number of sampled populations for a study ranged from one to 158, while sampled individuals ranged from 28 to 779. Most studies used buccal swabs for further deoxyribonucleic acid (DNA) extraction. Following tissue samples also were used: tail and toe clips, skeletal muscles, and liver samples.

For sample storage two studies eluted buccal swabs in a 200µl Qiagen Buffer AE (Broquet et al., 2010; Stöck et al., 2012), one study resuspended swabs in 100 µl Invitek Elution buffer (Auffarth et al., 2017) and one study diluted swabs with ddH20 in a ratio of 1:5 (Oswald et al., 2017). All previously mentioned studies stored samples at -18°C or -20°C. For the extraction of DNA from tree frog buccal swabs the following commercial kits were used: DNeasy Tissue kit (Qiagen) (Broquet et al., 2010; Dufresnes et al., 2013, 2016; Stöck et al., 2012) or Invisorb Spin Swab Kit (Invitek) (Auffarth et al., 2017; Oswald et al., 2017). For DNA extraction from frog tissues following protocols and/or kits were used: standard CTAB buffer (Andersen et al., 2004; Dubey et al., 2009), proteinase K procedures (Andersen et al., 2004), standard phenol-chloroform protocol (Arens et al., 2006; Verardi et al., 2009), QIAamp DNA Mini Kit (Qiagen) (Dubey et al., 2009) or DNeasy Blood and Tissue Kit (Qiagen) (Car et al., 2022).

Genetic diversity was assessed with microsatellites in ten studies (Table 3). Number of analysed microsatellites per study ranged from 6 to 30. Three studies assessed genetic diversity with enzymes (Table 4). Number of analysed loci per study ranged from nine to 18 loci, and most frequently used enzymes were aspartate aminotransferase (Aat), esterase (Est) and superoxide dismutase (Sod). Three types of buffer systems were used with standard horizontal gel or 10% starch gel.

**Table 2.** Characteristics of the eligible studies included in the literature review of genetic diversity of *Hyla arborea* group. NR - not reported.

| Authors | Sample location (country/-ies) | N. of populations sampled | N. of individuals sampled | Year(s) sampled | Species studied | Sample type |
|---|---|---|---|---|---|---|
| Auffarth et al., 2017 | Germany | 1 | 28 | 2005-2008 | *Hyla arborea* | Buccal swab |
| Oswald et al., 2017 | Germany | 3 | 91 | 2015 | *Hyla arborea* | Buccal swab |
| Kyriakopoulou-Sklavounou et al., 1992 | Greece | 2 | 51 | 1991 | *Hyla arborea* | Blood, skeletal muscle |
| Dufresnes et al., 2013 | Albania, Austria, Croatia, France, Germany, Greece, Hungary, Kosovo, Macedonia, Montenegro, Netherlands, Poland, Romania, Serbia, Switzerland | 65 | 779 | NR | *Hyla arborea* | Buccal swab, tail clip |
| Stöck et al., 2012 | Albania, Algeria, Azerbaijan, Belgium, Belarus, Croatia, Cyprus, France, Georgia, Germany, Greece, Hungary, Iran, Iraq, Israel, Italy, Japan, Moldavia, Montenegro, Morocco, Netherlands, Poland, Portugal, Romania, Russia, Serbia, Spain, Switzerland, Syria, Tunisia, Turkey, Ukraine, Yemen | 158 | 462 | 1994-2011 | *Hyla arborea, Hyla orientalis, Hyla molleri, Hyla savignyi* | Buccal swab, tail clip |
| Dufresnes et al., 2016 | Azerbaijan, Bulgaria, Belarus, Georgia, Greece, Moldavia, Poland, Russia, Serbia, Turkey, Ukraine | NR | 557 | NR | *Hyla orientalis* | Buccal swab |
| Broquet et al., 2010 | Germany, France | 12 | 539 | 2006-2007 | *Hyla arborea* | Buccal swab, tadpole tissue |
| Edenhamn et al., 2000 | Sweden | NR | 319 | 1991 | *Hyla arborea* | Skeletal muscle, liver |
| Andersen et al., 2004 | Denmark | 8 | 494 | 1991-2001 | *Hyla arborea* | Tail clip |
| Luquet et al., 2011 | France | 4 | NR | 2007-2008 | *Hyla arborea* | Buccal swab |
| Arens et al., 2006 | Netherlands | 12 | 175 | 1998 | *Hyla arborea* | Tail clip |
| Verardi et al., 2009 | Italy, Slovenia, Croatia | 16 | 282 | NR | *Hyla arborea, Hyla intermedia* | NR |
| Dubey et al., 2009 | Switzerland | 2 | 235 | 2002-2003 | *Hyla arborea* | Tadpole tissue |
| Gvoždík et al., 2010 | Azerbaijan, Cyprus, Georgia, Iran, Iraq, Israel, Japan, Jordan, Lebanon, Spain, Syria, Turkey, Yemen | NR | >200 | NR | *Hyla orientalis, Hyla meridionalis, Hyla japonica, Hyla felixarabica, Hyla savignyi* | Buccal swab, tail, and toe clips |
| Gvoždík et al., 2015 | Austria, Azerbaijan, Bulgaria, Croatia, Czech Republic, Denmark, France, Georgia, Germany, Greece, Hungary, Iran, Italy, Japan, Montenegro, Netherlands, Poland, Portugal, Romania, Russia, Slovakia, Slovenia, Spain, Switzerland, Turkey, Ukraine | NR | 198 | NR | *Hyla arborea, Hyla orientalis, Hyla intermedia, Hyla molleri, Hyla sarda* | Unspecified tissue sample |
| Car et al., 2022 | Ukraine | 19 | 216 | 2016-2018 | *Hyla orientalis* | Tibia muscle |

**Table 3.** Microsatellites used in studies for assessment of genetic diversity of *Hyla arborea* populations. NR - not reported.

| Authors | N. of microsatellites used | Microsatellites |
|---|---|---|
| Auffarth et al., 2017 | 8 | WHA1-9, WHA1-60, WHA1- 67, WHA1-104, WHA1-140, WHA1-20, WHA1-25, WHA1-103 |
| Oswald et al., 2017 | 12 | Ha-A130, Ha-B12, Ha-B5R3, Ha-D115, WHA1-9, WHA1-20, WHA1-25, WHA1-60, WHA1-67, WHA1-103§ WHA1-104, WHA1-140 |
| Dufresnes et al., 2013 | 30 | WHA1-20, WHA1-25, WHA1-103, WHA1-67, Ha-B12, Ha-A130, Ha-A11, Ha-A127, Ha-B5R3, Ha-D115, Ha-A119, Ha-E2, Ha-A136, Ha-A110, Ha-D104, Ha-H116, Ha-A139, Ha-T32, Ha-T41, Ha-T49, Ha-T50, Ha-T56, Ha-T58, Ha-T60, Ha-T63, Ha-T64, Ha-T66, Ha-T67, Ha-T68, Ha-T69 |
| Dufresnes et al., 2016 | 12 | WHA1-103, Ha-T49, Ha-T64, Ha-T41, Ha-T69, Ha-T54, Ha-T55, Ha-T50, Ha-T58, Ha-T53, Ha-T60, Ha-T68 |
| Broquet et al., 2010 | 21 | Ha-A-110, Ha-A-136, Ha-A-139, Ha-A11, Ha-A119, Ha-A127, Ha-A130, Ha-B5R3, Ha-D-104, Ha-D-106, Ha-D115, Ha-D3R3, Ha-E2, Ha-H-116, WHA1-103, WHA1-104, WHA1-140, WHA1-20, WHA1-25, WHA1-67 WHA1-9 |
| Andersen et al., 2004 | 12 | NR |
| Luquet et al., 2011 | 15 | Ha-A11, Ha-A119, Ha-A127, Ha- A130, Ha-B5R3, Ha-D3R3, Ha-D115, WHA1–20, WHA1– 25, WHA1–67, WHA1–103, Ha-D-104, Ha-D-106, Ha-H- 116, Ha-A-136 |
| Arens et al., 2006 | 8 | WHA5-22A, WHA5-201, WHA1- 60, WHA1-104, WHA1-09, WHA1-20, WHA1-25, WHA1-140 |
| Dubey et al., 2009 | 6 | WHA1- 9, WHA1-20, WHA1-25, WHA1-103, WHA1-104, WHA1-140 |
| Car et al., 2022 | 21 | Ha-T50, Ha-T53, Ha-T54, Ha-T55, Ha-T56, Ha-T58, Ha-T60, Ha-T61, Ha-T63, Ha-T66, Ha-T67, Ha-T68, Ha-A11, Ha-A127, Ha-B5R3, WHA1-67, Ha-D104, Ha-D115, Ha-E2, Ha-A110, Ha-A119 |

**Table 4.** Electrophoretic analysis method for assessing genetic diversity with enzymes of *Hyla arborea* group. NR - not reported.

| Authors | Enzyme | Locus | Buffer system | Gel |
|---|---|---|---|---|
| Edenhamn et al., 2000 | Aspartate aminotransferase | Aat-1, Aat-2 | Tris-citrate buffer pH 8.2 | Standard horizontal gel |
| | Isocitrate dehydrogenase | Idh-1, Idh-2 | | |
| | Malate dehydrogenase (NADP+) | Mdhp-1, Mdhp-2 | | |
| | Esterase | Est-1, Est-2, Est-3, Est-4, Est-5 | N-(-3- aminopropyl) morpholine/ citratebuffer pH 6.1 | |
| | General protein | Gp- 1, Gp-2 | | |
| | Glucose-6-phosphate Isomerase | Gpi-1, Gpi-2 | | |
| | Malate dehydrogenase | Mdh-1 | | |
| | Phosphoglucomutase | Pgm- 1 | | |
| | Superoxide dismutase | Sod-1 | | |
| Kyriakopoulou-Sklavounou et al., 1992 | Aspartate aminotransferase | Aat-1, Aat-2 | N-(-3- aminopropyl) morpholine/ citratebuffer pH 6.1 | 10% starch gel |
| | Malate dehydrogenase | Mdh-1, Mdh-2 | | |
| | Creatine kinase | Ck-1 | Tris-citrate buffer pH 8.2 | |
| | Lactate dehydrogenase | Ldh-i | | |
| | Superoxide dismutase | Sod-1 | Tris-LiOH-Boric buffer pH 8.2 | |
| | Esterase | Est-1, Est-2 | | |
| | Haemoglobin | Hb-1 | | |
| Verardi et al., 2009 | Isocitrate dehydrogenase | Idh-1 | NR | Standard horizontal gel |
| | Xanthine dehydrogenase | Xdh | | |
| | Superoxide dismutase | Sod-1 | | |
| | Aspartate aminotransferase | Aat-2 | | |
| | Esterase | Est-2 | | |
| | Aminopeptidase | Pep-1, Pep-2, Pep-4 | | |
| | Adenosine deaminase | Ada | | |

For calculating genetic diversity of *H. arborea* group populations, several different softwares were used (Table 5). For calculating allelic richness, allelic frequencies, gene diversity and genetic differentiation, the software used most frequently was FSTAT (Goudet, 1995). On the contrary, the software STRUCTURE (Pritchard et al., 2000) was the most frequently used for analysing population structure based on genetic data. The software ARLEQUIN (Excoffier et al., 2005) was used for calculating mitochondrial gene haplotype and nucleotide diversity, deviations from Hardy-Weinberg equilibrium, genetic similarity between populations (pairwise $F_{ST}$) and determining population differentiation. Studies performing bottleneck analysis often used BOTTLENECK (Cornuet & Luikart, 1996).

**Table 5.** Softwares used for analysis of *Hyla arborea* group genetic diversity and population genetics (software developers or authors in brackets). NR - Not reported.

| Analysis of genetic diversity* | Population structure* | Population differentiation* | Bottleneck analysis* | Authors |
|---|---|---|---|---|
| FSTAT 2.9.3.2 (Goudet, 1995)<br>GENALEX 5.6 (Peakall and Smouse, 2006)<br>ARLEQUIN v.3.5.2.2 (Excoffier et al., 2005)<br>Package DEMETICS in RStudio (Gerlach et al., 2010) | STRUCTURE v.2.3.4 (Pritchard et al., 2000) | NR | BOTTLENECK v.1.2.02 (Cornuet and Luikart, 1996) | Oswald et al., 2017 |
| BIOSYS-1 (Swofford and Selander, 1989) | NR | NR | NR | Kyriakopoulou-Sklavounou et al., 1992 |
| ARLEQUIN (Excoffier et al., 2005)<br>FSTAT (Goudet, 1995) | TCS 1.21 (Clement et al., 2000)<br>STRUCTURE 2.3.3 (Pritchard et al., 2000)<br>STRUCTURE HARVESTER 0.6.92 (Earl and vonHoldt, 2012) | PCAGEN 1.2 (Goudet, 1999) | NR | Dufresnes et al., 2013 |
| FSTAT 2.9.3 (Goudet, 1995) | STRUCTURE 2.3.4 (Pritchard et al., 2000)<br>STRUCTURE HARVESTER (Earl and vonHoldt, 2012) | NR | NR | Dufresnes et al., 2016 |
| Package HIERFSTAT (Goudet, 2005) in R v2.6.1 (R Development Core Team, 2017) | NR | NR | BOTTLENECK (Cornuet and Luikart, 1996) | Broquet et al., 2010 |
| FSTAT (Goudet, 1995) | STRUCTURE v.2 (Pritchard et al., 2000) | ARLEQUIN v. 2.0 (Schneider et al. 2000) | *M* ratio (Garza and Williamson, 2001) | Andersen et al., 2004 |
| FSTAT 2.9.3 (Goudet, 1995) | NR | NR | NR | Luquet et al., 2011 |
| FSTAT 2.93 (Goudet, 1995) | STRUCTURE (Pritchard et al., 2000) | Fst-estimator (Weir, Cockerham, 1984).<br>ARLEQUIN (Excoffier et al., 2005) | BOTTLENECK (Cornuet and Luikart, 1996),<br>*M* ratio (Garza and Williamson, 2001) | Arens et al., 2006 |
| FSTAT 2.9.3.2 (Goudet, 2001),<br>GENEPOP 3.3 (Raymond, Rousset, 1995) | STRUCTURE 2.1 (Pritchard et al., 2000) | GENETIX 4.05 (Belkhir et al., 1996–2004) | NR | Verardi et al., 2009 |
| FSTAT 2.9.3.2 (Goudet, 1995) | STRUCTURE 2.1 (Pritchard et al., 2000) | NR | NR | Dubey et al., 2009 |
| FSTAT (Goudet, 1995)<br>ARLEQUIN (Excoffier et al., 2005),<br>GENETIX (Belkhir et al., 2004),<br>ADZE (Szpiech et al., 2008) | ARLEQUIN (Excoffier et al., 2005) | ARLEQUIN (Excoffier et al., 2005) | NR | Car et al., 2022 |

Five studies performed phylogenetic analysis to assess *H. arborea* group genetic diversity (Table 6). The most common gene used for genetic diversity analyses was the mitochondrial *cytochrome b* gene. Most studies performed maximum likelihood reconstructions with the software PHYML (Guindon & Gascuel, 2003) and Bayesian phylogeographic and species tree reconstructions with the software BEAST (Drummond & Rambaut, 2007) and its multiple packages and modules (Table 7).

### 3.3. Genetic diversity results of Hyla arborea group populations

Overall, the genetic diversity was assessed based on microsatellite analyses (Table 8) and enzymes (Table 9) only in three species: *H. arborea* s.s., *H. orientalis* and *H. intermedia*. The sampled populations were mainly from Central and Eastern Europe.

**Table 6.** Sequenced genes and used primers in studies assessing *Hyla arborea* group genetic diversity. NR - not reported.

| Authors | Gene name | F primer name or sequence | R primer name or sequence |
|---|---|---|---|
| Dufresnes et al., 2013 | Cytochrome b | L0 | H1046 |
| | partial D-loop | Ha-Dloop-Int | Ha-Control-PH |
| | rag-1 intron | Ha-Rag1f | Ha-Rag1r |
| Stöck et al., 2012 | Cytochrome b | L0 | H1046 |
| | Fibrinogen A, alpha-polypeptide | MVZ4 | MVZ48 |
| Dufresnes et al., 2016 | Cytochrome b | NR | NR |
| Verardi et al., 2009 | Cytochrome b | L14841 | H15149 |
| Gvoždík et al., 2010, Gvoždík et al., 2015 | 12S | 12Sa | 12Sbs |
| | 16S | 16SL1 | 16SH1 |
| | Rhodopsin, exon 1 | Rhod1A | Rhod1C |
| | Tyrosinase precursor, exon 1 | Tyr1C | Tyr1G |
| Car et al., 2022 | Cytochrome b | L0 | H1046 |

**Table 7.** Data analysis methods used for phylogenetic studies of *Hyla arborea* group. ML – Maximum-likelihood, cyt-b – cytochrome b, BI – Bayesian inference.

| Authors | Analysis method* | Software used for analysis |
|---|---|---|
| Dufresnes et al., 2013 | ML phylogenetic reconstructions (1) with the selected model (2) and 1000 bootstrap replicates. Divergence time estimated between major haplogroups from cyt-b data set (3), using a strict molecular clock and a coalescent prior. Ran 3 independent chains of 30 million iterations each and checked for convergence (4). Phylogeographic history was constructed by a Bayesian phylogeographic analysis of mtDNA data set using spatial continuous diffusion models (5). | 1) PHYML 3.0 (Guindon and Gascuel, 2003)<br>2) JMODELTEST 0.1.1 (Posada, 2008)<br>3) BEAST 1.6.2 (Drummond and Rambaut, 2007)<br>4) TRACER 1.5 (BEAST package)<br>5) BEAST 1.7.5 (Drummond et al., 2012) |
| Stöck et al., 2012 | ML phylogenies (1) using the GTR model for cyt-b and selected model for the Fibrinogen alpha nuclear marker. | 1) PHYML 3.0 (Guindon et al., 2010) |
| Dufresnes et al., 2016 | ML reconstructions (1) and Bayesian phylogenetic reconstructions (2) of cyt-b haplotypes, using a selected model (3) of sequence evolution. | 1) PHYML 3.0.1 (Guindon and Gascuel, 2003)<br>2) MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001)<br>3) MrAIC 1.4.4 (Nylander, 2004). |
| Gvoždík et al., 2010 | ML tests (1) with chosen model from (2), BI analysis (3) with two runs and four chains for each run for six million generations and sampling every 100th tree. Maximum parsimony (4) was also analysed. | 1) PHYML 3.0.1 (Guindon and Gascuel, 2003)<br>2) JMODELTEST 0.1.1 (Posada, 2008)<br>3) MrBayes 3.2. (Huelsenbeck and Ronquist, 2001)<br>4) PAUP 4.0b10 (Swofford and Sullivan, 2003) |
| Gvoždík et al., 2015 | Gene trees were reconstructed by BI (1) and ML criterion. Best-fit partitioning schemes and nucleotide substitution models selected using (2). A species tree was inferred using (3). Alignments of all individuals were uploaded into (4) where they were assigned separate and unlinked substitution, clock, and tree models. Five independent (3) runs were performed, each for 200 million generations, sampling every 20,000th generation to obtain a posterior sample of 10,000 trees. The likelihoods were inspected using (5). The post burn-in samples of the five runs were combined in (6) The output of 45,000 sampled trees was uploaded to (7), to infer the final species tree as a maximum clade credibility tree. For species delimitation coalescent-based Bayesian species delimitation analyses were conducted in (8). | 1) MrBayes 3.2. (Ronquist et al., 2012)<br>2) PartitionFinder v1.1.0 (Lanfear et al., 2012)<br>3) BEAST (Heled and Drummond, 2010)<br>4) BEAUti v1.8.0 (BEAST package)<br>5) TRACER 1.5 (BEAST package)<br>6) LogCombiner v1.8.0. (BEAST module)<br>7) TreeAnnotator v1.8.0 (BEAST module)<br>8) Bayesian Phylogeny and Phylogeography v2.2 (Yang, 2013) |

*Numbers in brackets correspond to the software in the adjacent column used for performing mentioned analysis

**Table 8.** Genetic diversity results of *Hyla arborea* group populations based on microsatellites. NR - not reported.

| Species | Region or country | N. of alleles | Allelic richness | Allelic frequencies | Loci polymorphism | Genetic diversity | | | Authors |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | $H_{exp}$ | $H_o$ | Consensus | |
| *Hyla arborea* | Germany | 4 to 7 | NR | NR | Moderately to highly polymorphic | 0.50 | 0.56 (mean) | High | Auffarth et al., 2017 |
| | | 2 to 9 | 3.81 - 5.41 | 0.01 - 0.93 | 11 polymorphic, 1 monomorphic | 0.62 - 0.72 | 0.65 - 0.72 | High | Oswald et al., 2017 |
| | Central Europe | NR | 1.90 - 4.20 | NR | NR | NR | 0.26 - 0.62 | Low to high | Dufresnes et al., 2013 |
| | | NR | 0.54 - 0.94 | NR | NR | NR | 0.76 - 0.96 | High | Broquet et al., 2010 |
| | Denmark | 6 to 21 | NR | NR | Most polymorphic, few monomorphic | 0.35 - 0.53 | NR | Low | Andersen et al., 2004 |
| | France | 4 to 6 | 3.56 - 5.52 | NR | NR | 0.37 - 0.46 | 0.38 - 0.46 | Low | Luquet et al., 2011 |
| | Netherlands | 2 to 10 | 1.90 - 6.00 | 0.01 - 0.13* | All polymorphic | 0.39 - 0.58 | NR | Low | Arens et al., 2006 |
| | Switzerland | 7 to 17 | 4.41 - 5.29 | NR | NR | 0.35 - 0.86 | 0.44 - 0.68 | Low to high | Dubey et al., 2009 |
| *Hyla orientalis* | East Europe | NR | NR | NR | NR | 0.20 - 0.50 | NR | Low | Dufresnes et al., 2016 |
| | | NR | 1.53 - 1.73 | NR | NR | 0.19-0.27 | 0.17-0.25 | Low | Car et al., 2022 |

**Table 9.** Genetic diversity results of *Hyla arborea* group populations based on enzymes. NR - not reported.

| Species | Study site | Loci polymorphism | Nr of alleles (avg) | Allelic richness | Allelic frequencies | Enzyme genetic diversity | | Authors |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Heterozygosity | Consensus | |
| *Hyla arborea* | Greece | 5 systems polymorphic, 4 monomorphic | 1.54 | NR | 0.10 - 1.00 | 0.13 - 0.14 | High | Kyriakopoulou-Sklavounou et al., 1992 |
| | Sweden | 1 out of 18 systems was polymorphic | 1.06 | NR | 0.09 - 0.10 | 0.08 - 0.11 | Low | Edenhamn et al., 2000 |
| | Italy, Croatia, Slovenia | 5 of 9 systems were bi-allelic, 2 systems were mostly bi-allelic, 2 systems polymorphic | NR | 1.0 - 1.2 | NR | 0.00 - 0.05 | Low | Verardi et al., 2009 |
| *Hyla intermedia* | | | | 1.1 - 1.7 | | 0.05 - 0.22 | High | |

The genetic diversity of the European tree frog *H. arborea* s.s. was assessed on microsatellite analyses in eight studies and on enzymes in three studies. In most cases the genetic diversity was described as low, however in some studies it ranged from low to high or high overall. The genetic diversity of enzymes in two of the three studies was reported very low, however it should be noted that these results can't be compared between studies, because different enzymes were used. The genetic diversity of the Italian tree frog *H. intermedia* was assessed on individuals collected from the Italian Peninsula and had higher diversity than *H. arborea* s.s. from the same region (Table 9).

The genetic diversity of the Eastern tree frog *H. orientalis* was assessed in two studies (Car et al., 2022; Dufresnes et al., 2016) from populations in Eastern Europe (Table 8). The microsatellite diversity in both studies was low, however the mitochondrial nucleotide diversity of populations from Ukraine was high at a value of 0.002 (Car et al., 2022).

Eight studies reported population genetics results of three species in the *H. arborea* group (Table 10). Few deviations from the Hardy-Weinberg equilibrium (HWE) were found in populations. However, the number of analysed individuals in three of these studies (Auffarth et al., 2017; Kyriakopoulou-Sklavounou et al., 1992; Oswald et al., 2017) was lower than 100, possibly reducing the accuracy of HWE analysis. Population structures often corresponded to isolated geographic locations, however, were genetically low differentiated. Bottleneck effects were reported in multiple populations.

**Table 10.** Population genetics results of *Hyla arborea* group. NR - not reported.

| Species | Deviations from Hardy-Weinberg equilibrium | Population structure | Population differentiation | Bottleneck effect present | Authors |
|---|---|---|---|---|---|
| *Hyla arborea* | 1 (WHA1-60) out of 7 loci | NR | NR | NR | Auffarth et al., 2017 |
| | 1 (WHA1-60) out of 12 loci | Three genetically different clusters, corresponds to the three geographical populations | Low genetic differentiation between three populations | Yes | Oswald et al., 2017 |
| | 1 (EST-2) out of 20 loci | NR | Low genetic differentiation between two populations | NR | Kyriakopoulou-Sklavounou et al., 1992 |
| | NR | NR | NR | Yes | Broquet et al., 2010 |
| | In 12 out of 144 tests at the single locus level | 11 genetically different populations | NR | Yes | Andersen et al., 2004 |
| | In 1 out of 88 tests at the single locus level | About 5 genetically different populations from 5 ponds, one subpopulation | Low to high genetic differentiation between populations | Yes | Arens et al., 2006 |
| | None | Two populations | Low between metapopulations | NR | Dubey et al., 2009 |
| | None | No ongoing gene flow between *H. arborea.* and *H. intermedia* populations | NR | NR | Verardi et al., 2009 |
| *Hyla intermedia* | None | | NR | NR | |
| *Hyla orientalis* | NR | Genetic structure corresponds to geographic distribution | Low microsatellite genetic differentiation, high mitochondrial genetic differentiation between 19 populations | No | Car et al., 2022 |

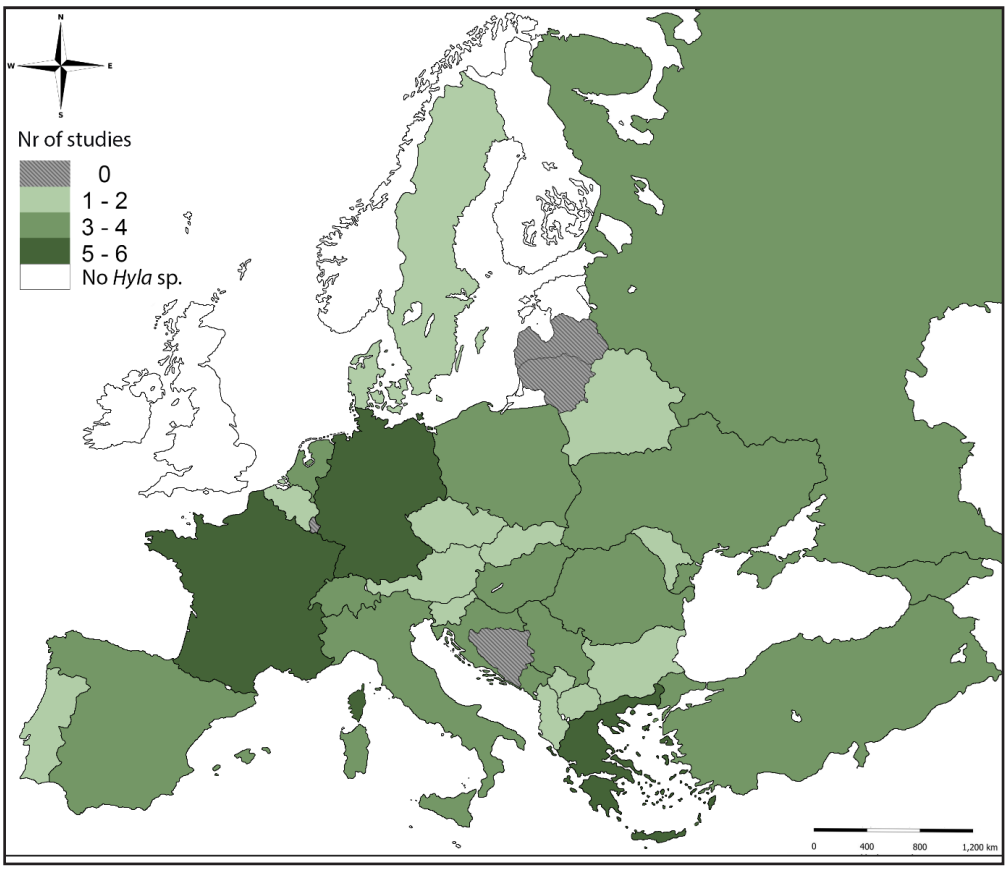


**Fig. 2.** Genetic studies on *Hyla arborea* group populations per country across the groups distribution area in Europe (map from https://worldmapblank.com/, adjusted)

Areas with no genetic diversity data on any *Hyla* species in their distribution range currently are Bosnia and Herzegovina, Lithuania and Latvia (Figure 2). The most intensively studied geographic area appears to be Central Europe (Germany, France, Greece) which overlaps with the distribution area of the most researched species - *H. arborea* s.s.

### 3.4. Phylogeographic diversity results of the Hyla arborea group

Studies that performed phylogeographic analysis covered seven species in the *Hyla arborea* group: *H. arborea* s.s., *H. orientalis*, *H. molleri*, *H. meridionalis*, *H. felixarabica*, *H. intermedia* and *H. sarda* (Table 11). These studies covered tree frog populations across Europe and the Middle East.

The earliest *Hyla* genus species divergence was detected in the late Miocene between *H. arborea* s.s., *H. sarda* and *H. savignyi* (Stöck et al., 2012) and between *H. orientalis*, *H. savignyi* and *H. felixarabica* (Gvoždík et al., 2010). During the Pliocene, *H. molleri*, *H. intermedia* and *H. sarda* diverged into distinct lineages (Gvoždík et al., 2015). While Stöck et al. (2012) suggested that *H. orientalis* might have diverged from *H. molleri* in the Pliocene

**Table 11.** Results of phylogenetic diversity and history analysis of the *Hyla arborea* group. NR - not reported, HA - *Hyla arborea* s.s., HO - *Hyla orientalis*, HMo - *Hyla molleri*, HMe - *Hyla meridionalis*, HSr - *Hyla sarda*, HSv - *Hyla savignyi*, HF - *Hyla felixarabica*, HI - *Hyla intermedia*, Mya - million years ago, kya - thousand years ago.

| Divergence time or epoch | Hybridization | Genetic diversity | Population expansion | Clades | Genetic differentiation | Authors |
|---|---|---|---|---|---|---|
| 180 kya (HA Adriatic clade) to 90 kya (HA most recent ancestor) | NR | HA - haplotype diversity greatly variable throughout range, nucleotide diversity high in southern Balkans and low in western Europe | Demographic and spatial expansions of HA | HA - main clade in the Adriatic coast | HA eastern, western, southern, and central-northern populations high differentiation | Dufresnes et al., 2013 |
| Pliocene (species HO - HMo) Late Miocene, low Pliocene (species HA - HSr - HSv) | HMo x HMe in France<br><br>HA x HO in Poland | HA – low<br><br>HO – high<br><br>HM – low | Postglacial range expansion of HA and HO (northern clade) | HO - five clades | HO lineages high differentiation | Stöck et al., 2012 |
| 1.2 Mya (HO main clades) 0.7 to 0.4 Mya (HO subclades) | NR | HO - overall high, higher in southern populations than northern. | Recent and postglacial range expansion of HO populations | HO - four clades, multiple subdivisions | High differentiation between eastern and western HO populations and admixture in Crimea and western Anatolia. Ring like pattern around Black Sea | Dufresnes et al., 2016 |
| 8.4 Mya (species HSv - HF) 11.1 Mya (species HO - Hsv) | HF x HSv in Israel | HO – high<br><br>HSv – high<br><br>HF – low to high | HO and HSv expansion from middle to late Pliocene | HSv - two main clades<br><br>HF - two main clades<br><br>HO - one main clade | NR | Gvoždík et al., 2010 |
| 1.4 Mya (species HO - HMo)<br><br>Pliocene (species HA - HI - HSr - HMo) | HA x HO in Greece, Bulgaria, Romania, Poland<br><br>HA x HM in France | HO – high | Recent HA expansion | HO and HMo - sister clades<br><br>HSr - sister clade to rest of *Hyla* taxa<br><br>HI - sister clade to HA sensu lato | HA high differentiation,<br><br>HI northern and southern populations low differentiation,<br><br>HO and HMo low differentiation | Gvoždík et al., 2015 |
| 5.5 Mya (species HA - HI) | HA x HI in Italy in the past | (see Table 9) | NR | NR | NR | Verardi et al. 2009 |

Gvoždik et al. (2015) postated such the divergence of the two species in the Pleistocene. i.e. about 1.4 million years ago. The genetic diversity of *H. arborea* s.s. across Europe was greatly variable, and particularly low in Western Europe and high in the southern Balkans (Dufresnes et al., 2013; Stöck et al., 2012). Multiple demographic, spatial, postglacial range and recent population expansions for this species were reported across the studies. For *H. orientalis* a high genetic diversity centre was found around the Black Sea (Dufresnes et al., 2016). The genetic differentiation of *H. arborea* group populations were reported mostly strong with few low differentiated clades. Hybridization events were reported between multiple pairs of *H. arborea* group species (Table 11).

## 4. Discussion

Overall, we observed the genetic diversity of species in the *H. arborea* group in Europe and Middle East most often being low. Genetic diversity can be used as an important marker of the persistance of native individuals (DeWoody et al., 2021; Gaitán-Espitia & Hobday, 2021) and therefore can show the necessity of conservation measures (Dufresnes et al., 2016; Oswald et al., 2017).

In this review we summarised the methods used and genetic diversity data of species in the *H. arborea* group. For the genetic studies DNA samples were taken mostly from buccal swabs which is a recommended method for amphibian genetic studies (Pidancier et al., 2003). DNA extraction was done most often with commercial kits. The best has been shown to be the CTAB phenol-chlorophorm extraction method, however kits are the best alternative in reducing time and usage of hazardous chemicals (Schiebelhut et al., 2017). From the most used methods – microsatellite analysis, enzyme electrophoresis, phylogenetic methods – for assessment of *Hyla* population genetic diversity microsatellites seem to be most effective and popular and their use is becoming more widespread in genetic research. Studies that used microsatellites often chose markers that were first described by Arens et al. (2000) and Berset-Brändli et al. (2008) for *H. arborea* s.s. Use of enzymes for genetic diversity has become less common, also represented in this review by the low number of studies and the last one being more than ten years ago. The several disadvantages of enzyme electrophoresis nowadays are replaced by DNA markers (Freeland, 2020; Jehle & Arntzen, 2002). Another disadvantage of this method is enzyme sensitivity and complicated replicability, making the results comparable only between the same enzyme loci. Another method of genetic diversity analysis that was not included in the studies of this review are single nucleotide polymorphisms (SNPs). A study on Iberian tree frogs concluded that SNPs provide more reliable genetic diversity pattern results than microsatellites (Camacho-Sanchez et al., 2020). SNPs are currently used for genetic studies with many other amphibian groups and can therefore be applied to *Hyla* group studies. SNPs analysis adds additional advantages to the previously described methods being even more timely and cost-effective (Freeland, 2020).

Phylogeographic studies in this review covered multiple tree frog species per study and presented not only genetic diversity data, but also their genetic history offering valuable insight. The five studies that performed phylogeographic analysis on tree frog species show valuable results explaining the plausible reasons for the variability of *Hyla* genetic diversity (Dufresnes et al., 2013, 2016; Gvoždík et al., 2010, 2015; Stöck et al., 2012). The variability of genetic diversity in a species can be affected by population expansions which was the case for the European tree frog *H. arborea* s.s. population with high genetic diversity in the Balkan peninsula, but the lineage that expanded to recolonize Northern and Western Europe lost its genetic diversity in the process (Dufresnes et al., 2013, 2016; Gvoždík et al., 2010, 2015; Stöck et al., 2012). This observation aligns with studies that reported bottleneck effects (Andersen et al., 2004; Arens et al., 2006; Broquet et al., 2010; Oswald et al., 2017) and deviations from HWE (Andersen et al., 2004; Arens et al., 2006; Auffarth et al., 2017; Kyriakopoulou-Sklavounou et al., 1992; Oswald et al., 2017) in populations from Western Europe. However, some of the *H. arborea* s.s. populations were affected by habitat fragmentation decreasing their genetic diversity even more (Andersen et al., 2004; Arens et al., 2006). Fragmented populations with low genetic diversity should be at higher risk of extinction (Frankham et al., 2010). The Eastern tree frog *H. orientalis* has a high genetic diversity in the area surrounding the Black sea, but lower diversity in northern populations, most likely due to a recent range expansion (Dufresnes et al., 2016). For five pairs of species, hybridization events have been reported. Hybridization occurs between genetically close species and increases the populations genetic diversity (Freeland, 2020). While sometimes hybridization can have deleterious effects, it can be also beneficial (Stelkens et al., 2014). A study on toads found that the enhanced genetic variation by hybridization might have enabled their expansion in novel habitats (Pierce et al., 2017). Currently there is no detailed information available on the genetic diversity in *Hyla* species hybrid zones and could have potential for future studies.

European tree frog *H. arborea* s.s. is the most studied from the *H. arborea* species group, while rest of the species from the group (*H. orientalis*, *H. intermedia*, *H. molleri*, *H. sarda*, *H. felixarabica*, *H. meridionalis*, *H. savignyi*) were mostly analysed in phylogeographic studies. Therefore, genetic diversity studies on species other than *H. arborea* s.s would be beneficial to improve the knowledge of local factor influence to genetic diversity. Two species – *H. intermedia perrini* and *H. carthaginiensis* – which were added to the *H. arborea* group in the last few years (Dufresnes et al., 2018, 2019; Speybroeck et al.,

2020) are not represented in any of the studies included in present systematic review. However, the follow up of the eventual changes in genetic diversity of these new species would be a valuable addition for research in the future. The map of genetic diversity research (Figure 2), shows how many times a genetic diversity analysis has been made in each country of *Hyla* sp. distribution in Europe (one study could sample populations in multiple countries). However, many of the counts in the map are from the phylogeographic studies that cover many countries, which notes the lack of regional studies on *Hyla* populations. There is a noticable lack of studies in Northern Europe, especially Latvia and Lithuania. However, a study on *H. orientalis* published since the systematic review has partially filled this gap (Birbele et al., 2023). Lithuania possibly has a population of *H. orientalis* that is expanding from the Latvian population, but no studies have yet been made.

The variability of *H. arborea* group population genetic diversity shows a rich history of species evolution and expansion over Eurasia, but also vulnerability to anthropogenic factors. Climate change is a current factor that can drive amphibian populations to expand or lose their range and occupy new niches (Alves-Ferreira et al., 2022; Enriquez-Urzelai et al., 2019). Human activities like accidental introductions of *Hyla* species in novel habitats or spreading of amphibian pathogens like chytridiomycosis and Ranavirus into native populations could drastically affect already genetically weakened *Hyla* populations (Allentoft & O'Brien, 2010). Notably studies of the *H. orientalis* population in the Chornobyl exclusion zone (Ukraine) have found unusual responses to radiation. Ongoing microevolutionary processes in the population have been detected rising the question of long-term impact of ionizing radiation on the species (Car et al., 2022). The absorbed rates of radiation in tree frogs were also generally lower than the harmful tresholds (Burraco et al., 2021). These findings of unusual environmental factors could be the reasoning behind the genetic variability in the area.

Multiple authors (Andersen et al., 2004; Arens et al., 2006; Dubey et al., 2009; Dufresnes et al., 2013, 2016; Luquet et al., 2011; Oswald et al., 2017) point out the contribution of their studies for species conservation management further proving the importance of genetic and phylogeographic studies. Monitoring genetics helps inform the health and sustainability of a population (Freeland, 2020). Endangered species and amphibians in general are at a higher risk of losing genetic diversity, because of their breeding strategies, declines in population sizes, habitat fragmentation and low dispersal capabilities (Allentoft & O'Brien, 2010). Despite many conservation efforts the role of genetic diversity of populations is overlooked and is not included in international and regional policies (Hoban et al., 2021; Laikre, 2010; Laikre et al., 2010). Therefore, comprehensive genetic studies can highlight important signals, where conservation efforts are lacking. From the ecological point of view conservation actions like reintroduction or population supplementation can improve ecological processes in habitats (ecosystem connectivity, biodiversity etc.) (Strange et al., 2021). In these cases it is necessary to understand the genetic backstory of species like *Hyla* frogs to inform the next steps.

## 5. Conclusions

The knowledge base of species and the necessary conservation applications is becoming more accessible and affordable with genomic technologies (Segelbacher et al., 2022). This review highlights the geographic regions and species in the *H. arborea* group (*H. orientalis*, *H. meridionals* etc.) where there are still gaps of knowledge. The compiled methods of genetic diversity analysis provides a useful overview of the available information. This review can guide future studies in choosing the tree frog species and appropriate methods for genetic diversity analysis.

**Data availability**

The data used in this study are provided in the Supplement.

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