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ENVIRONMENTAL SUSTAINABILITY AND WELLBEING

CYCLE XXXVI

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A pond of knowledge

A multi-disciplinary perspective on amphibian conservation

Scientific/Disciplinary Sector (SDS) BIO/18

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WHY POETRY?

John Laue

(…) There were
poems that shouted,
poems that whispered,
poems that cried,
and poems that
appeared to say,
“O well. What the hell.
Let’s have poetry
today
instead of toast.”

Why poetry?
Don’t you see
the possible combinations:

real toads
in imaginary gardens;

real gardens
in imaginary toads;

imaginary toads
in real gardens;

imaginary gardens
in real toads?

It may not
save you,
serve you,
soothe you,
or amuse you.

But you might
as well ask,

Why life?

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ABSTRACT

As the dramatic decline of amphibian populations worldwide is becoming increasingly alarming, the question arises of whether a sustainable coexistence between humans and these ‘foul and loathsome beasts’ is possible at all. To address this issue, this doctoral thesis focuses on the Province of Trento as a case study to investigate the presence and wellbeing of amphibian communities in protected areas as well as artificial and human-impacted sites applying different molecular and statistical approaches, including an innovative multidisciplinary workflow based on landscape planning methods. First, I developed a new, non-invasive protocol based on environmental DNA samples, testing it on the model species *Rana temporaria*. This study proved that the new protocol can be used to obtain information on both species presence and genetic diversity, as I found that standard genetic diversity indices calculated with this approach strongly correlated with that estimated with traditional methods. Secondly, I therefore extended this innovative protocol to all amphibian species living in the study area, testing the new workflow both in the field, across a variety of natural and artificial habitats, and during bioinformatic analysis, successfully retrieving mitochondrial haplotype data of ten amphibian species. Thirdly, I included these amphibian presence data, along with a series of ecological and landscape indices characterizing the considered sites, into a multidimensional analysis approach derived from landscape planning theory. The first results of this study highlighted the important role of protected wetlands for amphibian communities’ conservation, and also indicated farm ponds as potential additional breeding sites for amphibians. Lastly, I characterized the composition of both bacterial and fungal skin communities in *Bombina variegata* populations from habitats with varying degrees of anthropogenic impact, showing that these microbial communities are indeed affected by human presence and activities, but showing discording patterns between different diversity indices (i.e., alpha and beta diversity). In conclusion, despite confirming the influence of anthropogenic pressures on presence and wellbeing of amphibians, this thesis also highlighted the existence of possible ‘intermediate’ habitats where human-amphibian cohabitation is possible (i.e., farm ponds), as well as the necessity of integrating approaches from different disciplines to achieve a unified conservation framework aimed at a truly sustainable development.

RIASSUNTO

Mentre il drammatico declino globale delle popolazioni di anfibi diviene sempre più allarmante, sorge la domanda se sia effettivamente possibile una convivenza sostenibile tra gli esseri umani e queste “bestie immonde e ripugnanti”. Nel tentativo di affrontare questa questione, questa tesi di dottorato si concentra sulla Provincia di Trento come caso di studio per indagare la presenza e il benessere delle comunità di anfibi all’interno di aree protette e siti artificiali e soggetti a impatto antropico, applicando diversi approcci molecolari e statistici, incluso un innovativo workflow multidisciplinare basato su metodi di pianificazione del paesaggio. Innanzitutto, ho sviluppato un nuovo protocollo non invasivo basato su campioni di DNA ambientale, testandolo sulla specie modello *Rana temporaria*. Da questo studio è emerso che il protocollo può essere utilizzato per ottenere informazioni sia sulla presenza delle specie che sulla loro diversità genetica, poiché ho dimostrato che gli indici standard di diversità genetica calcolati con questo approccio e con i metodi tradizionali sono fortemente correlati. In secondo luogo, ho quindi esteso questo protocollo a tutte le specie di anfibi nell'area di studio, testando il nuovo workflow sia sul campo, in una varietà di habitat naturali e artificiali, sia durante l'analisi bioinformatica, ottenendo con successo dati sugli aplotipi mitocondriali di dieci specie di anfibi. In terzo luogo, ho incluso questi dati di presenza di anfibi, insieme ad una serie di indici ecologici e paesaggistici che caratterizzano i siti considerati, in un approccio di analisi multidimensionale utilizzato in pianificazione del paesaggio. I primi risultati hanno evidenziato l’importante ruolo delle zone umide protette per la conservazione delle comunità di anfibi e hanno anche indicato le pozze d’alpeggio come potenziali ulteriori siti di riproduzione per gli anfibi. Infine, ho caratterizzato la composizione delle comunità cutanee sia batteriche che fungine nelle popolazioni di *Bombina variegata* da habitat con vari gradi di impatto antropico, dimostrando che queste comunità microbiche sono in effetti influenzate dalla presenza e dalle attività umane, seppur presentando pattern discordanti tra diversi indici di diversità (cioè, diversità alfa e beta). In conclusione, pur confermando l'influenza delle pressioni antropiche sulla presenza e sul benessere degli anfibi, questa tesi ha anche evidenziato l'esistenza di possibili habitat 'intermedi' dove la convivenza uomo-anfibi è possibile (ad esempio, le pozze d’alpeggio), nonché la necessità di integrare approcci provenienti da diverse discipline per ottenere un quadro di conservazione unificato mirato a uno sviluppo veramente sostenibile.

MACBETH IV.i 10-19; 35-38

William Shakespeare

Double, double toil and trouble;
Fire burn and caldron bubble.
Fillet of a fenny snake,
In the caldron boil and bake;
Eye of newt and toe of frog,
Wool of bat and tongue of dog,
Adder's fork and blind-worm's sting,
Lizard's leg and howlet's wing,
For a charm of powerful trouble,
Like a hell-broth boil and bubble.

Double, double toil and trouble;
Fire burn and caldron bubble.
Cool it with a baboon's blood,
Then the charm is firm and good.

CHAPTER 1

INTRODUCTION

1.1 AMPHIBIAN CONSERVATION

1.1.1 GLOBAL AMPHIBIAN DECLINE

The Class Amphibia is an ancient taxonomic group, with origins in the early Mesozoic, about 250 million years ago (Mya; Wake & Koo, 2018) or even 309 Mya according to other authors (Lu, 2023). Thus, amphibians have already survived two of the ‘Big Five’ mass extinctions: the Triassic–Jurassic (Tr-J) extinction event, that caused an estimated loss of 37- 73 % of all marine genera (Sepkoski, 1996; McGhee et al., 2013) and the Cretaceous–Paleogene (K–Pg) extinction event, for which most authors estimate a diversity loss of 40% marine genera (Sepkoski, 1996; McGhee et al., 2013; Stanley, 2016).

This Class comprehends three main Orders: Anurans (frogs and toads, 88% of the species), Caudata or Urodeles (salamanders and newts, 9%) and Gymnophiones (caecilians, 3%), as shown in Figure 1.1.1.1. These three groups are characterized by moist skin that serves as an auxiliary respiratory organ and also produces a variety of secretions, many of which are toxic to other organisms (Jørgensen, 2000; Prates et al., 2012; Larsen, 2021). The aquatic environment is fundamental to the life cycle of the vast majority of amphibians, even though a few species have developed extreme specializations, such as viviparity, an adaptation to living in conditions of water scarcity (Vences & Köhler, 2008; Wake & Koo, 2018). Otherwise, animals of these three Orders have very different appearances (McDiarmid, 1994). For instance, most anurans have short trunks, and all adults lack tails. Frogs and toads have relatively short forelimbs and strong hind limbs, that allow them to leap long distances compared to their body size. Salamanders and newts, on the other hand, typically have moderately elongated trunks, all limbs of about the same length, and a tail that is about as long as the head plus the body. Exceptions include aquatic amphiumas and sirens (salamanders with reduced or missing limbs), or other species whose adults have retained the aquatic larval morphology, a phenomenon called neoteny (e.g., the axolotl *Ambystoma mexicanum* Shaw, 1789). Caecilians are worm-like animals, having elongated bodies marked by rings, and often no tail nor limbs. They have burrowing or aquatic lifestyles and possess a unique chemosensory organ, the tentacle, on their heads (Wake & Koo, 2018).

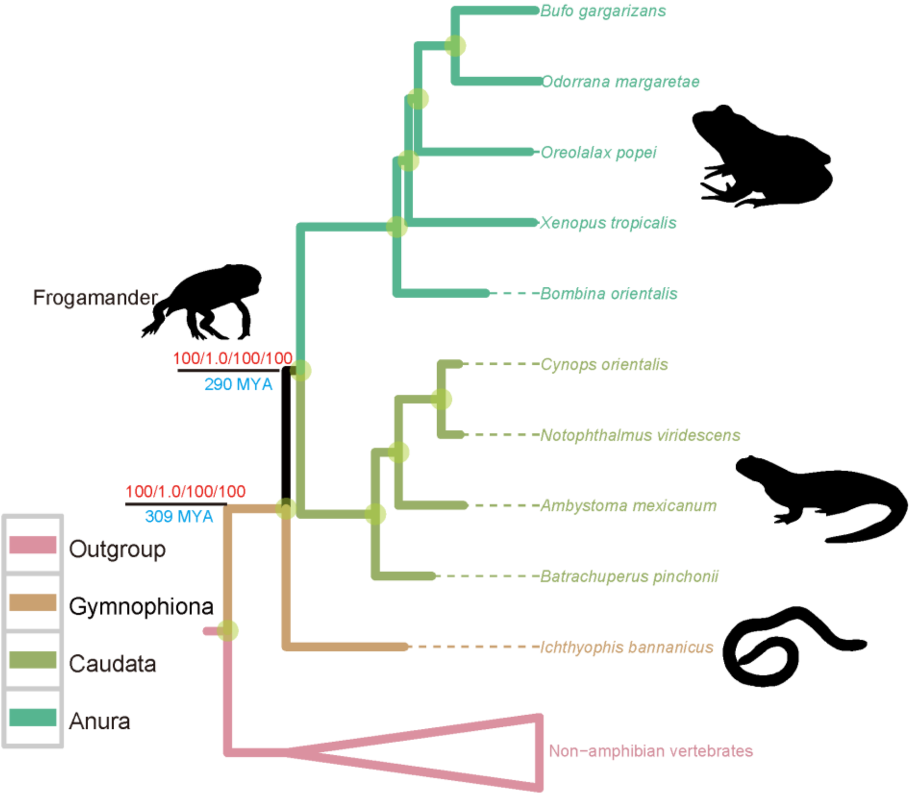

Figure 1.1.1.1 The amphibian tree of life (modified from Lu, 2023). Red numbers on the main nodes refer to support values from several phylogenomic methods, including, respectively, maximum likelihood (ML), Bayesian (PB), maximum pseudo-likelihood estimates (MPE), and maximum parsimony "Genes as characters" (GAC); blue numbers highlight the origin date of extant amphibians (309 Mya) and in particular extant frogs and salamanders (290 Mya).

Amphibians are currently widespread worldwide, being present on every continent except Antarctica, although with different trends according to Order. Their species biodiversity is higher in tropical regions, with the most notable hotspots being Central and South America (see Wake & Koo, 2018). Despite their global distribution, amphibians are currently one of the most threatened yet least well studied groups among Vertebrates. Out of the 8,715 known species of amphibians, 8,020 are included in the International Union for the Conservation of Nature (IUCN) Red List assessments, and 41% of these are considered 'threatened' with extinction, as shown in Figure 1.1.1.2 (IUCN, 2024). Even within the worldwide biodiversity crisis scenario, which has been referred to as Sixth Mass Extinction by many authors (e.g., Barnosky et al., 2011; Cafaro, 2015; Ceballos et al., 2015; McCallum, 2015; Rampino & Shen, 2021), amphibian population decline is particularly alarming because several drivers seem to be occurring simultaneously and over much of their distribution, affecting even populations living in protected areas (Collins & Storfer, 2003; D'Amen et al., 2011). This severe biodiversity loss has recently led to the second Global Amphibian Assessment (GAA2, 2004-2022), that updated the threat status of amphibians including 2,286 more species (Luedtke et al., 2023) with respect to the first Global Amphibian Assessment (GAA1, 1980-2004). The GAA2 highlighted that this decline is particularly strong in the Neotropics, although the Palearctic is the biogeographic area showing the greatest increase in risk compared to GAA1. As mentioned above, several causes seem to play a major role in amphibian declines, including land use changes and habitat fragmentation, the spread of new pathogens, the climate crisis, and overexploitation (Collins & Storfer, 2003; Luedtke et al., 2023).

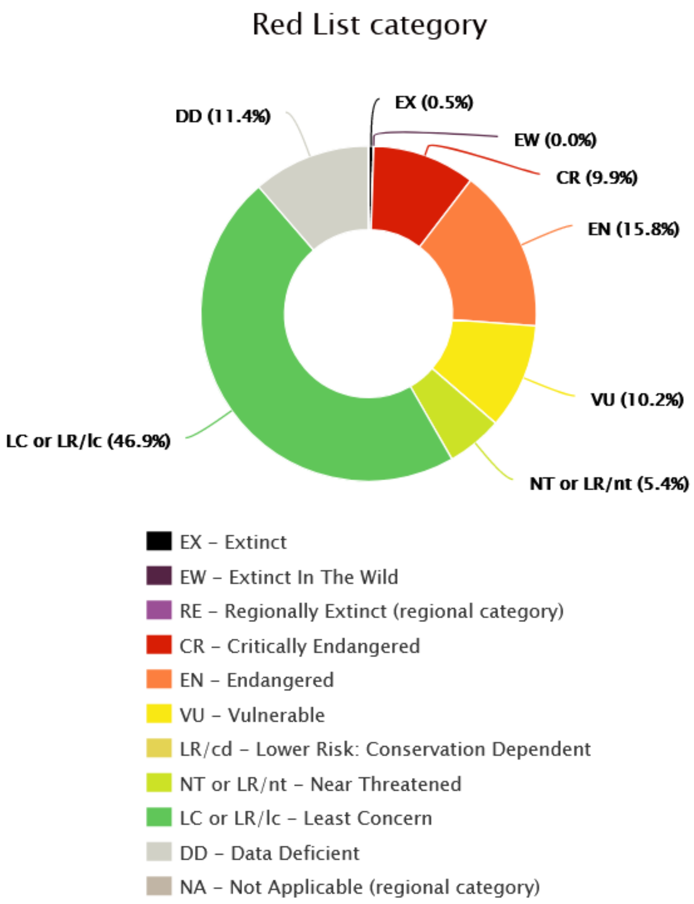

Figure 1.1.1.2. Percentage of amphibians by Red List category (IUCN, 2024).

Among these, habitat degradation is possibly the most important contributing factor to amphibian decline (Whittaker et al., 2013), as changes in land use have in many cases led to local extinctions either by direct killing of individuals, deteriorating and fragmenting their habitat, or even just limiting access to their reproductive sites or terrestrial refuges (Collins & Storfer, 2003; see also Cordier et al., 2021 for a more recent review). Amphibians are highly sensitive to environmental pollutants due to their skin permeability and the absence of a hard shell protecting their eggs (Blaustein & Kiesecker, 2002). Moreover, during their peculiar life cycle they occupy both aquatic and terrestrial habitats, being thus exposed to threats present in both environments (Nolan et al., 2023). These characteristics, that make amphibians excellent bioindicators of ecosystem health, are often also the cause of their decline. In general, species living in temperate areas are especially affected by wetland loss, while deforestation is a major threat for tropical species (Lehtinen et al., 1999; Azevedo-Ramos & Galatti, 2000).

As a consequence of wetland isolation, temperate amphibian species are often genetically highly structured as metapopulations due to combined effects of isolation-by-distance and isolation-by-environment (Van Buskirk & van Rensburg, 2020). Therefore, the decimation of big source populations can cause considerable losses of genetic biodiversity for the surrounding populations as well (Whittaker et al., 2013; Zamberletti et al., 2018). Moreover, because of their biological characteristics such as small body size, poor tolerance of desiccation and limited dispersion capacity, urban and extensively farmed landscapes can severely affect their ability to re-colonize habitats after a local extinction (Hamer & McDonnell, 2008). Rural and urban landscapes potentially limit amphibian migration and dispersal due to the high mortality associated to the urban road networks (e.g., Jochimsen et al., 2004), environmental pollution linked to intensive pesticide use, and the general lack of suitable habitats (Hayes et al., 2006; Egea-Serrano et al., 2012; Pinelli et al., 2019). In particular, the impact of pesticide use has been demonstrated even in GMO-based agriculture (Agostini et al., 2020), while traditional and organic farming could in some cases be more easily adapted to take account of amphibian habitat needs (Schuler et al., 2013; Hartel et al., 2014). In general, gene flow and genetic diversity of urban amphibian populations is expected to be lower than natural populations (Goldberg & Waits, 2010), an occurrence that can lead to lower adaptability in the former. However, as urban environments are very different from natural wetlands, it should be noted that cases of local adaptation to the former have also been reported, even while the urban population still retained genome-wide genetic diversity levels similar to the natural ones (Homola et al., 2019). With about 49% of the species currently threatened by extinction, compared to 41% worldwide, amphibians of the Mediterranean area are especially at risk (Nadin, 2008). In this area, in fact, several species use water bodies as reproductive sites that are highly sensitive to modifications in land use and to the increasingly severe droughts linked to the climate crisis, such as temporary ponds and small streams (Caballero-Diaz et al., 2020).

The GAA2 evaluation confirmed that habitat degradation is still the primary cause of amphibian decline worldwide, and their conservation status could be improved with appropriate forest conservation and management policies (Pabijan et al., 2023). However, the ongoing climate crisis has also been proven to affect both directly and indirectly amphibians, at individual, population and species levels, shaping both functional diversity and species richness patterns (Tsianou et al., 2021). Climate warming can in fact cause changes to their habitats (affecting vegetation, soil and hydrology), alter food accessibility and in general both prey-predator and host-pathogen interactions, thus impacting the overall community composition. More specifically, survival, growth, timing of hibernation and breeding, and dispersal capacity can be negatively affected, causing variations even in altitudinal and longitudinal ranges (Blaustein et al., 2010). For instance, the increasingly early reproduction of some temperate species in the northernmost part of their range has long been demonstrated be an effect of climatic shifts (Beebee, 1995), a phenomenon that could in some cases lead to hybridization even in deeply divergent species (Canestrelli et al., 2017). In Italy, climate change has been linked to local populations disappearances (D’Amen & Bombi, 2009), and these variations and new constrictions on their dispersal range seem to result in a decrease in the efficiency of national protected areas, an occurrence that has also been reported in other Parks and reserves worldwide (e.g., Whitfield et al., 2007; McMenamin et al., 2008; D’Amen et al., 2011; Xu et al., 2022). Recent studies have added more evidence of the role of the climate crisis in amphibian decline, as well as highlighted the need for more accurate estimates of its effects, since the current literature is still scarce and geographically biased (Winter et al., 2016; Luedtke et al., 2023).

Amphibian-specific diseases caused by viruses, bacteria, fungi and parasitic flukes have been linked to amphibian population declines across the globe, including in Europe (Whittaker et al., 2013). Iridoviruses of the genus *Ranavirus* and a chytrid fungus, *Batrachochytrium dendrobatidis* (Longcore, Pessier & D.K. Nichols, 1999) are currently the two most widespread and studied amphibian pathogens. Several long-term studies point to the hypothesis that Ranaviruses cause fluctuations, and not long-term declines, in the affected populations (Collins & Storfer, 2003). Instead, *B. dendrobatidis* (Bd) has long been recognized as the main agent responsible for mass mortality of amphibians on a global scale (e.g., Stuart et al., 2004; Luedtke et al., 2023). The disease resulting from Bd infections, chytridiomycosis, can be deadly to some amphibian species (Vredenburg et al., 2010), even though some species have been reported to host Bd without manifesting the disease (Briggs et al., 2010; Reeder et al., 2012). This fungus seems to also be affected by higher temperature, as it mainly impacts tropical species at high altitudes (Collins & Storfer, 2003). This could explain, for instance, why amphibian populations in urban areas seem to experience less severe disease than those living in natural environments (e.g., Karvemo et al., 2019). The authors of a study based in the US suggested that the lower rates of occurrence of this pathogen in urban areas can be explained by the warmer temperatures, along with lower animal densities and overall lower species richness (Saenz et al., 2015). According to an increasingly consistent body of research, skin bacterial communities (microbiota) also play a major role in host immune function and determining the probability of survival of Bd-infected amphibians (e.g., Becker et al., 2015, 2017; Madison et al., 2017; Muletz Woltz et al., 2017; Woodhams et al., 2017).

Bd is increasingly widespread in Europe and has been associated with the decline of populations of several amphibian species. In Italy, Bd was reported for the first time in 2001 in some populations of *Bombina pachypus* Bonaparte, 1832 in the Northern Apennines (Stagni et al., 2004; Lanza et al., 2009), but has currently spread to the entire peninsula, including the northern regions (Federici et al., 2008; Tessa et al., 2013; Zampiglia et al., 2013). More recently the emergence of the fungus *B. salamandrivorans* (Martel et al., 2013; Bsal) has caused significant losses in the northern European populations of *Salamandra salamandra* Linneaus, 1758 (Burns et al., 2016; Fisher et al., 2020). Peaks in the spread of Bd between the 1990s and early 2000s recorded by the GAA2 have been the cause of declines in amphibian populations in the Neotropics. For this reason, recent guidelines are advocating for the implementation of measures to control the spread of both Bd and Bsal (Luedtke et al., 2023).

Lastly, among the other factors affecting amphibian populations worldwide, it is worth mentioning the introduction of alien species, that can become predators or competitors of native species, as well as transmitting new pathogens or hybridizing with residents (Collins & Storfer, 2003). In some areas of Italy, for instance, the alien bullfrog *Lithobates catesbeianus* (Shaw, 1802) has been shown to effectively compete with the autochthonous anurans (Lanza et al., 2009). Another cause of decline is harvesting of individuals from natural populations for food or trade purposes, although in Italy the overexploitation of some species (e.g. the green frog, *Pelophylax* kl. *esculentus*) is a threat only for already declining populations (Lapini, 2005). These problematic interactions of human activities and the local fauna are often intimately connected to specific economic circumstances and traditions, and their resolution thus depends mostly on effective public education (Whittaker et al., 2013).

In summary, the amphibian decline appears to be related to complex interactions between multiple factors, often acting synergistically. It may be therefore overly simplistic to look at single risk factors to explain and address this global phenomenon. In fact, even the conservation status of individual amphibian populations tends to vary widely depending on local combinations of these factors (Blaustein & Kiesecker, 2002; Collins & Storfer, 2003). For this reason, increased habitat protection and comprehensive conservation policies are needed to achieve the agreed global and national biodiversity conservation targets (Luedtke et al., 2023; see also sections 1.2.2 and 1.2.3). In particular, studies addressing the effects of land use changes and the climate crisis on amphibian biodiversity, along with the community-level (rather than single species-level) drivers of declines, and rapid monitoring and screening protocols, are among the priority research questions recently highlighted by a comprehensive review (Campbell Grant et al., 2023).

1.1.2 ECOLOGICAL, ECONOMIC AND CULTURAL RELEVANCE OF AMPHIBIANS

The protection of amphibian biodiversity is critical to both ecosystem health and human wellbeing, as these animals contribute to regulating and provisioning services, as well as increasing human quality of life by serving as a source of medical treatments and food, but also through recreation, spirituality, and aesthetics.

Firstly, amphibians provide regulatory ecosystem services by reducing the spread of mosquito-borne diseases by competing with or preying on mosquitoes (during larval and adult stages, respectively), and more generally acting as invertebrate pest control agents (see Hocking & Babbitt, 2014, and references therein). For instance, the frog *Lysapsus limellus* (Cope, 1862) frequently feeds on Ephydridae, a family of flies of the neotropics that are vectors of zoonotic pathogens (Valencia-Aguilar et al., 2013). Amphibians also have an important influence on the structure of ecosystems thanks to their soil-burrowing and aquatic bioturbation activities, as well as on ecosystem functions, such as nutrient and decomposition cycles, directly through waste excretion and indirectly through predation (Hocking & Babbitt, 2014). In addition, by preying on a plethora of arthropods such as flies, butterflies, moths, and beetles, amphibians display a great potential to affect pollination and seed dispersal (Abrol, 2012). In rare cases, some frog species have also been reported to consume fruit and disperse their seeds (Da Silva & Britto-Pereira, 2006).

Furthermore, amphibians have been and are still widely used as ‘model organisms’ in medical research and are a potential source of pharmaceutical compounds (Gardner, 2001). The axolotl, for instance, has been the subject of several studies of limb and tail regeneration, while the African clawed frog (*Xenopus laevis* Daudin, 1802) and its diploid relative *X. tropicalis* (Gray, 1864) are widely used in developmental and cell biology research (Wake & Koo, 2018). Several other anuran species, including the genera *Rana* and *Lithobates*, have been studied as a source of antidiabetic peptides (Soltaninejad et al., 2021). The fluids secreted by salamanders such as *Hynobius dunni* (Tago, 1931) have several applications as medical bioadhesives, being biocompatible, non-toxic, and with mechanical properties on different surfaces (von Byern et al., 2021). The secretions of tropical frogs such as *Phyllomedusa bicolor* (Boddaert, 1772) have been used for medical purposes by the indigenous populations for centuries, and their exploitation and eventual commercialization by the ‘Global North’ pharmacological industry is now the center of debates concerning epistemic justice, biopiracy and biocolonialism (Gebara et al., 2023). Their susceptibility to environmental pollution, despite being one of the causes of their decline as mentioned in section 1.1.1., makes amphibians particularly useful as bioindicators of ecosystems health, and therefore, in some cases, also of human wellbeing in polluted landscapes. For instance, a recent study in southern Italy has applied an approach based on biomonitoring of DNA damage in *Pelophylax* kl. *esculentus* (Linnaeus,

1758) populations as a means of estimating the danger on human health posed by illegal dumping of toxic substances in the environment, as it was suspected to increase the incidence of cancer in local populations, both human and non (Maselli et al., 2019).

Even though this tradition has almost disappeared in Italy (as mentioned in section 1.1.1), amphibians are still a source of food in many countries worldwide. In Nigeria, for instance, a survey reported that 73.3% of the 900 respondents had seen someone eating amphibians, and that 43.0% had done so themselves. These results thus highlighted the need to move towards frog farming or other sustainable harvesting strategies (Onadeko et al., 2011). Frog legs are still widely consumed also in Southeast Asia as well as in Central and South America (Hocking & Babbitt, 2014).

Amphibians (especially the brightly colored, often poisonous, tropical species) are also traded worldwide as pets, a commerce the volume of which is particularly difficult to establish with precision but that was estimated to be nearly 85,000 tons in 2005, worth a third of a billion U.S. dollars (Garner et al., 2009). A more recent study tried to calculate the economic value of all amphibian individuals captured in a year from a single, isolated wetland, finding that the 392,605 animals corresponded to $3,605,848 (DeGregorio et al., 2014). As global amphibian trade is also the main cause for the spread of deadly pathogens such as Bd and Bsal from Asia to Australia, Central America and temperate areas including Europe, the appropriateness of bans or restrictions is the subject of unending debates (Garner et al., 2009).

Last but not least, because amphibians are a rich and diverse class of vertebrate, they also play a prominent role in human culture, such as in mythology, literature and art (Hocking & Babbitt, 2014). The anthropic perspective on this species should be integrated as a fundamental component of comprehensive conservation plans, as it can greatly affect amphibian conservation initiatives in the current human-shaped landscapes (Hartel et al., 2020). Both knowledge of amphibian species and their perceptual favorability seem to vary widely across the globe. In China, for instance, the general public shows good identification skills and an overall appreciation of toads and frogs, that are considered beautiful animals (Jimenez & Lindemann-Matthies, 2015a). In Chinese mythology, in fact, a toad/frog is traditionally associated with the Moon and the *yin* (dark, slow, soft, cold, and wet; Paterska-Kubacka, 2019), and legends claim that the world rests on the back of a frog (Crump, 2015). In South Korea dreaming about a frog is thought to bring hope for the birth of a son

(Paterska-Kubacka, 2019). The Wa, an ethnic group living in Myanmar, even consider themselves descendants of tadpoles (Crump, 2015). Amphibians, in fact, appear very frequently on Chinese and Japanese wooden screens and pottery (e.g., see Figure 1.1.2.1), but even more so in pottery, basketry, beadwork and ornaments from Central and South America (Frost, 1932), where amphibians are associated with rain and, therefore, fertility (Crump, 2015). Because of their life cycle (from eggs to tadpoles to hopping adults) and their ability to appear and disappear as the weather changes, frogs are also a symbol of rebirth and immortality in many cultures, from ancient Egyptians to Romans (i.e., Pliny the Elder) to the Olmec in Mexico and again Chinese and Japanese people (Crump, 2015).

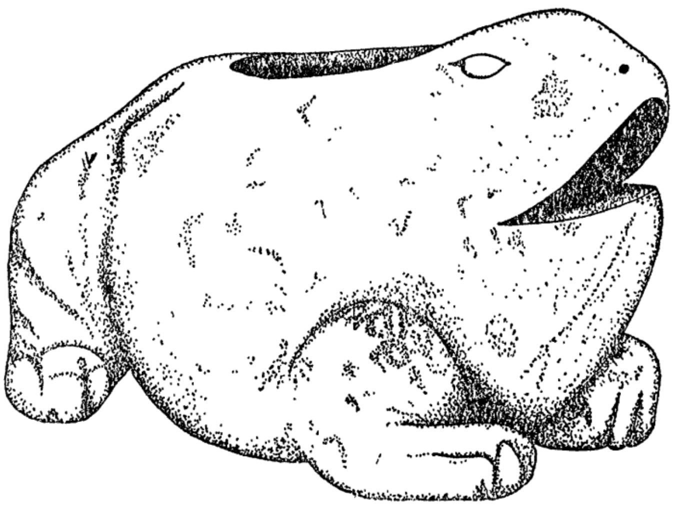

Figure 1.1.2.1. A jade sacrificial vessel from China of the Hsia of Shang Dinasty (2205- 1122 B.C., image from Frost, 1932).

On the other hand, a survey performed in California showed that both the public's attitudes to, and knowledge of frogs, were rather poor, although their conservation value and importance for medical and ecological purposes were still recognized by most (Jimenez & Lindemann-Matthies, 2015b). Based on zoo visitor perceptions of animal welfare as well as their willingness to support conservation efforts, the connection between likeability of these animals, understanding of their diversity, and interest in their conservation has been recently highlighted also by Ogle and Devlin (2022). Moreover, even the ‘Global North’ public’s perception of amphibians cognitive and emotive traits seems to be heavily biased with respect to other animals such as mammals (Callahan et al., 2021), despite the scientific consensus being that they are to be considered capable of a wide range of feelings and states (Lambert et al., 2022). In 'Western' culture, in fact, toads and frogs are usually associated with sorcery and fear of the unknown, a tradition that might explain the relative rarity of amphibians' artistic representations with respect to, for instance, fish (Frost, 1932). Medieval European folklore, in fact, associate frogs and toads with filth and evil, probably because they live in mud. The Catholic Church, in particular, claimed that amphibians are a witch's 'familiars', into which they could also transform, or are otherwise ingredients for their brews (Crump, 2015). Even salamanders, that previously enjoyed the fine reputation of being invulnerable to fire and were therefore linked to purity, courage and rebirth, became witches' pets during the Middle Ages (Crump, 2015, shown in Figure 1.1.2.2). The fear and repulsion that these animals generated in European people is visible through their artistic representations, including those driven by scientific curiosity (Etheridge, 2007). This negative depiction of amphibians is shared across all Africa, where several legends tell that toads are the main culprits for human mortality (Crump, 2015). Considering the plausible impact that the public's perception has on wildlife conservation, amphibian-friendly activities such as creating frog ponds in the backyards has been suggested as a way for people to approach these species with a sustainable mindset (White, 2008).

Figure 1.1.2.2. A salamander unharmed in the fire, 1350. Source: Wikipedia Commons: Numerisation par Koninklijke Bibliotheek KB, KA 16, Folio 126r, Netherlands, Public domain, via Wikimedia Commons.

Beyond the ecological, economic and cultural reasons listed above, it could also be argued that amphibians, like all other living beings, have an inherent right to be respected and protected as much as possible (Singer, 2023). Such is, in fact, the position of the philosophical-political movement called ‘antispeciesism’ in opposition to speciesism. Since the latter can be defined as a mental attitude based on the assumption that humans are the center of the universe and all other living creatures are therefore completely subservient to them, the former instead promotes, for example, the extension of several rights to non-human animals (Maurizi, 2021). Antispeciesist is in fact a broad term which includes people with different ideologies, from welfare proponents to defenders of animal rights and finally to animal liberationists (Best, 2006). Although the antispeciesism philosophical instances are usually applied to animal farming, they are increasingly accepted, also in Italy (Losi & Bertuzzi, 2020), and could become an important ethical rationale to boost amphibians (and all wild animal) conservation.

1.4. WETLAND CONSERVATION

1.4.1. CAUSES AND IMPACT OF DISAPPEARING WETLANDS

Wetlands can be conceived as lands of transition, intermediate between terrestrial and aquatic systems, and include marshes, swamps, ponds, but also areas near rivers, streams, and lakes (see Scott & Jones, 1995 for an overview of wetlands classification; Keddy, 2010; see also Figure 1.4.1.1 for examples of alpine wetlands). According to several studies, wetlands can be considered the richest ecosystems after tropical rainforests, hosting high levels of biological diversity (see Desta et al., 2012, and references therein). However, these ecosystems only cover about 1.5% of the Earth’s surface, or 5–10% considering only the terrestrial surface (Aryal et al., 2021), while providing a disproportionately high 40% of global ecosystem services (Zedler & Kercher, 2005). Despite their importance, they have been systematically drained and ‘reclaimed’ to satisfy policies focused on agriculture, urban expansion, and other developments. It has been estimated that about 50% of the original wetland area has been lost globally, with extreme losses of >90% in some areas of Europe (Clarkson et al., 2013).

The ecosystems services provided by wetlands are numerous and varied, touching areas such as the defense of territory, food production, freshwater supply, and even culture

(Gokce, 2018; Prete, 2018). The dense forests in wetlands located near the coasts, for instance, constitute a natural barrier to erosion, stabilizing the sand substrate and slowing down the force of currents (Törnqvist et al., 2021). More in general, wetlands act as buffer zones for containing river levels fluctuations due to seasonal snow melting or heavy rains, while during the dry season help in balancing humidity in the surrounding areas (Keddy et al., 2009). Larger bodies of water, in particular, help to regulate climate stability through their thermoregulatory properties and their ability to increase atmospheric humidity, thus contributing significantly to the cooling of agricultural landscapes (Huryna et al., 2013; Pokorný et al., 2018). The presence of water bodies has *per se* an economic value, as they provide a reserve of water for agriculture, fishing and grazing activities (Schuyt, 2005). These ecosystem services are vital, especially for communities of the 'Global South', in the cases where subsistence agriculture and utilization of wetland resources are the basis of everyday life (Silvius et al., 2000; Schuyt, 2005; Bezabih & Mosissa, 2017). They are also pollution 'filters', thanks to the metal-absorbing capacity of several macrophyte and other aquatic species (Marchand et al., 2010), therefore improving water quality for irrigation and human consumption. The major role played by wetlands in flood control as well as carbon absorption and management has recently attracted attention (Pokorný et al., 2018; Kolka et al., 2021), leading to the re-evaluation of these ecosystems in light of extreme climate events and net greenhouse gas emissions reduction targets, releasing a plethora of funding for restoration and 'rewilding' (see section 1.2.2 and 1.2.3).

Figure 1.4.1.1. Examples of alpine wetlands (left: Natural Reserve of Roncegno, right: alpine farm pond on Monte Baldo- Province of Trento, Italy; pictures by the author).

Several food products such as fish and shellfish, cranberries, blueberries, rice, and timber, as well as medicinal ingredients derived from plants are provided by wetlands (Keddy et al., 2009; Aryal et al., 2021). Moreover, wetlands constitute a cultural and social heritage, considering the number of educational and recreational activities that can be carried out near them, their landscape value and potential to attract tourists even outside the conventional 'tourist season' (Keddy et al., 2009). In some cases, the direct non-consumptive (recreational) service has been reported to be the most beneficial service, holding up to 85% of the total economic value (Mitsch & Gosselink, 2000; Thapa et al., 2020). Lastly, they are important hotspots of biodiversity, hosting a wide variety of fungal, plant and animal species. The species living in these transitional areas between terrestrial and aquatic environment often display very specific adaptations, such as the ability to tolerate short-term anoxia (Bobbink et al., 2006), that are currently of particular interest for the scientific community in the context of possible crop adaptations to the climate crisis (Anderson et al., 2020; Zahra et al., 2021). Overall, the total economic value per hectare per year of inland wetland ecosystem services has been estimated at up to US$ 44,000 (see Clarkson et al., 2013 for detailed information on the economic value of each ecosystem service), or, more recently, to about US$ 47.4 trillion per year globally, that is the 43.5% of the total value of all natural resources (Davidson et al., 2019). Nonetheless, it has been argued that the full economic value of the benefits provided by these ecosystems, even in the case of internationally important wetlands, is probably underestimated, as some services, such as those associated with mental health and well-being, might not be recognized at all (McInnes, 2013).

The main historic cause of wetland disappearance in Europe, and more specifically in Italy, is water drainage and land reclamation (Kingsford et al., 2016). In the past, marshes and swamps were considered 'waste' lands, unproductive or worse, merely harbingers of diseases such as malaria (so-called 'marsh fever'). This vision of such water bodies took hold especially in the XIX century, when important changes in both demography and available technologies led to new models of land use based on the expansion of cultivable lands coupled with innovative agricultural methods (Kingsford et al., 2016). In the Italian peninsula, several major reclamation campaigns took place following the Unification of Italy, and especially during the fascist period from 1922- 1943 (Pace, 1982). Despite the recent recognition of both importance and vulnerability of wetlands, these areas are still threatened by construction, water channeling for irrigation and production of hydroelectric power, air and water pollution, tourism, and increased agricultural pressure on water reserves (see Asselen et al., 2013 for a comprehensive meta-analysis). In addition, the climate crisis is also expected to heavily affect wetland ecosystems (Erwin, 2009) resulting, for instance, in less predictable rainfall and decreased summer water levels leading to further habitat fragmentation and reduction of its temperature-regulating properties. Overall warmer temperatures also imply an increase of evaporative loss and consequently, a decrease of carbon storage (Erwin, 2009; Desta et al., 2012). An increase in intensity and frequency of storms and other extreme climate events is also expected, with subsequent flooding that could increase wetland area, but damage the reproductive sites of wild fauna such as waterfowl (Erwin, 2009). Moreover, an increase in atmospheric CO_2 could lead to changes in leaf litter quality and consequently impact aquatic food webs (Desta et al., 2012). Coastal wetlands will most likely be subjected to a reduction in size due to the climate-driven accelerated relative sea-level rise (Erwin, 2009; Törnqvist et al., 2021). Increased agricultural water consumption, that is in itself a consequence of the climate crisis in several geographical regions, is another cause of wetlands area loss (Li et al., 2021).

Because climate changes affect wetlands differently across the globe, it has been suggested that specific restoration and management plans should be developed based on the habitat and local geographic area (Erwin, 2009). As most wetlands ecosystem services proportionately increase with the wetland area, it is fundamental for human societies to protect not only their existence but also their extension (Keddy et al., 2009).

1.2.2. LEGAL PROTECTION OF WETLANDS

Although a complete dissertation on the body of law concerning the protection of wetlands and other freshwater habitats goes beyond the scope of this thesis, this section nonetheless aims at critically summarizing the main international, European, national (Italian) and provincial (Autonomous Province of Trento) contributions.

The protection of natural habitats and ecosystems falls within a more general global concern related to the ongoing worldwide biodiversity loss. This issue has officially been addressed in the Convention on Biological Diversity (CBD), a document first signed on 5 June 1992 at the United Nations Conference on Environment and Development (the Rio De

Janeiro ‘Earth Summit’) and ratified on 29 December 1993. The text of the Convention provides a definition of ‘biological diversity’ (art. 2) and sets out a series of fundamental goals; i.e. the conservation of biodiversity, its sustainable use, and the equal and just distribution of any benefits deriving from it. In its vision, the strong relationship and interconnectivity running along each level of biological diversity, from genes to ecosystems and the biosphere, are recognized, as well as the need for action at all levels in order to preserve global biodiversity is underlined (Russo, 2018).

In light of the failure to reach the objectives set by the international Convention (Russo, 2018), and the concomitant acceleration of biodiversity decline (Barnosky et al., 2011), the Kunming-Montreal Global Biodiversity Framework (GBF) was very recently adopted during the fifteenth meeting of the Conference of the Parties (COP 15; CBD/COP/DEC/15/4, 19 December 2022). The GBF brings together the UN Sustainable Development Goals and the Convention’s previous Strategic Plans, delineating four goals for 2050 and 23 targets for 2030. Some of these targets refer to the protection and restoration of freshwater habitats, among others, advocating for instance “that by 2030 at least 30 per cent of areas of degraded terrestrial, inland water, and marine and coastal ecosystems are under effective restoration” (target 2), or “that by 2030 at least 30 per cent of terrestrial and inland water areas, and of marine and coastal areas, especially areas of particular importance for biodiversity and ecosystem functions and services, are effectively conserved and managed through ecologically representative, well-connected and equitably governed systems of protected areas and other effective area-based conservation measures” (target 3). Target 7 also recommends to “reduce pollution risks and the negative impact of pollution from all sources by 2030, to levels that are not harmful to biodiversity and ecosystem functions and services”, another measure that could positively impact wetlands considering that pollution and habitat degradation are among the most threatening factors (e.g., Kingsford et al., 2016).

Despite the definition of specific targets addressing tools and solutions for implementation of said measures (see targets 14-23 and section I of the same document), all mechanisms are to be undertaken in a “facilitative, non-intrusive, non-punitive manner”. Therefore, similar to the Convention itself (Russo, 2018), GBF Decision 15/4 does not appear to be particularly stringent in terms of the obligations imposed on the Parties. In addition, considering also the lack of a sanctions system, this document acts more as a political declaration than a legal text. Moreover, since the publication of the GBF, several scientific articles have pointed out the weaknesses of the Decision and the possible obstacles to its implementation. For instance, some authors have argued that the goals and targets addressing the subject of genetic diversity monitoring and conservation are not sufficiently clear for an effective implementation, and lack the broadness of scope that would be needed to maintain genetic diversity within and among populations of all species (Hoban et al., 2020, 2023). According to another review, freshwater habitats and biodiversity appear to be underprioritized with respect to the marine and terrestrial biota, considering their current vulnerability (van Rees et al., 2020). Williams et al. (2021) criticized the original formulation of Goal B on species conservation, while more recently Meng et al. (2023) highlighted that cropland have expanded globally inside protected areas since the 2000s, which challenges the post-2020 GBF examples. The management of protected areas with stakeholder involvement, and how to soften emerging tensions between various interest groups, are discussed with global case examples by Raymond et al. (2022). Despite the plausible need to strengthen this political document (Priyadarshini et al., 2022), however, it remains a fundamental starting point for future biodiversity conservation-oriented policies.

Concerning wetlands, the first international treaty to focus on these ecosystems was the Convention on Wetlands of International Importance, adopted in the Iranian city of Ramsar in 1971 (and consequently named the Ramsar Convention), in force since 1975. This Convention was signed by a group of countries, scientific institutions, and international organizations at the ‘International Conference on Wetlands and Waterfowl’, which was promoted by the International Wetlands and Waterfowl Bureau (IWRB) in collaboration with the IUCN and the International Council for Bird Preservation (ICBP). The Ramsar Convention is the first truly intergovernmental treaty with a global scope, in the most modern sense of the word, on the conservation and management of natural ecosystems. Compared to the more recent conventions such as the CBD, the Ramsar Convention designations are very precise, but often limited in scope since they refer to specific sites. In fact, this Convention is based on the identification of a list of wetlands of international relevance, to which are to be applied a number of measures ranging from the creation of natural reserves to the obligation of ‘wise use’ for the signatory countries. The Ramsar Convention was signed by Italy and ratified with the D.P.R. 448/1976 and subsequently with the D.P.R. 184/1987. In addition to the participation to all common international activities of the Convention, these decrees sanction several national commitments, including wetland monitoring and management activities, and the writing of a ‘National Report’ to be presented to each Conference of Parties. Europe, overall, has the highest number of Ramsar sites, with a total of 1,004, although the area of these sites occupies just 6% of the total area protected by the Ramsar Convention (Xu et al., 2019).

The European Council also adopted its own legal instruments for biodiversity protection simultaneously with the CBD. In fact, the European Habitats Directive (Council Directive 92/43/EEC), together with the Birds Directive (Directive 79/409/EEC), are to date a cornerstone of EU biodiversity policy. These two Directives have established the creation of the Natura2000 network, which is currently the largest coordinated network of protected areas in the world. The ambitious aim of the ‘Habitat’ Directive is, in particular, to protect not only over a thousand wild species, including vertebrates, invertebrates, and plants, but also characteristic habitat types, listed in Annex I and selected on the basis of scientific criteria described in Annex III. The need to protect the habitats themselves, along with the endangered species living there, stems from the recognized importance of allowing these species to recover and thrive in the long term, instead of merely surviving into the near future. The latest amendment of this Directive dates to 2013, after the accession of the Republic of Croatia (Council Directive 2013/17/EU). Annex I of the Directive lists freshwater habitats among those in need of legal protection, including both standing and running water (par. 3, coded respectively as 31xx and 32xx), and raised bogs (par. 7).

More recently, the European Green Deal also addressed the topic of biodiversity conservation in the context of preserving and increasing the habitats that are particularly efficient in capturing and storing carbon, and/or preventing and reducing the impact of natural disasters. This goal is to be achieved thanks to two main strategies: biodiversity conservation and land use management. The first one is described in the ‘Biodiversity strategy for 2030’ (COM/2020/380 final), that aims at putting “Europe’s biodiversity on the path to recovery by 2030 for the benefit of people, climate and the planet” and includes steps such as enlarging the EU network of protected areas, implementing EU’s first ever ‘Nature Restoration Law’ (COM/2022/304 final), and adopting a series of measures towards a global biodiversity framework in accordance with the Convention on Biological Diversity. In particular, the objectives of the EU ‘Nature Restoration Law include both enabling the long-term and sustained recovery of biodiversity and its ecosystem services, and achieving EU’s climate mitigation and adaptation goals. In particular, this law focuses on the improvement of existing regulations concerning biodiverse habitats, building on the habitat types protected under the Habitats Directive (including wetlands, river and lakes), as well as the monitoring of pollinators, and dedicates separate sections to forest, urban, agricultural and marine ecosystems. In this context, the agricultural ecosystem is the most connected to wetland management, as the law sanctions the necessity of sharing of agricultural land with high-diversity landscape features and the restoration of peatlands drained for agricultural use (art. 9; see also section 1.2.3.). These provisions are complementary to the second EU strategy, that concerns land use management and is outlined in the ‘Regulation on land, land use change and forestry’ (LULUCF, Regulation (EU) 2018/841 of the European Parliament and of the Council). After its revision in 2023, this regulation aims to achieve a net land-based carbon removal of 310 million tonnes of CO_2 equivalent by 2030, while promoting strong synergies between climate change mitigation and environmental protection measures, both on agricultural and forestry land and in natural or semi-natural areas (Regulation (EU) 2023/839; see also section 1.2.3.). However, it should be noted that the proceedings for approval of this Law are still ongoing, and recent modifications have gone in the direction of weakening its provisions (i.e., many exemptions were included, along with increased flexibility regarding obligations for Member States).

Regarding Italy in particular, even before the birth of the European Habitats Directive, the approval of the ‘Legge Quadro sulle Aree Protette’ (L.N. 394/1991) had defined the national areas subject to a special protection and management regime, thereby also preserving all fauna and flora species living within them. This framework (‘quadro’) delineates the general principles to be observed by regional legislation, which is responsible for detailed regulation. In addition to the instructions regulating local protected areas, this law also sanctions the management of national protected areas (see Graziani, 2018). Several national parks already existed at the time, such as the Gran Paradiso National Park (1922) and the National Park of Abruzzo (1921), but the ‘Legge Quadro sulle Aree Protette’ had a fundamental role in defining the responsibilities of National and Regional governments, a matter that was previously unestablished. Following a constitutional reform in 2001, the Italian Constitutional Court established that the Regions have jurisdiction in instituting and regulating protected areas, but only in a context of strengthening the sphere of action of the State (Graziani, 2018). The protected areas recognized by Italian law include: **parks**, which can be national or regional and host one or more intact or partially altered ecosystems; **reserves**, areas that contain one or more protected fauna or flora species, or physical, geological or geomorphological formations, or other systems of high natural, landscape, scientific or cultural value where anthropic impact is absent or very minor; and **areas protected by international law** such as the sites listed in the Ramsar Convention or included in the Natura2000 network. Before the introduction of the ‘Legge Quadro sulle Aree Protette’, the ‘legge Galasso’ also indirectly served as a protection tool for wetlands, as it subjected all Ramsar areas to landscape restrictions (L.N. 431/1985, art.1, (i)). At present in Italy, there are 57 sites listed in the List of Wetlands of International Importance (the ‘Ramsar List’), covering a total of 73,982 ha.

Furthermore, Italy has collaborated redacting and financing the 1st triennial action program of the international project ‘Mediterranean Wetlands’ (MedWet), cofinanced by the European Union, that aims at developing specific methodologies and strategies for managing Mediterranean wetlands. At present, Italy is still member of the MedWet Committee (MedWet/Com) together with 26 States of the Mediterranean area, activating collaborations on the local, regional and international scale in order to halt and invert the loss and degradation processes concerning the Mediterranean wetlands.

After ratifying the Habitats Directive with the D.P.R. 357/1997, a network of Natura2000 sites was also established in Italy, 2,637 sites to date. The basic idea behind the Natura2000 network is that, as Europe's habitats continue to deteriorate and wild species are increasingly threatened, the causes of this decline are mostly transboundary. This is particularly relevant for migratory birds (see Directive 79/409/EEC), but generally affects natural areas extending across borders. Therefore, an integrated approach that can be applied across all States of the European Union and also takes into account each country’s economic and recreative needs has been implemented thorough the Natura2000 network (Brocca, 2018). The designation of the Special Areas of Conservation (SAC) is the responsibility of the Member States, that first draft a list of proposed Sites of Community Importance (pSCI), and then designate them as SAC after approval of the European Commission. An important concept in the Habitats Directive is 'conservation status', which is both a constitutive element for a new SAC and a parameter for evaluating the conservation measures implemented in the site after its designation. The conservation status of a habitat is considered to be sufficient if its area is stable or increasing, the specific structure and functions necessary for its long-term maintenance are present and will remain so for the foreseeable future, and the conservation status of its species is satisfactory. The latter is considered to be the case if data on population fluctuations indicate that the species is a vital element of the habitat and will continue to be so for the foreseeable future, the range of the species is not declining and is not likely to do so in the foreseeable future, and habitat sufficient for the survival of wild populations exists and will continue to exist for the foreseeable future (Brocca, 2018).

More recently, a major change in national law has been determined by the Constitutional Law 1/2022, approved in 2022, which amended articles 9 and 41 of the Italian Constitution. In detail, this law introduced in article 9 the express provision of protection of the environment and biodiversity, and the need to take animal welfare into consideration. Concerning article 41, which concerns the private economic initiative, it included the protection of health and the environment as further limits to its exercise. Since these are modifications of the Constitution itself, and all subsequent legislation will need to conform to these principles, the impact of this law is highly significant.

Additionally, a first 'Biodiversity National Strategy' was implemented for the period 2011-2020, followed by the '2030 Biodiversity National Strategy' (Strategia Nazionale Biodiversità 2030), adopted with the Ministerial Decree n. 252. This latter Strategy builds on the objectives of the European 'Biodiversity strategy for 2030' (COM/2020/380 final) and of the Ecological Transition Plan, outlining a vision of future development focused on reversing the current trend of biodiversity loss and ecosystem collapse. The two strategic goals aim at creating a coherent network of terrestrial and marine protected areas (goal A) and restoring terrestrial and marine ecosystems (goal B). In detail, the first goal includes as sub-targets: expansion of the existing network of protected areas; improvement of the ecological-functional connectivity between said areas; efficient management of these areas with clear objectives and protection measures as well as proper financing. The second goal is more articulated, involving additional measures to preserve and restore degraded ecosystems, especially those useful as 'carbon sinks', as well as several provisions related to agriculture (protection of wild pollinators, reducing the use of pesticides, boosting biological agriculture) and forestry management. Objective B10 specifically addresses urban biodiversity, encouraging the creation of green areas, while B11 concerns hydrological network restoration and B12 marine ecosystems. Lastly, target B13 aims at achieving neutrality of land degradation and a zero net increase in land consumption. Somewhat unfortunately, these objectives focus more on preserving ecosystem biodiversity rather than species or genetic biodiversity. The latter, in particular, is considered only in the context of protecting and restoring genetic resources of agricultural interest, agroecosystems and forests. The lack of consideration for wildlife genetic diversity preservation had already been highlighted in the case of the CBD Convention (see Hoban et al., 2020, 2023), and unfortunately, the National Strategy confirms this trend.

The Autonomous Province of Trento (Provincia Autonoma di Trento, PAT), together with the Autonomous Province of Bolzano, has regulatory powers similar to those of a Region with special set of statutes. For PAT, the main regulation addressing the subjects of both land use and protected habitats management is the Provincial Law L.P. 11/2007 (modified with the L.P. 11/2016). The general aim of this law is to "[improve the physical stability and ecological balance of forest and mountain areas, as well as conserve and improve the biodiversity expressed by habitats and species]" (art. 1, par. 1). In addition to ecosystem conservation, PAT is committed to preserve natural biodiversity levels through monitoring activities, the application the EU 'Habitats' and 'Birds' Directives (see art. 33- 41 for dispositions regarding specifically the Natura2000 sites), and the implementation of specific measures and interventions aimed at preserving single species, habitats or overall landscape (art. 21-22). All conservation measures applied to Natura2000 sites are approved by the Provincial Council after consultation with the Council of Local Authorities, subject to the obligatory opinion of the municipalities and local authorities concerned, as well as forest owners with an area of at least 100 hectares (art. 38). Furthermore, this law also outlines dispositions on the management of provincial parks (art. 42- 44), the Stelvio National Park (art. 44 bis-44 undecies), and provincial and local natural reserves (art. 45- 49). Concerning wetland management, the law promotes a conservative use of water bodies, with the declared aim of preserving their stability in order to maintain the peculiar hydrogeological balance of alpine ecosystems (art. 1, par. 1 and 4; art. 8, par. 2 and 3).

The regulation concerning the procedures for the identification of Natura2000 sites and the adoption and approval of the related conservation measures and management plans for the provincial protected areas is detailed in the Provincial Decree D.P.P. 50-157/Leg/2008.

Similarly, the Provincial Decree D.P.P. 3-35/Leg/2010 regulates the organization and functioning of Provincial natural parks.

At present, in PAT there are 155 Natura 2000 network sites, 75 nature reserves and 223 local reserves. About 30% of PAT's territory is within the network of protected areas and 80% of the province's municipalities include at least one protected area. Between 2012 and 2017 the Province has been involved in the 'LIFE Project T.E.N. (Trentino Ecological Network): a focal point for a Pan-Alpine Ecological Network' (Reference: LIFE11/NAT/IT/000187 'TEN'). The LIFE Programme is an EU's instrument for environment and climate action, and in this specific case the overall objective was to "plan an integrated long-term management system and restoration programme that targets the Natura 2000 network under the jurisdiction of the Autonomous Province of Trento". The project's layman report has been published in 2021, and it includes several conservation actions specifically targeting wetlands and peat bogs (see C7- C8). The project also led to the creation of the 'Trentino Living Atlas', an online portal featuring observations of 1,483 species living inside the protected areas network.

Despite the presence of these regulations, studies from all around the world have pointed out the many limits still existing with respect to protected areas' effectiveness. Among these, it is worth mentioning the lack of regular monitoring of protected areas, including also systematic social science studies (Stoll-Kleemann, 2010; Wang et al., 2016; Starnes et al., 2021), or its limit in taking into account individual sites (Gaston et al., 2006; Le Saout et al., 2013). Other authors have suggested improving quality before quantity, cautioning against rapidly establishing new protected areas without first addressing the conditions necessary for a favorable outcome (Geldmann et al., 2019). Regarding Europe, an evaluation of the coverage of 300 threatened species by the Natura2000 network showed that a large proportion of the former is still poorly covered by the Natura2000 sites, and designation of sites is strongly influenced by governmental politics, socio-economic and cultural criteria (Trochet & Schmeller, 2013).

Overall, despite some positive results in terms of improved planning and enforcement measures, measures on biological condition assessments are still inadequate (Geldmann et al., 2015), leading to a lack of knowledge especially concerning the wider landscape beyond target areas (Loos et al., 2021). For this reason, studies focusing on assessment of both endangered species actually living inside protected areas and ecological and landscape characteristics of these areas and their surroundings are fundamental to improving functionality and effective connectivity of these areas.

1.2.3. SUSTAINABLE LANDSCAPE PLANNING

Although problems and challenges of human- wildlife relationships are already at the center of a vast body of research accumulated in recent years (e.g., Nyhus, 2016; König et al., 2020, 2021; Treves & Santiago-Ávila, 2020), increasing numbers of international treaties and organizations are addressing the topic of environmentally sustainable development as an urgent target. Among the UN Sustainable Development Goals, the protection, restoration and sustainable use of terrestrial ecosystems, the halting and reversing of land degradation and stemming biodiversity loss are all targets of Goal 15, and are among the objectives that have and are shaping recent policies for sustainable landscape planning.

As mentioned in the section above, the European Green Deal includes a set of proposals that aim at reversing the trend of greenhouse gas emissions as well as of climate changes. This goal is in itself is highly valuable, as it addresses human responsibilities on the climate crisis, a cause of decline for many wildlife species, including amphibians (see section 1.1.1.). In addition, the Green Deal explicitly recognizes that securing biodiverse ecosystems and tackling climate change are intrinsically intertwined (‘Nature Restoration Law’, COM/2022/304 final, par. 15), and therefore, much of its policy focuses on preserving the existing biodiversity and planning future development with a sustainable mindset. Building on the knowledge collected in the Special Reports of the Intergovernmental Panel on Climate Change (IPCCs), it states, in fact, that ecosystems restoration, especially those that have been identified as important ‘carbon sinks’ due to having great potential of carbon absorption, plays a fundamental role in helping to counter the climate crisis. Beyond restoring all habitats defined by the European ‘Habitats’ Directive (92/43/EEC), the EU ‘Restoration Law’ also gives instructions for sustainable landscape planning that include urban (see art. 6) and agricultural ecosystems (art. 9). In the latter case, in particular, the law prescribes the sharing of agricultural lands with high-diversity landscape features, as well as the restoration of any organic soils in agricultural use constituting drained peatlands, recognized as potential ‘carbon sinks’ (art. 9, par. 4).

The ‘Regulation on land, land use change and forestry’ (LULUCF, Regulation (EU) 2023/839 of the European Parliament and of the Council) further underlines that “peatlands are the largest terrestrial store of organic carbon, and improving peatland management and protection is an important aspect contributing to climate change mitigation” (Regulation (EU) 2023/839, par. 2). It should be noted, however, that wetlands and managed wetlands are only marginally taken into account in this regulation, as their inclusion in the scope of commitments of the Member States is left to discretion of each (see art. 2, par. 1 (f); art. 7, par. 3).

The vision of sustainable development is also the foundation of the World Green Building Council (WorldGBC), a global network of national Green Building Councils (GBCs) in more than 70 countries around the world dedicated to transforming the built environment into one that is environmentally sensitive, economically viable, socially just and culturally meaningful. The WorldGBC has developed a set of green building rating tools (also known as certifications), that are used to assess and identify buildings meeting certain environmental requirements or standards. These rating tools vary widely in their approach, being appliable to all phases of building, from planning and design, to construction, operation and maintenance, renovation and eventual demolition. There are also specific tools or subsets of tools to evaluate different building types such as homes, commercial buildings or even whole neighborhoods. These instruments can be developed by the single national GBCs according to the specific conditions and needs of each country and might therefore differ among countries.

According to the Italian GBC Neighborhood Manual, for instance, there are several prerequisites and credits linked to endangered species and habitats, and/ or wetlands conservation, that can affect the building rating from a point of view of site localization. In the first case, as an example, it is required to the construction company to first carry out a screening study or evaluation of environmental impact in accordance with Annex G of the Presidential Decree D.P.R. 357/1997 or the methodological guide ‘Assessment of plan and project affecting Natura2000 sites’. The second case, instead, concerns a series of instructions ranging from banning on all interventions that cause direct impacts on wetlands and water bodies to allowing only minor interventions with the aim of promoting accessibility in the case of ‘buffer zones’, defined in the same point. A third credit even actively promotes the restoration of natural habitats, wetlands and surface water bodies that might have been damaged by previous human actions.

To date, several studies have attempted to untangle the complex relationship between the human-shaped landscape and wildlife. Łopucki and colleagues (2020), for instance, estimated the effect of city size on wild ground-dwelling small mammals, and found that, to maintain the initial biodiversity of native wildlife, appropriate measures should be taken in the early stages of urban development. A study investigating database-derived data of 25,985 vertebrate species suggested that species that apparently thrive in an urban landscape possess adaptations to cope with environmental disturbances and more in general are able to adopt a generalist strategy which permits them to flourish even in disturbed conditions (Ducatez et al., 2018). Schell (2018) summarized several cases of adaptations to urban-related selective pressures, while also highlighting the need for genetic and genomic studies of said adaptations. Regarding specific engineering improvements, a recent meta-analysis showed that constructed green infrastructures (GIs) significantly improve biodiversity over conventional infrastructure equivalents, although there is still a lack of information on taxa specificity, multi-trophic interactions and landscape-level patterns (Filazzola et al., 2019). The authors thus argue that taking into account these data could help deepen the understanding of GI effects on biodiversity. A previous review that considered both urban and agricultural landscapes found that anthropogenic waterbodies, if in possess of specific local-scale attributes, could act as refuges for freshwater biodiversity (Chester & Robson, 2013). The authors also reported some cases where moderate levels of management intervention were linked to higher biodiversity at the sites.

Overall, these examples suggest that a respectful coexistence of human and wildlife is achievable, but more studies are needed in order to properly understand the specific needs connected to local contexts, including economic and social peculiarities, as well as to the wild species considered.

1.3. AMPHIBIAN SPECIES OF THE PROVINCE OF TRENTO

1.3.1. TAXONOMY, CURRENT DISTRIBUTION AND GENETIC DIVERSITY

In PAT, 12 species of Amphibians have been recorded as having resident breeding populations, seven Anurans and five Urodeles (Caldonazzi et al., 2002; Trentino Living Atlas).

The most common Anuran is the European common frog *Rana temporaria* (Linneaus, 1758), a generalist species widespread in the Province that can be found in a great range of habitats from the valley floor to 2,500 m a.s.l. *R. temporaria* has been a model species for several genetic studies, which highlighted the highly structured nature of its alpine populations and the existence of 'refugia-within-refugia' across the Alps (e.g., Stefani et al., 2012; Vences et al., 2013; Marchesini et al., 2017; Dufresnes et al., 2022).

The other brown frog is *Rana dalmatina* (Fitzinger, 1839), the agile frog, occurring mainly in the central part of the Province. This species is fairing relatively well in Italy with respect to central and northern Europe, but is quite rare in the study area due to its preference for low altitude sites (Di Nicola et al., 2019). Contrary to *R. temporaria*, *R. dalmatina* shows a comparatively homogeneous genetic diversity across all Europe, having a unique strongly differentiated population in southern Italy (Vences et al., 2013).

The yellow-bellied toad *Bombina variegata* (Linneaus, 1758) also lives predominantly at low altitudes. It is considered a pioneer species for its capability to colonize seasonal-dependent and/ or artificial pools, but despite being widespread in Europe *B. variegata* is fairly rare in the Province, and declining (Di Nicola et al., 2019; Caldonazzi et al., 2002). This species was one of the targets of LIFE Project T.E.N. actions (Trentino Ecological Network; LIFE11 NAT/IT/000187, mentioned also in section 1.2.2), which included the recovery of existing sites, as well as creation of new ones, to safeguard its populations (see points A5 and C12 of the LIFE report). Nonetheless, a very recent report highlighted the slightly negative trend of *B. variegata* populations (Endrizzi et al., 2023). According to a recent study on the genetic diversity of *B. variegata*, gene flow between the small populations living in the study area is quite limited but still present, thus resulting in moderate genetic structuring (Cornetti et al., 2016), especially compared to the more structured northern populations (Hantzschmann et al., 2021).

The common toad *Bufo bufo* (Linnaeus, 1758) is another generalist species, living even in urbanized areas, although rarely above 1,000 m a.s.l. This species can be found in all the Regions of Italy, but nonetheless it is now considered vulnerable to extinction nationally due to major population declines in the last decade (Di Nicola et a., 2019). In the study area, however, according to recent reports, *B. bufo* is still the second most common amphibian species (Trentino Living Atlas). *B. bufo* displays overall low levels of genetic diversity in the alpine region, with only one mitochondrial lineage present in the north-eastern Alps (Chiocchio et al., 2021).

The green toad *Bufotes viridis* (Laurenti, 1768), easily recognizable from the previous species thanks to the smaller size and colorful skin, has a more localized distribution at low altitude. Local declines and even extinctions have been reported for this species in Italy (Di Nicola et al., 2019). Despite the lack of genetic data in the alpine region, studies conducted in other areas have found that *B. viridis* populations tend to be highly structured (e.g., Vences et al., 2019; Dufresnes et al., 2021).

PAT is also home to the common water frog, a species complex (*Pelophylax* kl. *esculentus* Linnaeus, 1758) that constitutes of the *P. lessonae* (Camerano, 1882) and *P. ridibundus* (Pallas, 1771) species and their hybridogenetic form *P*. kl. *esculentus*. As *P. lessonae* and the hybridogenetic form are usually sympatric and morphologically almost indistinguishable (Bovero et al., 2013; Lanza et al. 2009), in the present work they are treated as a single species, according to the classification suggested by Caldonazzi et al. (2002). *P*. kl. *esculentus* occurs mainly in the central part of the Province, in the wetlands of the valley floors, and is currently declining due to habitat loss, although the hybrids seem to be more resistant (Caldonazzi et al., 2002; Di Nicola et al., 2019). Hoffmann et al. (2015) demonstrated that this species complex shows a gradient of genetic diversity across Europe, depending on both geography (latitude and longitude) and the presence/absence of parental species.

Lastly, the Anurans also include the treefrog *Hyla intermedia* (Boulenger, 1882), or *H. perrini* according to a recent genetic study that separates this species from *H. intermedia* (Dufresnes et al., 2018). The treefrog is currently the rarest Anuran in PAT, and is declining across Italy (Trentino Living Atlas; Di Nicola et al., 2019).

As for Urodeles, the most common in the Province of Trento is the fire salamander *Salamandra salamandra* (Linnaeus, 1758), even though it is still quite rare in the more isolated valleys, and completely absent from the higher elevations (above 1,700 m a.s.l.; Caldonazzi et al., 2002; Trentino Living Atlas). *S. salamandra* is generally considered at low risk due to its widespread distribution and stable populations (Di Nicola et al., 2019). Studies of the *B. bufo* of this species in the alpine region are currently lacking, although research on populations in the Prealpine and foothill areas of northern Italy noted a moderate genetic population structure, probably due to urbanization separating the populations from the two study areas (Pisa et al., 2015).

Above 1,300 m a.s.l. the alpine salamander *Salamandra atra* (Laurenti, 1768) is present, a species characterized by the absence of aquatic life stages. The nominal subspecies, *S. a. atra*, is not particularly in decline at present, although its limited distribution means populations of *S. a. atra* are the target of annual monitoring (Roner et al., 2023). The subspecies *S. a. aurorae*, also present in the Province, has a much more limited distribution and is even more threatened by human activities and climate change (Romanazzi & Bonato, 2014; Di Nicola et al., 2019). For this reason, and especially considering the reduction and fragmentation of its habitat following the Vaia Windstorm (in 2018), this subspecies is also being closely monitored annually (Romano et al., 2023). Genetic studies of *S. atra* showed an overall lack of genetic structuring (e.g., Riberon et al., 2002, 2004), and a very recent study also confirmed high within-population diversity and inter-population gene flow (Dufresnes et al., 2022). Other authors additionally highlighted the existence of highly differentiated peripheral populations in the southern Prealps (Bonato et al., 2018).

The alpine newt *Ichthyosaura alpestris* (Laurenti, 1768), the second most common Urodele in PAT, is irregularly distributed and lives generally above 1,500 m a.s.l. Overall this species is not particularly threatened at present (Di Nicola et al., 2019). Although few genetic studies have been performed on this species in northern Italy, a comprehensive study showed that *I. alpestris* is characterized by ancient and highly divergent lineages across Europe (Recuero et al., 2014).

The smooth newt *Lissotriton vulgaris* (Linnaeus, 1758) can only be found in southern Trentino, at low altitudes, while the crested newt (*Triturus carnifex* Laurenti, 1768) has been reported in only one location, in the 'Laghetti di Marco' area (Caldonazzi et al., 2002). These two species are rare in the study area due to their preference for relatively low altitudes but are otherwise widespread both in Italy and in Europe (Di Nicola et a., 2019). Maura et al. (2014) found ten differentiated and geographically restricted *L. vulgaris* lineages distributed mainly in the southern Alps of northern Italy, indicating possible multiple refugia during the last glacial maximum. A recent study suggested a ‘refugia-within-refugia’ phylogeographic scenario in the case of *T. carnifex* (Wielstra et al., 2021).

1.3.2. LEGAL PROTECTION

Regarding the conservation measures concerning amphibians of PAT, all species are included in the Convention on the Conservation of European Wildlife and Natural Habitats, or ‘Bern Convention’ (ETS No. 104, Bern 19/09/1979), the first European Convention to take into consideration the protection of herpetofauna. The ‘Bern Convention’ is a treaty binding by international law, that covers most of the natural heritage of Europe and extends to some States of Africa. Signed by fifty countries and the European Union, it came into force on 1st June 1982 and has been ratified five times.

The Convention is a cooperation agreement based on the recognition of the “aesthetic, scientific, cultural, recreational, economic and intrinsic value” of wild flora and fauna, that “needs to be preserved and handed on to future generations”. The treaty also takes heed of the serious decline of numerous species of wild flora and fauna and their current risk of extinction, as well as recognizing that the conservation of natural habitats is of fundamental relevance for the protection and conservation of said species. All Contracting Parties (that is, signatory countries) are thus legally required to promote national policies for the preservation of wild flora, fauna and natural habitats (including educating their citizens on the need of protecting endangered species, art. 1-3), by taking all appropriate and necessary legislative and administrative measures to ensure the conservation of the species listed in Appendices I, II and III of the Convention (art. 4-7). Article 9 of the Convention, however, states that exceptions on the provisions mentioned above can be made in consideration of economic, public health, research and education interests, if “the exception will not be detrimental to the survival of the population concerned”. To monitor the adequacy of any undertaken exceptions, a Standing Committee is also set up (art. 13-15), to which all Contracting Parties are required to send a biennial report (art. 9, par. 2). Of the four Appendices of the Convention, the first one lists the strictly protected flora species, the second the strictly protected fauna species, the third the fauna species that require less severe protection measures, and the fourth is a summary of all the prohibited means and methods of killing, capture and exploitation of protected flora and fauna populations.

Among the species listed in Appendix II there are five amphibian species with distributions including the Province of Trento, namely *B. variegata*, *B. viridis*, *R. dalmatina*, *S. atra* and *T. carnifex*, while all remaining species are included in Appendix III. As Appendix II species are subject to special protection measures, such as all forms of deliberate capture, confinement or killing; damage to or destruction of breeding or resting sites; disturbance, in particular during periods of breeding, rearing and hibernation; destruction or removal of eggs; and the captive breeding and trade in these animals, alive or dead, are strictly prohibited (art. 6). Appendix III species, instead, are only subjected to a regulated exploitation in order to ensure the preservation of satisfactory population levels (e.g., temporary or local prohibition of exploitation might be enacted to allow reproduction of the populations, art. 7).

Biodiversity loss has not significantly decreased the measures listed in the Bern Convention were adopted and instead, is accelerating at an alarming rate. Therefore, the Standing Committee has recently signed a statement, the ‘Vision for the Bern Convention for the period to 2030’ (T-PVS(2021)14), that summarizes the objectives that the Contracting Parties are expect to achieve by 2030. The Vision states as follows: “By 2030, declines in biodiversity will be halted, leading to recovery of wildlife and habitats, improving the lives of people and contributing to the health of the planet”.

In order to define the guidelines to accomplish this objective, a Strategic Plan has been published very recently (Strategic Plan for the Bern Convention for the period to 2030, adopted by the Standing Committee, December 2023; T-PVS(2023)18). The Plan aligns with the Kunming-Montreal Global Biodiversity Framework (discussed in section 1.2.2.) and sets out both a list of specific goals and targets and the respective indicators that can be used to track progress toward these goals, thereby building a monitoring and evaluation framework based on objective data. Annex 2 of the Strategic Plan defines ownership and responsibilities of implementation, calling to action “all areas of sectoral responsibility, including, for example, agriculture, forestry, fisheries, energy, transport, infrastructure planning, water resources management, industry, urban development, climate change and finance, with education also playing a role”. Despite the broad range of possible actors, governments of the Contributing Parties are still formally accountable for achieving the Convention's goals.

Seven amphibian species and one subspecies of the Province of Trento are also included in the European Habitats Directive (Council Directive 92/43/EEC; see also section 1.2.2.). In particular, *B. variegata, S. a. aurorae* and *T. carnifex* are included in Annex II of the European Habitats Directive, a list consisting of all species that require the designation of special areas of conservation. Among them, the subspecies *S. a. aurorae* is additionally indicated as a priority species. Moreover, *S. atra*, *R. dalmatina* and *B. viridis* are mentioned in Annex IV as species in need of strict protection, and *R. temporaria* e *P.* kl. *esculentus* in Annex V, that refers to "species of community interest whose taking in the wild and exploitation may be subject to management measures".

None of the amphibians living in PAT are included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), an international agreement aiming to ensure that international trade in wild animals and plants' specimens does not threaten species survival. This Convention is enforced in the European Union through the 'EU wildlife trade regulations', and in particular the Council Regulation (EC) No 338/97 (amended recently by the Commission Regulation (EU) 2023/966 of 15 May 2023), that includes a series of Annexes stricter than CITES itself. For instance, a species that may be listed on CITES Appendix II could appear in EU Annex A, thus obtaining higher protection priority; Annex B considers also species that, if introduced in the European Union territories, could become an ecological threat to the native species; Annex D includes non-CITES species for which the EC is independently monitoring the level of imports, and so on.

The absence of the alpine species from this Convention can be explained by considering both the IUCN evaluation of their conservation status (briefly summarized below) and the absent or moderate trade concerning these species (Di Nicola et al., 2019). However, the CITES Convention still represents a legal tool for indirectly protecting EU native species, thanks to the control exercised on the introduction of non-autochthonous species and on the health status of the traded flora and fauna, consequently containing the spread of new pathogens. Both these phenomena are in fact causes of biodiversity loss, amphibians included (see section 1.1.1.).

Despite numerous cases of populations local declines reported worldwide (e.g., Daszak et al., 1999; Carrier & Beebee, 2003; Araújo et al., 2006; Bosch & Martínez Solano, 2006; D'Amen & Bombi, 2009; Spitzen van der Sluijs et al., 2013), most of the target species of this thesis are classified as Least Concern (LC) in the IUCN Red List (IUCN, 2024) in light of their still wide distribution. The only exceptions are represented by *S. salamandra* and *T. carnifex*, both classified as Vulnerable (VU). These assessments are all quite recent, dating between 2019 (*H. intermedia*) and 2022 (*S. salamandra*, *L. vulgaris*, *T. carnifex*). In Italy, however, the latest assessment shows some differences with respect to the global evaluation (Rondinini et al., 2022). For instance, *L. vulgaris* was classified as Near Threatened (NT) in the first evaluation (2013) and is the same in the latest one (2022). The subspecies *S. a. aurorae* conservation status was already considered VU in 2013 and has now worsened to Endangered (EN), despite habitat loss during the Vaia Windstorm. *B. bufo* was also nationally evaluated as VU, both in 2013 and 2022, while *H. intermedia* was not included in either assessment. However, according to the Red List of the Province of Trento *S. atra* is considered NT, *B. variegata* VU and *T. carnifex* EN.

In Italy, both the 'Bern Convention' and 'Habitat' Directive have been ratified with two National laws (L.N. 503/1981 and D.P.R. 357/1997). Nevertheless, at present there is no unified National law for the protection of the so-called 'minor fauna' (including amphibians, reptiles, fish and invertebrates), therefore the Regions and Provinces have enacted specific local laws to ensure the preservation of these animals.

In PAT, the protection of wild flora and fauna is addressed in L.P.N. 11/2007, art. 25-26, that ratifies the Directives 79/409/EEC and 92/43/EEC. Regarding amphibians, this law limits harvesting of specimens belonging to the genus *Rana* (l.p. n. 11/2007, art. 26, par. 1) to 1 kg per person and per day, and forbids the collection or capture of amphibian eggs and tadpoles (L.P.N. 11/2007, art. 26, par. 2). The Decree of the President of the Province n. 23-25/Leg/2009 further underlines that all amphibian species of the Province are under legal protection (art. 5, par. 1) and fixes new limits on the exploitation of *Rana* specimens, thereby excluding the species *R. dalmatina* from legal harvesting, as well as the period between March, 1st and April, 30th for the remaining species (*R. temporaria*). The 'provincial law on hunting' (L.P.N. 24/1991), last updated in 2023, whose main purpose is to regulate hunting activities, also deliberates on the introduction in nature of fauna for repopulation purposes, capture and use of animals for scientific research, and wild fauna detention, all fields that have a potential impact on wild amphibian conservation. Lastly, the LIFE project T.E.N. (LIFE11 NAT/IT/000187) mentions amphibians in Action 8 section, which aimed at drawing up management guidelines for several key species; in addition, the demonstrative conservation action C12 specifically aimed at improving and increasing reproductive habitats for *B. variegata* populations.

1.4. CONSERVATION GENETICS AND GENOMICS

1.4.1. WHY CONSERVE GENETIC DIVERSITY?

The aim of the Convention on Biological Diversity and the Global Biodiversity Framework is to promote the conservation of biological diversity, its sustainable use and the equitable sharing of the benefits arising from its conservation (CBD, 1992; CBD/COP/DEC/15/4, 2022), and acknowledging three main levels of biodiversity: ecosystem, species and genetic diversity. The importance of the latter in the context of wildlife conservation, however, is still worryingly underrepresented (Hoban et al., 2023).

Genetic diversity can be defined as the total gene-level variation within species. This diversity is fundamental for the survival and evolution of wildlife, as it represents a pool of genetic characteristics that have been or might be useful for coping with, and finally adapting to, changing environments (Booy et al., 2000; Frankham et al., 2002; Gaston & Spicer, 2004; Höglund, 2009). Consequently, the loss of genetic diversity can lead to a reduction in fitness and capability to adapt to new environmental conditions, including the introduction of new pathogens (Frankham et al., 2002). Moreover, in small and/ or genetically isolated populations (but also in captive populations, see Willoughby et al., 2017), the resulting inbreeding can have severe deleterious effects on reproduction and survival at the individual level, a phenomenon known as inbreeding depression that ranges from an increased homozygosity to a higher likelihood of fixation of deleterious alleles (Frankham et al., 2002, 2017). For this reason, a reduction in genetic diversity of a population can in some cases lead to a rapid demographic decline, with important consequences at both species and ecosystems levels (Frankham et al., 2017; DeWoody et al., 2021).

As the main drivers for genetic diversity loss include habitat fragmentation and, consequently, population isolation, the development of rapid genetic characterization of natural populations is now considered an impelling concern (see Hoban et al., 2013, 2021, 2023). Firstly, this research provides important information on how the target species have already, and might in the future, respond to changing environment, including the climate crisis and human-related land use changes (Hoban et al., 2013; DeWoody et al., 2021). Secondly, results can also help shed light on existing taxonomic uncertainties and on the identification of management units (Frankham et al., 2002; Coates et al., 2018). Overall, genetic diversity studies are currently considered fundamental to developing effective long-term conservation strategies (Hoban et al., 2013).

1.4.2. INNOVATIVE AND NON-INVASIVE APPROACHES

In order to address the increasingly urgent need to safeguard natural genetic diversity in addition to species and ecosystems biodiversity, thanks to technological advancements, several new approaches have been developed in recent years.

In particular, conservation studies are now supported by the rapid development of non-invasive genetics, the analysis of genetic material extracted from organic remains rather than from whole organisms. The non-invasive genetic approaches allow genetic information to be obtained without killing, harming or even capturing the target species, a fundamental consideration when the aim is, for instance, to preserve small populations. Among these approaches, the use of environmental DNA (eDNA) is a particularly promising field (Taberlet et al., 2018). The definition of 'eDNA' includes all genetic material that can be extracted from environmental samples such as soil, water, ice and even air (Barnes & Turner, 2016). Such DNA is shed into the environment by organisms through cells contained in feces, urine, fur, skin fragments, gametes as well as other body fluids (Herder et al., 2014; Barnes & Turner, 2016).

Since the advent of Next Generation Sequencing (NGS) in 2005 (Margulies et al., 2005), the use of these samples has been coupled to the possibility of mass-processing DNA sequences derived from several samples and/or several individuals (metabarcoding and metataxonomy) in a less expensive and time-consuming fashion. Metabarcoding derives from the concept of a ‘DNA barcode’, or one or more short DNA sequences used for identification of any taxonomic level (Kress et al., 2015). Species identification by DNA barcoding typically involves the following steps: a DNA sequence (potential barcode) is chosen based on the specific aim and taxonomic scope of the study, extracted and amplified from the samples to be examined, compared with a database of reference barcode sequences (generated from individuals of known identity), and is considered identified if it closely matches a database sequence (Hebert et al., 2003; Hajibabaei et al., 2007). The metabarcoding approach extends this DNA-based identification at species level to a community of species that play different roles in the ecosystem (Cristescu, 2014; Taberlet et al., 2018). This innovation has paved the way for a plethora of applications, from single species detection (e.g. Buxton et al., 2017, 2018; Franklin et al., 2019; Katano et al., 2017; including invasives species, see Tingley et al., 2019) to characterization of entire communities (e.g., Cannon et al., 2016; Valentini et al., 2016; Vences et al., 2016; Bálint et al., 2018), diet analysis (e.g., Deagle et al., 2010; Pompanon et al., 2012), and even to monitor the post-release survival of translocated populations (Rojahn et al., 2018).

The eDNA metabarcoding approach, however, has its own limits and unresolved issues. For example, when analyzing systems in which interspecies hybridization is possible, this method may fail to distinguish between the parental species and the hybrids, (e.g., for the hybridogenetic pool frog genus *Pelophylax*, Ficetola et al., 2019). Another source of taxonomic bias is the potential lack of a complete reference database of species for the chosen barcode, and local databases need to be created (e.g., Valentini et al., 2016). In addition, the reliability of results obtained with this approach can be compromised by false positives and/or false negatives (type I and type II errors), which include: non-specific primers amplifying non-target DNA; contamination between samples or sites; prolonged persistence of DNA after the death of an individual; or poor sampling design (Ficetola et al., 2015, 2019; Renan et al., 2017). False negatives are particularly problematic for cryptic target species that also occur at low densities (Hobbs et al., 2019). To help reduce the probability of these errors, common guidelines for eDNA metabarcoding workflows have been suggested by several authors (e.g., Ficetola et al., 2015; Goldberg et al., 2016; Harper et al., 2019). In this study, we have followed most of these guidelines; in particular, to minimize false positives, all recognized procedures for sample contamination management were followed (e.g., addition of negative controls to each extraction use of BSL2 hoods for sample processing; bioinformatic decontamination); barcode sequences were selected from gene regions for which comprehensive databases were already available online (Cytochrome Oxidase I, Cytochrome B) to ensure taxonomic coverage of all target species;

DNA fragments assigned to *Pelophylax* were assigned to the species complex *P*. kl. *esculentus* as suggested by previous observations in the study area (see section 1.3.1.). To reduce false negatives, temporal and spatial replicates were collected for the pilot study on *R. temporaria* (see Chapter 2), and spatial replicates were collected thereafter for each site.

In addition to metabarcoding, metataxonomy has recently gained attention as a way to obtain information about microbial communities and their impact on wildlife conservation (e.g., Hauffe & Barelli, 2019; Wei et al., 2019; Akoijam & Joshi, 2022). In the case of amphibians, for instance, skin microbial communities (microbiota) have been shown to have an important role in regulating the host's response to pathogens (see Woodhams et al., 2023 for a comprehensive review), but also to be affected by environmental conditions and human disturbances (Bernardo-Cravo et al., 2020; Hernández-Gómez & Hua, 2023). Metataxonomy shares some technical challenges with eDNA metabarcoding, such as the risk of contamination, both cross-sample and from external sources (Glassing et al., 2016; Cando-Dumancela et al., 2023). Therefore, in this study, negative controls were included at each laboratory step, and special consideration was given to bioinformatic data cleaning. Again, metataxonomic barcodes were chosen while considering the most complete reference databases available (i.e., variable regions of the 16S rRNA gene for bacterial taxa, and of the ITS region for fungal taxa).

In summary, the innovative and non-invasive approaches presented here and applied throughout this thesis have the potential to be greatly useful for conservation studies, especially considering that they do not require harming (and sometimes not even handling) of the target animals while at the same time providing relevant data for informing monitoring and conservation plans (Taberlet et al., 2018).

1.5. AIM AND STRUCTURE OF THE THESIS

Considering the alarming decline of amphibian populations both worldwide and in the Alps, and in light of the recently renewed interest in conservation of their habitats through both expansion of existing protected areas and creation of suitable reproductive sites in a context of sustainable landscape planning, this doctoral thesis aimed at investigating the presence and wellbeing of amphibian communities in protected areas as well as artificial and human-impacted sites using various molecular and statistical approaches. First, I developed new, completely non-invasive monitoring protocols in order to obtain information on amphibian species presence and genetic diversity, testing them initially on one common species, and then validating them for all species present in the Province of Trento, where observational databases were available. Secondly, I then used this presence data in combination with a series of ecological and landscape indices and landscape planning methods to identify meaningful correlations between amphibian species richness, human presence and activities, and climate-related factors. Lastly, I investigated the diversity and composition of skin microbial communities of *B. variegata* populations living in habitats with varying degrees of human-related impact, to further understand the role of microbiota in the conservation status of amphibian taxa. The thesis is therefore structured as follows:

Chapter 2: A validated protocol for eDNA-based monitoring of within-species genetic diversity in a pond-breeding amphibian

PUBLISHED SCIENTIFIC ARTICLE*: Zanovello, L., Girardi, M., Marchesini, A., Galla, G., Casari, S., Micheletti, D., Endrizzi, S., Fedrigotti, C., Pedrini, P., Bertorelle, G. & Hauffe, H. C. (2023). A validated protocol for eDNA-based monitoring of within-species genetic diversity in a pond-breeding amphibian. Scientific Reports, 13(1), 4346. https://doi.org/10.1038/s41598-023-31410-4*

Here I tested the possibility of using non-invasive environmental DNA (eDNA) samples to i) detect the common frog (*R. temporaria*), across different seasons and life stages; and ii) estimate genetic diversity indices, comparing these results with the same indices calculated from traditional, invasive, tissue sampling data in a previous genetic study. The results of this study showed that species detectability varies across seasons, with peaks corresponding to spring-summer (breeding season). I also demonstrated that there is a strong correlation between genetic diversity indices calculated with the two approaches. Therefore, provided that eDNA samples are collected at peak reproductive activity of the species, presence and genetic data generated from eDNA can in principle be used to estimate standard measures of genetic diversity in this, and potentially any other, amphibian species.

Chapter 3: Validation of an eDNA-based workflow for monitoring inter- and intra-specific CytB haplotype diversity of alpine amphibians

ACCEPTED SCIENTIFIC ARTICLE: *Zanovello, L., Martins, F. M. S., Girardi, M., Casari, S., Galla, G., Beja, P., Pedrini, P., Bertorelle, G., & Hauffe, H. C. (2024). Validation of an eDNA-based workflow for monitoring inter- and intra-specific CytB haplotype diversity of alpine amphibians. Accepted for publication by Environmental DNA.*

Here I expanded the eDNA method presented in Chapter 2 to detect all amphibian species living in the Province of Trento using the same simple water collection and filtering field methods. Using a more variable mitochondrial gene (Cytochrome B) and a seven-marker approach targeting amphibian families and genera, I showed that it was possible to retrieve data on both species and genetic diversity. These new primer pairs were tested a) *in silico* using a reference database including all target genera; b) *in vitro* on extracts from tissues of the target species; and c) *in situ* on water samples from 38 wetlands in the Province of Trento (Italy). The protocol successfully detected most target species, although some markers also amplified non-target amphibian species. Using the same metabarcoding sequences, I also compared the results from analyzing the data using three different bioinformatic pipelines, in terms of reads and exact sequence variants retrieved. Considering the number of reads retrieved, as well as a comparison of these sequences with previously known haplotypes, one pipeline (combining the software MICCA with VSEARCH) appeared to be the most appropriate for minimizing the probability of false positives, while at the same time retrieving the highest number of reads.

Chapter 4: A pond of knowledge: applying multi-disciplinary approaches to amphibian conservation in the Province of Trento

UNPUBLISHED MANUSCRIPT: *Zanovello, L., Pozzer, G., Girardi, M., Delucchi, L., Russo, L., Pedrini, P., Maragno, D., Bertorelle, G., & Hauffe, H. C. A pond of knowledge: applying multi-disciplinary approaches to amphibian conservation in the Province of Trento.*

This chapter is dedicated to exploring the possibility of using amphibian presence data from the metabarcoding dataset identified as most robust in Chapter 3 to determine the ecological and landscape factors impacting amphibian species richness. Using landscape planning methods, an innovative approach seldom applied in conservation biology, I applied a multivariate correspondence analysis, integrated with regression tests, to describe the relationship between species richness data, habitat quality, human pressures and climatic/environmental changes. The preliminary results of this study indicated that the protected wetlands considered here are fundamental for preserving amphibian communities, hosting the highest mean number of species. In addition, and more surprisingly, farm ponds showed a comparatively high species richness: this result is particularly significant, not only for evaluating the sustainability of human agricultural activities, but also because despite being very numerous, these habitats have very rarely been monitored up to now as key amphibian habitats, or as relevant components of ecological corridors maintaining gene flow between populations in larger, natural water bodies.

Chapter 5: More than meets the eye: unraveling patterns of skin microbiota diversity of an opportunistic amphibian species in different habitats

UNPUBLISHED MANUSCRIPT: *Zanovello, L., Galla, G., Girardi, M., Casari, S., Lo Presti, I., Pedrini, P., Bertorelle, G., Hauffe, H. C. More than meets the eye: unraveling patterns of skin microbiota diversity of an opportunistic amphibian species in different habitats.*

In this chapter, *B. variegata* was used as a model species for a highly comprehensive study addressing the composition of both their bacterial and fungal skin communities, as well as the relationship of this microbiodiversity with biotic and abiotic variables such as water microbiota, temperature, pH and dissolved oxygen. Unlike many microbiota studies to date, this study highlighted the specific patterns of biodiversity in bacterial and fungal communities, as well as any interactions between the two, in both natural and artificial water bodies. In fact, the populations sampled for this study originated from a variety of habitats, including natural pools along the Avisio River, ephemeral ponds near apple orchards, irrigation tanks in vineyards, and higher-altitude farm ponds. This work showed that alpha and beta diversity of bacterial and fungal communities present different patterns with respect to habitat and the environmental variables, despite both being affected by human activities.

Chapter 6: General conclusions

In the concluding chapter, I summarize the primary outcomes of my research, discuss their implications for amphibian and wetlands conservation, and provide suggestions for further investigations. In particular, I highlight the merits of a multidisciplinary approach to integrate amphibian conservation within a scenario of human sustainable development.

IN PRAISE OF FROGS

Cassandra Sagan Bell

Deep in the belly of winter
the tadpoles wait, frozen paisleys
plastered in place,
captured commas, crescent moons
unchanging at the bottom
of the pond. The ice and the silence
are their womb. Only spring
will birth them, only thaw
will release them. No mother
to teach them to leap
or to dive. They emerge
by the thousands,
dart wildly
in green water. They are the spitting
image of human sperm cells
magnified.
Had men never merged with woman,
with egg, we would still be frogs,
the Earth surrounded by moons.

Frogs- pure leaping
pure croaking
quick tongues and pure hunger
for live, moving objects.
they remember
when our planet was cool and her blood
ran green, pumped
by her reptilian heart.

Praise the frog! After all
we, too, are part frog,
part wriggling, urgent tadpole,
part waiting, part silence, part leaping.
I offer this praise
from the bottom
of my once-green heart.

CHAPTER 2

A VALIDATED PROTOCOL FOR eDNA-BASED MONITORING OF WITHIN-SPECIES GENETIC DIVERSITY IN A POND-BREEDING AMPHIBIAN

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ABSTRACT

In light of the dramatic decline in amphibian biodiversity, new cost-efficient tools to rapidly monitor species abundance and population genetic diversity in space and time are urgently needed. It has been amply demonstrated that the use of environmental DNA (eDNA) for single-species detection and characterization of community composition can increase the precision of amphibian monitoring compared to traditional (observational) approaches.

However, it has been suggested that the efficiency and accuracy of the eDNA approach could be further improved by more timely sampling; in addition, the quality of genetic diversity data derived from the same DNA has been confirmed in other vertebrate taxa, but not amphibians. Given the availability of previous tissue-based genetic data, here we use the common frog *Rana temporaria* Linnaeus, 1758 as our target species and an improved eDNA protocol to: i) investigate differences in species detection between three developmental stages in various freshwater environments; and ii) study the diversity of mitochondrial DNA (mtDNA) haplotypes detected in eDNA (water) samples, by amplifying a specific fragment of the COI gene (331 base pairs, bp) commonly used as a barcode. Our protocol proved to be a reliable tool for monitoring population genetic diversity of this species, and could be a valuable addition to amphibian conservation and wetland management.

INTRODUCTION

Of all taxa affected by the current biodiversity crisis, amphibians are the most endangered group of vertebrates [1], with 41% of globally-evaluated IUCN species included in ‘threatened’ categories (IUCN, 2021). Due to the general elusiveness of amphibian adults and limited detectability of early life stages, which often live in protected, difficult-to-access and/or season-dependent aquatic environments, accurate estimates of the presence and distribution of amphibian species are logistically difficult, costly and time-consuming to obtain [2, 3].

In addition to species richness, within-species genetic diversity is of crucial importance for the persistence and evolution of natural populations [4, 5], enabling them to adapt to environmental changes, and to the spread of new pathogens [6, 7]. Moreover, the loss of genetic variability may have detrimental effects on individual health due to inbreeding depression, reducing fitness and ultimately increasing the risk of population and species extinction [8]. Due to their particular breeding strategy (often r-strategists with small effective population sizes and high clutch mortality), low dispersal rates and high philopatry, all of which limit population connectivity, amphibians seem to be especially prone to genetic erosion [9]. Therefore, regular, cost-effective genetic monitoring should be considered a fundamental aspect of amphibian conservation strategies. Although the importance of protecting genetic diversity is widely recognized and has been addressed under the Aichi Biodiversity Targets [10], the development of standardized monitoring frameworks for an accurate surveillance of genetic diversity trends in natural populations is still limited to few charismatic or economically relevant species [5, 11].

For the last decade, environmental DNA (i.e. DNA that can be extracted from noninvasive samples such as soil, water, fecal pellets, hair or feathers; eDNA) has been used for accurate, cost-efficient species detection in aquatic environments [12, 13], including for amphibians (for a recent review, see [3]). Specifically, single species eDNA surveys (mainly using quantitative PCR; qPCR) have increased the speed and efficiency of amphibian detection, compared to traditional observational monitoring [14, 15], and have proven to be particularly useful for detecting rare or elusive amphibian species (e.g., [16, 17, 18]), improving the knowledge of habitat requirements and species distributions (e.g. [19, 20, 21]), and tracking invasive alien amphibians (e.g.[22, 23, 24]). In addition, the eDNA metabarcoding approach is increasingly used as a cost-effective method for the simultaneous assessment of species composition in amphibian communities [3, 25]. However, to our knowledge, only one study aiming at the development of a long-term eDNA-based monitoring program for an amphibian species has been published thus far [26].

Very recently, the possibility of inferring intraspecific genetic diversity from eDNA has also been explored [3, 27, 28, 29]; however, only a few pilot studies on amphibians have been published. For example, Gorički et al. (2017)[30] used two short CytB fragments (about 100-150 bp) for the discrimination of two putative olm (*Proteus anguinus)* subspecies, but the protocol was not designed to estimate population-level genetic diversity. Wang et al. (2021)[31] developed an eDNA metabarcoding protocol for the Chinese giant salamander (*Andrias davidianus*) to allow the detection of seven haplotypes corresponding to distinct evolutionary lineages (reporting the results of laboratory amplicon mixture), but again the study was not aimed at the assessment of within-population genetic variability.

Despite their great potential, eDNA methods are extremely sensitive to sampling design and field and laboratory protocols, as eDNA is not abundant and persists in aquatic environments for a limited time, from a few days to several weeks [32, 33, 34]. Therefore, developing and validating effective field sampling methods is essential to the application of eDNA-based survey methods [35, 36, 37].

In this study, we chose the common frog (*Rana temporaria* Linnaeus, 1758), a widespread European pond-breeding amphibian [38], showing high genetic diversity [39, 40], to develop and validate an eDNA metabarcoding protocol allowing rapid and standardized assessments of within-population genetic variability from water samples. Specifically, by selecting 10 wetland sites, for which we had previous information on *R. temporaria* haplotypes identified using traditional tissue sampling [41], we: (a) developed a metabarcoding protocol targeting a 331 bp long fragment of the COI region, which allowed discrimination of previously identified haplotypes; (b) optimized the sampling design, in terms of temporal and spatial replicates, for the collection of water samples in a variety of wetland habitats; (c) computed standard genetic diversity estimates for *R. temporaria* populations from eDNA metabarcoding, to compare eDNA-based results with previously available genetic data.

MATERIALS AND METHODS

Study species and study area

The common frog has been a model species for previous genetic diversity studies (e.g., [39, 40]); however, despite being the most widespread amphibian species in Europe [42], local population declines are frequent due to climate change and habitat degradation [43, 44]. Our study area covers the Autonomous Province of Trento (Italy), a mountainous region located in the eastern Alps; mtDNA haplotypes data are available for *R. temporaria* across the study area from a recent genetic survey [41].

Sample collection

For the present study, 10 sites were selected from Marchesini et al. (2017) [hereafter 'reference dataset'; 41] (Figure 1 and Supplementary Information, Table S1) that encompassed all three mitochondrial lineages of *R. temporaria* known to be present in the study area, as well as the majority of the haplotypes (8/12) noted in [41].

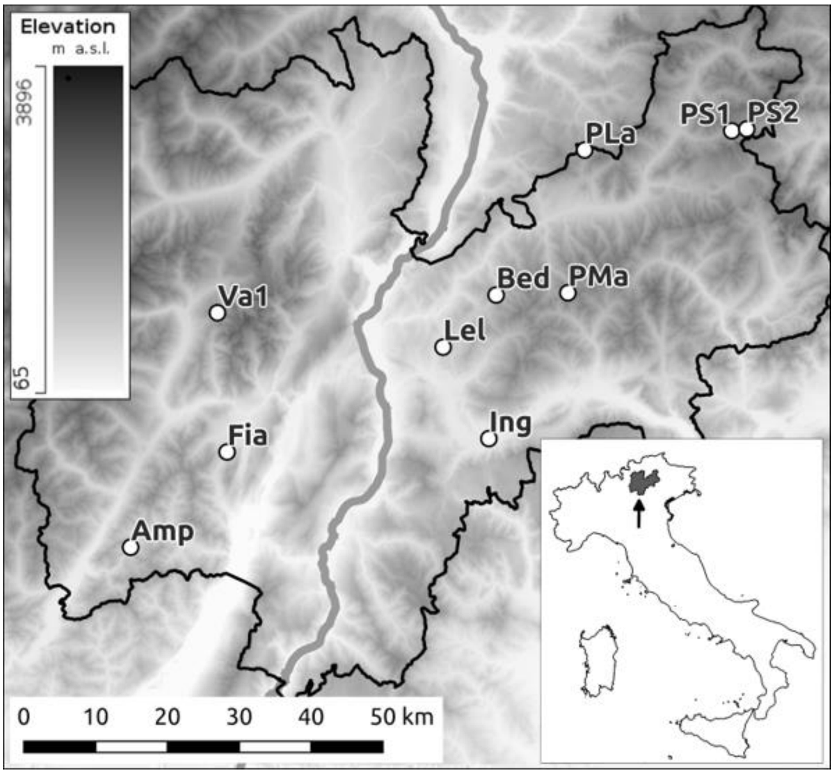

Figure 1. Map of the study region (Province of Trento, Italy) showing the 10 selected wetland sample sites, labeled according to Table 1. Site locations, abbreviations, coordinates, elevation and description are listed in Supplementary information, Table S1. The map was generated using QGIS version 3.20 (http://www.qgis.org); Digital Terrain Model (DTM) for the study area was extracted from the 20 m-resolution DTM of Italy, publicly available at the National Geoportal of Italy (http://www.pcn.minambiente.it/mattm/servizio-di-scaricamento-wcs/).

Water samples were collected at each of the 10 sites during the reproductive season (March-September depending on altitude) from 2019 to 2021. In order to define a standard protocol, three temporal (T1-T3) and three spatial (S1-S3) replicates were collected, where T1 corresponds to a replicate collected when eggs or early-stage larvae were present at the site (early spring), T2, when late-stage larvae were present (mid-summer), and T3, when both adults and juveniles had abandoned the sites following reproduction (end of summer/ early autumn). Some of the wetland sites became partially desiccated during the study, thus collecting the T3 replicates was not always possible. When possible (i.e. whenever spatially separated, accessible water microhabitats were available at the site), for each temporal replicate, three spatial replicates were sampled (5/10 sites) close to observed life stages. For three of these sites, for one of the three spatial replicates (Amp-T1-S2; Ing-T1-S3; PMa-T1-S1, Table 1 and in Supplementary Information, Table S2), no egg clutches or tadpoles were detected at T1; nonetheless, sampling was performed in any case, and all sampled microhabitats were included in the experimental design. In the remaining five sites, only two spatial replicates were possible. In order to capture as many haplotypes as possible from the same sampling site, the spatial replicates were collected at least 20 m apart (depending on the area of wetland on the sampling site).

Each replicate sample was collected manually from just under the surface of the water body using a sterile plastic canister. Stirring up sediment was carefully avoided. To minimize sample contamination, field workers wore laboratory gloves (changed between each replicate), collected samples from the shore without disturbing the water body, and wiped all equipment with bleach and alcohol between each site. The water collected in the canister was stirred before being drawn up with a 100 ml syringe and filtered through a Sterivex-GP Filter unit (pore size 0.22 µm, Millipore cat. no. SVGPL10RC); this step was repeated until the filter clogged. A second filter was also used for the same spatial replicate. The quantity of water that could be filtered through two Sterivex-GP Filter units varied widely among sites, ranging from about 100 ml to more than one liter, depending on the suspended organic matter. Each filter was drained and capped at both ends with the inlet and outlet caps. Following the manufacturer’s instructions, all filters were kept at ambient temperature out of direct sunlight until arrival in the laboratory later the same day, and archived at -20°C until DNA extraction.

Sample processing, including primer validation

All laboratory procedures were carried out at the Platform of Animal, Environmental and Antique DNA of the Conservation Genomics Research Unit, Fondazione Edmund Mach, following recommended guidelines for eDNA analyses, including separate pre- and post-PCR laboratories [45, 46]. All procedures were performed under BSL2 biological hoods. DNA extraction from filters was carried out using the DNeasy PowerWater Sterivex Kit (Qiagen), following the manufacturer’s instructions with these modifications: the heating step and bead beating tubes (PowerBead Tubes) were eliminated to reduce extraction of non-target genetic material from microorganisms. The two filters corresponding to the same spatial replicate S1, S2, or S3 were processed simultaneously and extracts were merged into a single tube at the 14th step of the protocol. DNA extraction was performed in batches of a maximum of 10 water replicate samples, including one negative control (extraction blank) for each extraction batch.

To design a primer pair for amplifying the diagnostic mtDNA region for *R. temporaria,* Primer3Plus [47] was used with the haplotype sequences available from Marchesini et al. (2017) [41]. The resulting primer pair, named Rt-aplo_COI F (GTAATAATTTTCTTTATGGT) and Rt-aplo_COI R (TCAAACAAAGAGGGGTGT), amplifies a fragment 331 bp long distinguishing 11 of the 12 mtDNA haplotypes listed in Marchesini et al. (2017) [41] for the Province of Trento. Specifically, haplotype pairs CA2 and TN4, and SA1 and PR7 (the latter not being previously found in the study area) are not distinguishable, and therefore, were named CA2_TN4 and SA1_PR7, respectively, in the Results section. To distinguish all known haplotypes for the study area, a 569 bp sequence plus adapters (thus about 690 bp in total) would have been required, but such a long fragment cannot be processed using the Illumina technology commonly adopted for metabarcoding (maximum length 600 bp). The primer pair was tested *in vitro* on DNA extracted from *R. temporaria* tissue samples available from previous studies, and amplification success of all samples was confirmed via screening on a Qiaxcel Advanced System (Qiagen). The amplification reaction took place in a final volume of 50µL, containing H_2O (22.25µL), Promega Flexi Buffer 5X (10µL), $MgCl_2$ 25mM (4µL), BSA 10 mg/mL (0.5µL), Rt-aplo_COI-F 10 ρmol/µL (1µL) and Rt-aplo_COI-R 10 ρmol/µL (1µL), dNTP's 10mM each (1µL total), Promega GoTaq G2 5U/µL (0.25µL) and template DNA (10µL). The PCR mixture was denatured at 95 °C for 2 min, followed by 40 cycles of 30 s at 95 °C, 15 s at 48°C and 40 s at 72 °C, and a final elongation step at 72 °C for 5 min. Only one PCR was run per replicate, and a second PCR was run only if the first one failed to produce results. One negative control (PCR blank) was included for each PCR reaction, along with all extraction negative controls.

Each amplification product was then purified with the MinElute PCR Purification Kit (Qiagen) following manufacturer's instructions. 20 µl of each purified product were loaded into a single 96-well plate and sequenced at the FEM Sequencing and Genotyping Platform using paired-end sequencing (2 × 300 bp) on an Illumina Miseq (Illumina, San Diego, CA) with a 30 000 bp coverage.

Bioinformatics and statistical analysis:

All bioinformatic analyses were performed with the software MICCA [48]. Overlapping paired-end sequences were merged to obtain consensus sequences using the command 'mergepairs' with a minimum overlap length of 100 bp and maximum of one mismatch in the overlap region. Reads that did not contain the forward or reverse primers were discarded, and primers were trimmed from the remaining fragments with the command 'trim'. Sequences were quality filtered using the command 'filter', assuming a maximum allowed expected error rate of 0.1% and a minimum length of 331 bp (exact length of the target sequence without primers, as is becoming common practice for genetic diversity studies based on eDNA metabarcoding, e.g., [27, 49]). The reference database for haplotype classification was assembled using published *R. temporaria* haplotype sequences found in Italy. The method 'otu open_ref' was used to cluster our sequences against this database with an identity threshold of 0.99, rejecting a sequence if the fraction of alignment to the reference sequence was lower than one, and discarding sequences with a read abundance value lower than 100 after dereplication. To avoid false positives, either from PCR or sequencing errors, or minor cross-contaminations between the replicate samples, OTUs represented by fewer reads than 5% of the total reads for a specific replicate sample were discarded [27].

To calculate the diversity indices, haplotype relative frequencies were obtained across spatial and temporal replicates through simulated datasets created with a Linux shell script. The datasets were built such as that, for each site and each recorded haplotype, the haplotypes frequencies are equal to the haplotype total number of reads (sequence counts) divided by 100. The simulated datasets thus represent hypothetical populations in which absolute abundance (in terms of sequence counts) of each haplotype ideally correspond to the number of individuals carrying the haplotype. As the abundances calculated from eDNA data could be influenced by several limitations (i.e. non-exhaustive sampling in terms of spatial coverage of the site, potential preferential amplification between different haplotypes, potential differences in DNA particles release from different coexisting life stages of the species), these estimates may not be as accurate as more invasive methods. For a more precise estimate of the population genetic diversity, a traditional sampling is still desirable. However, the reproductive behavior of *R. temporaria*, similarly to other pond-breeding amphibians, is characterized by an 'explosive' reproduction, producing large numbers of egg masses sometimes crowded together. Furthermore, in our target species, generally one female lies one egg clutch, therefore the number of egg clutches roughly corresponds to the number of females in the population. Therefore, as eDNA samples were collected when larvae/eggs presence was visually confirmed, and by sampling multiple spatial replicates, the chances of obtaining a sufficiently representative sampling of the population's gene pool were increased.

Standard diversity indices were calculated for each site (number of haplotypes, n; haplotype diversity, h; nucleotide diversity, π) using the software DnaSP v6 [50], and Spearman rank correlations tests were performed using RStudio to compare these estimates with those reported in Marchesini et al. (2017) [41]. Since the estimates of haplotype frequencies and diversity indices from the two methods are not exactly the same (i.e. the tissue based measures were based on the number of individuals in which a haplotype was found, whereas eDNA haplotype frequencies and diversity are based on the number of samples through space in which each haplotype was detected), non-metric multidimensional scaling (NMDS), based on Bray-Curtis dissimilarity matrices and computed using the R package 'vegan' 2.6-4, was used to graphically represent differences between the eDNA metabarcoding and the reference datasets; these two datasets were then statistically compared with a Mantel test with 10000 permutations in RStudio. A correlation between the two measures is expected if eDNA is capable of detecting nearly all haplotypes (since common haplotypes would also be more widespread).

RESULTS

A total of 72 water samples were collected and filtered at the 10 sampling sites. *Rana temporaria* DNA was successfully amplified from 32 of these. T1 replicates were the most successful, with 21/25 eDNA-positive replicate samples, while T2 replicates yielded results in 10/24 replicates (Table 1). T3 replicates did not produce positive results for any sites except Va1 (data not shown). All negative controls from both the extraction and PCR steps were considered not contaminated, as none had more than 10 reads; therefore, these were removed during the OTU clustering step. The spatial replicates showed high variability in terms of amplification success, identified haplotypes and their relative frequencies for each sampling site (Tables 1 and 2).

Ten COI haplotypes (including CA2_TN4 and SA1_Pr7) were detected, all belonging to the three Alpine lineages already known to be present in the province of Trento [41]. The genetic diversity estimates calculated from the eDNA dataset are reported in Table 3. Considering the haplotype detected in all replicates from each site, the number of haplotypes detected with our eDNA protocol showed a strong and statistically significant correlation with the number of haplotypes found in the previous study (R= 0.78, p= 0.008). For eight out of 10 sites, in fact, the number of haplotypes detected by the two approaches was the same. The remaining two sites only differed for one haplotype. Similarly, nucleotide diversity for the eDNA data was strongly correlated with π of the reference dataset (R= 0.88, p= 0.002), as shown in Figure 2. Haplotype diversity (h) from the two datasets (Figure 3) showed a more moderate but statistically significant correlation (R= 0.63, p= 0.05). If only T1 (the temporal replicate with the highest number of positive samples) was considered, the correlation between the number of haplotypes calculated with the two datasets remained unchanged (R= 0.78, p= 0.008), while both h and π estimated from eDNA data showed a slightly higher correlation with the same indices from the reference dataset (R= 0.74, p= 0.014 and R= 0.9, p= 0.001, respectively).

The NMDS plot (shown in Figure 4) showed that, for each sampling site, the eDNA metabarcoding datapoints were closer to the reference datapoints from the same site, than to those of other sites, suggesting a reasonable match between eDNA metabarcoding results and the reference dataset, except for two sites (i.e.: Lel, PMa). Overall, the Mantel test consistently showed a statistically significant correlation between population genetic dissimilarities based on eDNA metabarcoding data and those based on the reference dataset (R=0.44, p= 0.005). In addition, the NMDS plot showed that three sites (Amp, Fia and Va1) cluster closely together with respect to the remaining seven, confirming the previously known distribution of *R. temporaria* haplotypes; that is, two clusters, one in western and one in eastern Trentino [41].

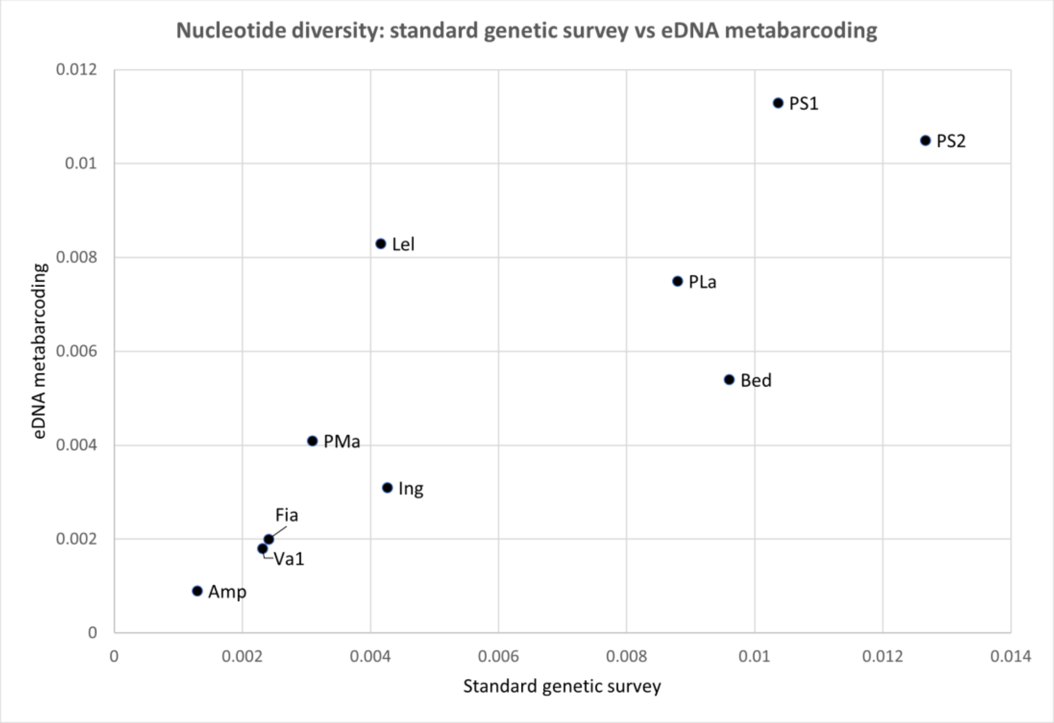

Figure 2. Scatterplot of the correlation between the nucleotide diversity index values for *Rana temporaria* in the Province of Trento Italy, for two datasets: x-axis: standard tissue-based genetic survey [41]; y-axis: current study (eDNA metabarcoding).

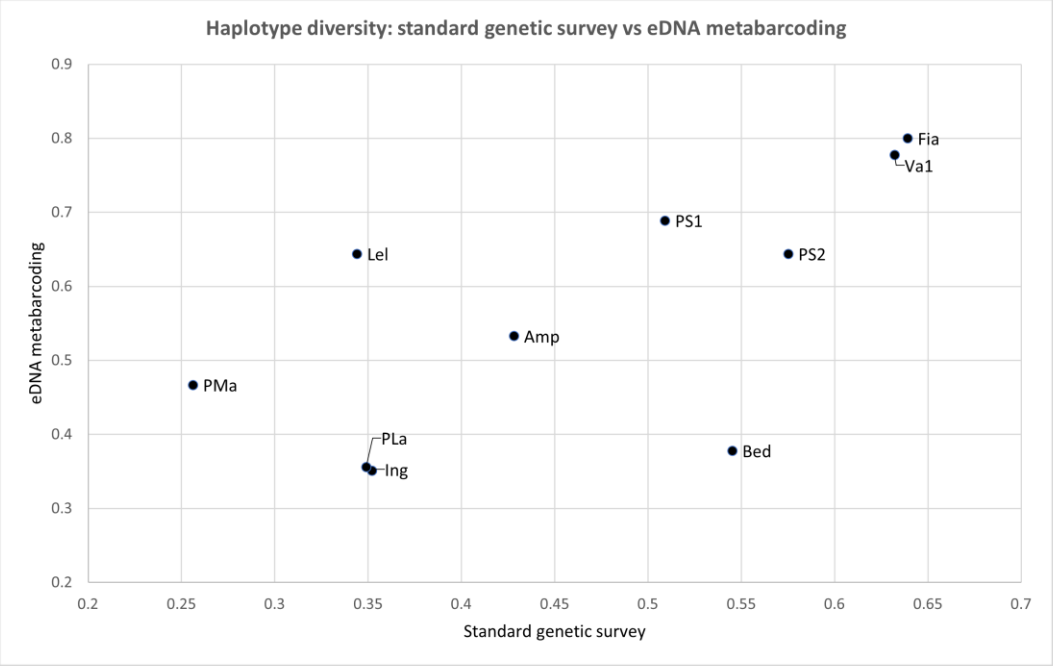

Figure 3. Scatterplot of the correlation between the haplotype diversity index values for *Rana temporaria* in the Province of Trento Italy, for two datasets: x-axis: standard tissue-based genetic survey [41]; y-axis: current study (eDNA metabarcoding).

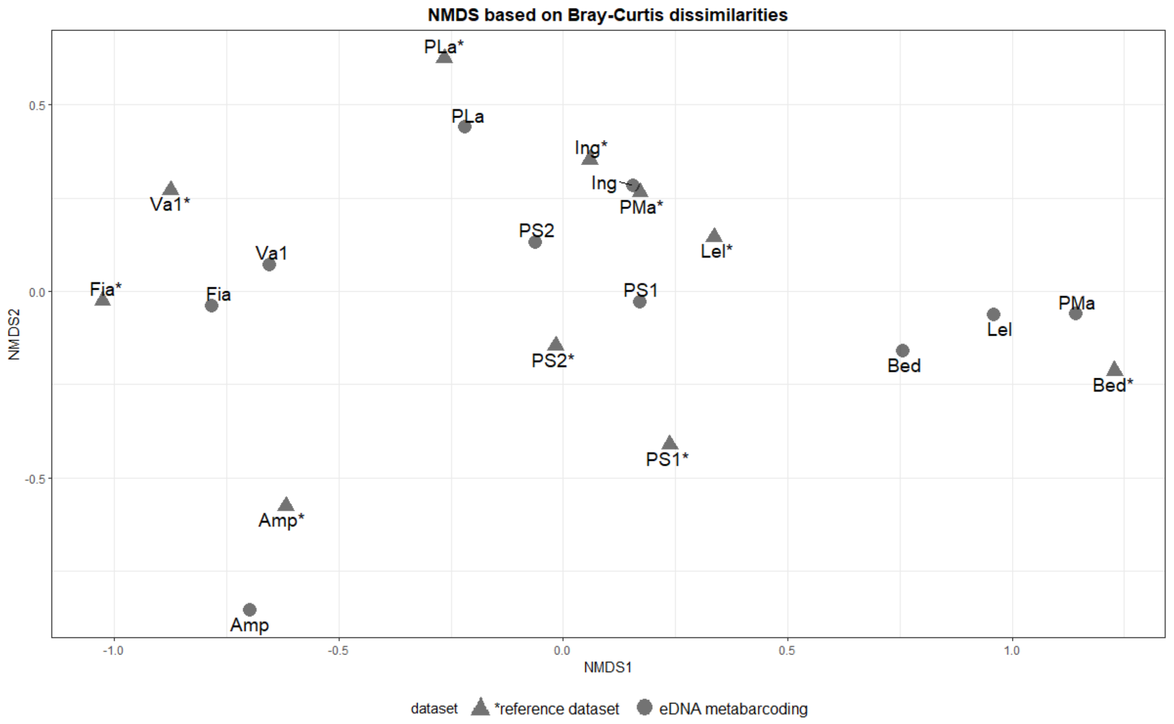

Figure 4. Non-metric multidimensional scaling (NMDS) plot, based on two Bray- Curtis dissimilarity matrices for *Rana temporaria* in the Province of Trento Italy, for two datasets: sites represented by a triangle and labeled with * refer to the standard genetic survey dataset [41]; sites represented with a circle and without * refer to the eDNA metabarcoding dataset.

Table 1. Spatial and temporal replicates included in the eDNA metabarcoding study of *Rana temporaria* in the Province of Trento, Italy. T1, T2, T3= temporal replicates 1, 2, and 3; Repl. T1, Repl. T2, Repl. T3: for every temporal point, the sum of spatial replicates per site is reported (positive/total); S1, S2, S3= spatial replicates 1, 2, and 3; x= sampling was performed and positive results were obtained after sequencing; nd= sampling was performed but amplification was not successful (no DNA bands visible on the Qiaxcel Advanced System (Qiagen) and/or several samples were sequenced for confirmation and no *R. temporaria* DNA was detected); /= sampling was not performed because the structure of the wetland site was not compatible with three spatial replicates (see text for details) or, (for T3) one of the previously selected water microhabitats was no longer physically accessible or non-existent (due to seasonal changes in wetland extent and hydrology).

	T1				**T2**				**T3**				
SITE	**S1**	**S2**	**S3**	**Repl. T1**	**S1**	**S2**	**s3**	**Repl. T2**	**S1**	**S2**	**S3**	**Repl. T3**	**Tot Repl.**
AMP	x	nd	x	2/3	nd	x	x	2/3	nd	nd	nd	0/3	4/9
BED	x	x	/	2/2	x	x	/	2/2	nd	nd	/	0/2	4/6
FIA	x	x	x	3/3	x	x	nd	2/3	nd	nd	nd	0/3	5/9
ING	x	x	nd	2/3	x	nd	nd	1/3	nd	nd	nd	0/3	3/9
LEL	x	nd	/	1/2	nd	/	/	0/1	/	/	/	0/0	1/3
PLA	x	x	x	3/3	nd	nd	nd	0/3	nd	nd	nd	0/3	3/9
PMA	nd	x	x	2/3	nd	nd	nd	0/3	nd	nd	nd	0/3	2/9
PS1	x	x	/	2/2	x	nd	/	1/2	nd	nd	/	0/2	3/6
PS2	x	x	/	2/2	nd	nd	/	0/2	nd	nd	/	0/2	2/6
VA1	x	x	/	2/2	x	x	/	2/2	x	nd	/	1/2	5/6
												TOT	**32/72**

Table 2. COI haplotype frequencies for *Rana temporaria* populations in the Province of Trento detected by eDNA metabarcoding across temporal replicates. For each wetland site, haplotype frequencies (mean of spatial replicates S1-3) detected by eDNA metabarcoding at the different temporal points (T1, T2 and mean T1+T2) are reported, together with available frequencies derived from traditional tissue-based genetic sampling (reference dataset: [41]). nd= no data.

		COI HAPLOTYPES (FREQUENCIES)							
SITE	**Dataset**	**CA2_TN4**	**VC6**	**PR4**	**SA1_PR7**	**TN2**	**TN3**	**TN5**	**MT5**
AMP	eDNA (T1)	0.644	0	0	0	0.74	0	0	0
	eDNA (T2)	0.317	0	0	0	0.68	0	0	0
	eDNA (T1+T2)	0.2907	0	0	0	0.71	0	0	0
	reference dataset	*0.4*	*0*	*0*	*0*	*0.6*	*0*	*0*	*0*
BED	eDNA (T1)	0.2637	0.61	0.122	0	0	0	0	0
	eDNA (T2)	0.2006	0.59	0.209	0	0	0	0	0
	eDNA (T1+T2)	0.2321	0.6	0.166	0	0	0	0	0
	reference dataset	*0.1*	*0.8*	*0.1*	*0*	*0*	*0*	*0*	*0*
FIA	eDNA (T1)	0.3871	0	0	0	0.16	0.43	0.0196	0
	eDNA (T2)	0.4415	0	0	0	0.06	0.49	0	0
	eDNA (T1+T2)	0.4089	0	0	0	0.12	0.46	0.0118	0
	reference dataset	*0.3*	*0*	*0*	*0*	*0.1*	*0.3*	*0.3*	*0*

ING	eDNA (T1)	0.9528	0.05	0	0	0	0	0	0
	eDNA (T2)	0.2345	0.77	0	0	0	0	0	0
	eDNA (T1+T2)	0.7133	0.29	0	0	0	0	0	0
	reference dataset	*0.8*	*0.2*	*0*	*0*	*0*	*0*	*0*	*0*
LEL	eDNA (T1)	0.2203	0.78	0	0	0	0	0	0
	eDNA (T2)	nd	nd	nd	nd	nd	nd	nd	nd
	eDNA (T1+T2)	0.2203	0.78	0	0	0	0	0	0
	reference dataset	*0.5*	*0.4*	*0*	*0.1*[1]	*0*	*0*	*0*	*0*
PLA	eDNA (T1)	0.8031	0	0.134[2]	0.0628	0	0	0	0
	eDNA (T2)	nd	nd	nd	nd	nd	nd	nd	nd
	eDNA (T1+T2)	0.8031	0	0.134[2]	0.0628	0	0	0	0
	reference dataset	*0.8*	*0*	*0*	*0.2*	*0*	*0*	*0*	*0*
PMA	eDNA (T1)	0.1754	0.82	0	0	0	0	0	0
	eDNA (T2)	nd	nd	nd	nd	nd	nd	nd	nd
	eDNA (T1+T2)	0.1754	0.82	0	0	0	0	0	0
	reference dataset	*0.7*	*0.3*	*0*	*0*	*0*	*0*	*0*	*0*
PS1	eDNA (T1)	0.3936	0.35	0.26	0	0	0	0	0
	eDNA (T2)	0.7635	0	0.237	0	0	0	0	0
	eDNA (T1+T2)	0.5169	0.23	0.252	0	0	0	0	0
	reference dataset	*0.3*	*0.2*	*0.5*	*0*	*0*	*0*	*0*	*0*
PS2	eDNA (T1)	0.6715	0.09	0.238	0	0	0	0	0
	eDNA (T2)	nd	nd	nd	nd	nd	nd	nd	nd
	eDNA (T1+T2)	0.6715	0.09	0.238	0	0	0	0	0
	reference dataset	*0.5*	*0.1*	*0.4*	*0*	*0*	*0*	*0*	*0*
VA1	eDNA (T1)	0.5235	0	0	0	0.24	0.19	0.0373[2]	0
	eDNA (T2)	0.4887	0	0	0	0.1	0.25	0.1544[2]	0
	eDNA (T1+T2)	0.5061	0	0	0	0.17	0.22	0.0959[2]	0
	reference dataset	*0.4*	*0*	*0*	*0*	*0.1*	*0.2*	*0*	*0.3*[1]

[1]= undetected by eDNA metabarcoding, but present in the reference dataset [41]; [2]= detected by eDNA metabarcoding but not in the reference dataset [41].

Table 3. Genetic diversity estimates for *Rana temporaria* populations in the Province of Trento: results of eDNA metabarcoding from the present study compared to the reference dataset [41]. eDNA (T1): eDNA metabarcoding considering only T1 replicates; eDNA (T1+T2): eDNA metabarcoding considering T1+T2 replicates; tissue DNA: estimates from the reference dataset [41].

	NO HAPLOTYPES (N)			**HAPLOTYPE DIV. (H)**			**NUCLEOTIDE DIV. (PI)**		
SITE	**eDNA (T1)**	**eDNA (T1+T2)**	**tissue DNA**	**eDNA (T1)**	**eDNA (T1+T2)**	**DNA**	**eDNA (T1)**	**eDNA (T1+T2)**	**tissue DNA**
AMP	2	2	2	0.368	0.428	0.533	0.001	0.001	9E-04
BED	3	3	3	0.543	0.545	0.378	0.009	0.01	0.005
FIA	4	4	4	0.644	0.639	0.8	0.002	0.002	0.002
ING	2	2	2	0.121	0.352	0.351	0.001	0.004	0.003
LEL	2	2	3	0.344	0.344	0.644	0.004	0.004	0.008
PLA	3	3	2	0.349	0.349	0.356	0.009	0.009	0.008
PMA	2	2	2	0.256	0.256	0.467	0.003	0.003	0.004
PS1	3	3	3	0.533	0.509	0.689	0.011	0.01	0.011
PS2	3	3	3	0.575	0.575	0.644	0.013	0.013	0.011
VA1	4	4	4	0.601	0.632	0.778	0.002	0.002	0.002

DISCUSSION

In this study, all statistical tests showed that our eDNA metabarcoding protocol targeting a 331 bp long fragment of the COI region could identify haplotypes of *R. temporaria* previously identified from tissue samples, most reliably in the spring season. In addition, our protocol allowed us, for the first time, to provide an estimation of standard genetic diversity indexes for an amphibian species from eDNA.

Our experimental design allowed us to identify an optimal sampling regime, in terms of temporal and spatial replicates, for the collection of eDNA water samples in a variety of small wetland habitats. Our results are in agreement with the recommendation from previous studies [51, 52] of sampling an adequate number of spatial replicates per site (up to one per 10-20 m perimeter), as this distribution of replicates allowed us to capture the full set of expected haplotypes from each sampling site. Regarding the temporal replicates, T1 alone allowed us to identify almost all haplotypes present per site in a given monitoring year. In fact, T1 yielded the same results, in terms of number of haplotypes identified, as those attained from the union of temporal replicates 1 and 2 (while no T3 replicates yielded target species DNA). Thus, if a multi-year monitoring program is being considered, we suggest implementing a simplified version of our protocol, with T1 as the optimal time point, as a strategy to maximize the cost/benefit ratio. However, as the combination of T1 and T2 replicates provided the most accurate data on haplotypes frequencies, for a more complete view of *R. temporaria* genetic diversity we propose that the protocol should be applied at least every three to five years. Replicate T3 did not provide any information on species presence and genetic diversity, and should not be considered for future eDNA protocol applications for amphibians that have a reproductive cycle similar to that of *R. temporaria*.

All the standard genetic diversity estimates computed here (namely, the number of haplotypes, h and π) proved to be statistically correlated with the same indices computed in the reference dataset from Marchesini et al. (2017) [41]. In agreement with the NMDS plot (Figure 4), the Mantel test shows a moderate, statistically significant, correlation between the dissimilarities computed based on eDNA metabarcoding data and those computed based on the reference dataset. Moreover, the NMDS plot based on eDNA data further confirms previous genetic distributions of *R. temporaria* haplotypes [41], identifying the same two geographical clusters formed by the three sites Amp, Fia and Va1 (western Trentino) and the remaining seven (eastern Trentino).

Only three of the 10 sampling sites consistently showed different haplotype counts and frequencies between the two approaches, namely sites Lel, PLa and PMa (Laghestel, Passo Lavazé and Passo Manghen; Table 2 and Table S1 in Supplementary Information). Interestingly, in these sites, the target species is known to breed in small temporary ponds near a bigger pond (Lel), or in small alpine lakes (PLa, PMa) [53]. These habitats are especially prone to seasonal fluctuations in water availability; therefore, it is possible that the differences in the haplotypes detected with the two methods, as well as in their relative frequencies, might be due to the ephemeral nature and repeated recolonization of these three sampling areas. In fact, the influence of short-term climatic fluctuations on demographic and genetic characteristics of wild populations has been demonstrated recently [54]. Even so, the eDNA protocol was able to detect two haplotypes out of the three found at Lel in the reference dataset. In the case of PLa, the most abundant haplotype shows the same relative frequency according to both datasets, and our protocol was also able to detect the less common haplotype. In addition, our eDNA metabarcoding approach detected another haplotype, not previously reported for this site but known to be present in eastern Trentino (PR4, see Table 2). For PMa, the eDNA protocol identified the same haplotypes as the reference dataset, but frequencies were reversed (see Table 2). Finally, the differences between the two datasets could also be explained by fluctuations of allele frequencies over time (with samples collected in 2017 and 2021, respectively), and considering that the reference dataset did not necessarily represent the entire gene pool of the considered populations, being based on only 10 samples per site.

The protocol developed here, which is completely non-invasive and less time-consuming than traditional observational or tissue-based genetic surveys, could be implemented routinely in amphibian monitoring programs that integrate genetic diversity estimates with confirmation of the target species presence. We are currently improving and extending this holistic approach to all European amphibian species, but also to even rarer and more elusive invertebrates such as the European freshwater crayfish *Austropotamobius pallipes*. The same approach could also be used to determine the presence of invasive species, such as the American bullfrog *Lithobates catesbeianus*, which occurs in the regions surrounding our study site, but has not been reported thus far in the Province of Trento. Finally, we advocate the integration of nucleotide diversity estimates in eDNA-based approaches, included in very few studies thus far (e.g., [28, 29], in order to obtain a detailed reconstruction of the potential adaptability of populations living in human- and/or climate change-impacted areas.

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AUTHOR CONTRIBUTIONS

Conceived and designed the study: L.Z., G.M., A.M., P.P, H.C.H.; acquired the funding: G.M., P.P, G.B., H.C.H.; collected the samples: L.Z., G.M., S.C., S.E., C.F.; performed laboratory analyses: L.Z., G.M., S.C.; completed bioinformatic and statistical analyses: L.Z., A.M., G.G., D.M; drafted the manuscript: L.Z., A.M., H.C.H; read, commented and approved the manuscript: all authors.

DATA AVAILABILITY STATEMENT.

Sequences available upon acceptance for publication of the manuscript or upon request. Correspondence and requests for materials should be addressed to H.C.H. (email: heidi.hauffe@fmach.it).

COMPETING INTERESTS

The authors declare no competing interests.

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SUPPLEMENTARY INFORMATION

Table S1. Sampling sites with mean geographic coordinates (UTM 32N; units in m), mean elevation (m a.s.l.) and wetland type.

SAMPLE SITE	ABBREV	EAST/LONG	WEST/LAT	ELEVATION (M A.S.L.)	SITE DESCRIPTION
LAGO D'AMPOLA	Amp	628457	5081277	795	small alpine lake with surrounding marshes
BEDOLLO	Bed	679355	5116467	1183	small alpine lake with surrounding marshes
TORBIERA DI FIAVÉ	Fia	641894	5094567	665	peatbog with small ponds and surrounding marshes
INGHIAIE	Ing	678339	5096436	444	peatbog with small ponds
LAGHESTEL	Lel	671966	5109197	876	peatbog with small ponds
PASSO LAVAZÉ	PLa	691672	5136691	1802	peatbog with small seasonal ponds
PASSO MANGHEN	PMa	689343	5116779	2083	small alpine lake with surrounding peatbogs
PASSO S. PELLEGRINO 1	PS1	712169	5139382	1838	peatbog with seasonal ponds
PASSO S. PELLEGRINO 2	PS2	714246	5139634	1940	small alpine lake and surrounding peatbogs
VALAGOLA	Va1	640550	5113989	1689	peatbog with seasonal ponds
LOMASONA	Lom	644431	5093917	510	peatbog with seasonal ponds

Table S2. *Rana temporaria* life stages and additional observations during sampling. For each spatial (S repl.) and temporal (T repl.) replicate, the presence of *R. temporaria* was recorded for the following life stages: freshly laid egg clutches (E1); egg clutches, hatching (E2); larvae, initial stages (lL1); larvae, intermediate and final stages (L2); neometamorphs (N); adults (A). For each replicate, the corresponding outcome of eDNA metabarcoding is also reported (eDNA outcome; no = no *R. temporaria* DNA was amplified; /= the replicate was not collected). Additional observations on changes in site extent due to water loss are reported as footnotes.

				***R. TEMPORARIA* STAGES**					
SITE	**T repl.**	**S repl.**	**eDNA outcome**	**E1**	**E2**	**L1**	**L2**	**N**	**A**
AMP	T1	S1	yes			x			
AMP	T1	S2	no						
AMP	T1	S3	yes	x	x	x			
AMP[A]	T2	S1	no						
AMP	T2	S2	yes				x		
AMP	T2	S3	yes				x		
BED	T1	S1	yes	x					
BED	T1	S2	yes	x					
BED	T2	S1	yes				x	x	
BED	T2	S2	yes				x	x	
FIA	T1	S1	yes	x					
FIA	T1	S2	yes	x					
FIA	T1	S3	yes	x					
FIA	T2	S1	yes			x	x		
FIA	T2	S2	yes			x	x		
FIA	T2	S3	no				x		
ING	T1	S1	yes		x	x			
ING	T1	S2	yes		x	x			
ING	T1	S3	no						
ING	T2	S1	yes				x		
ING	T2	S2	no						
ING[A]	T2	S3	no						
LEL	T1	S1	yes	x[b]					
LEL	T1	S2	no		x[b]	x[b]			
LEL	T2	S1	yes				x[b]		
LEL	T2	S2[c]	/						
PLA	T1	S1	yes				x		
PLA	T1	S2	yes						x
PLA	T1	S3	yes				x		
PLA	T2	S1	no						
PLA[A]	T2	S2	no						
PLA[C]	T2	S3	no						
PMA	T1	S1	no						x
PMA	T1	S2	yes				x		x
PMA	T1	S3	yes				x		
PMAA	T2	S1	no						
PMA	T2	S2	no					x	
PMA	T2	S3	no						
PS1	T1	S1	yes			x			x
PS1	T1	S2	yes			x			x
PS1	T2	S1	yes				x	x	

PS1	T2	S2	no						
PS2	T1	S1	yes		x	x			
PS2	T1	S2	yes		x	x			
PS2	T2	S1	no					x	
PS2	T2	S2	no						
VA1	T1	S1	yes	x					x
VA1	T1	S2	yes	x	x				
VA1	T2	S1	yes				x		
VA1	T2	S2	yes					x	
VA1	T3	S1	yes						

[a] site partially dried up

[b] live stages potentially *R. dalmatina*

[c] site completely dried up.

SONG OF THE SLENDER SALAMANDER

Lucille Day

He came out from under a log
on a clear winter night.
I was looking for worms
in soft earth, after a long rain.

He was so skinny and slithery
I thought he was a snake
until I saw his little feet.
I knew something was up

when he began to prod
and rub me. It felt so good
that I straddled his tail.
He moved forward, dropping

something shiny on the earth.
I retrieved it, and worlds
congealed in my body-
tiny planets, silken stones.

When the star tulip turns
the hillside purple,
I'll crawl beneath a rock,
crouch in the dark, and let go.

CHAPTER 3

VALIDATION OF AN eDNA-BASED WORKFLOW FOR MONITORING INTER- AND INTRA-SPECIFIC CYTB HAPLOTYPE DIVERSITY OF ALPINE AMPHIBIANS

STATUS: ACCEPTED

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ABSTRACT

Environmental DNA (eDNA) analysis is a promising tool for monitoring wild animal populations, and more recently, their genetic variability. In the present study, we used the mitochondrial Cytochrome B gene to develop and apply new eDNA metabarcoding assays targeting amphibian families and genera in order to estimate both inter- and intraspecific genetic diversity. We designed and tested seven new primer pairs a) *in silico* against an amphibian reference database based on the target genera; b) *in vitro* on tissue samples of the target species; and c) *in situ* on water samples from 38 wetlands in the Province of Trento (Italy). Overall, most target species were amplified successfully, although some markers also amplified non-target amphibian species. In addition, to complete the workflow, we also compared the performance of three different bioinformatic pipelines (namely, MICCA with VSEARCH, and OBITools using ecotag or metabinkit), in retrieving reads and exact sequence variants from the metabarcoding datasets. Overall, the MICCA based pipeline retrieved overall more reads, but less putative haplotypes of amphibians. After comparing these sequences with previously known haplotypes from tissue-based studies, when the aim is to both decrease the probability of detecting false haplotypes and retrieve the highest number of reads, we suggest using MICCA+VSEARCH, unless a direct comparison with tissue-based genetic data is possible.

KEYWORDS: environmental DNA, eDNA, metabarcoding, bioinformatics, genetic diversity, amphibians, freshwater, pipelines

INTRODUCTION

With 41% of known species assessed as ‘threatened’ by the IUCN (IUCN, 2022), amphibians are currently the most endangered vertebrate taxon on the planet (Hoffmann et al., 2010). Precise estimates of the abundances and distributions of amphibians are often difficult and time consuming to obtain, due to the cryptic lifestyle and small size of adults, but also to the challenges of detecting and correctly identifying their early life stages in secluded and season-dependent aquatic environments (Barata et al., 2017; Ficetola et al., 2019).

In the last 15 years, environmental DNA (eDNA) approaches have been increasingly used for accurate and cost-efficient detection of wetland-dependent amphibian species (see Ficetola et al., 2019, and references therein). Most studies have focused on monitoring single species, showing the potential for eDNA surveys to increase the speed and efficiency of amphibian detection in comparison to traditional observational methods (Fediajevaite et al., 2021; Moss et al., 2022; Zanovello et al., 2023). The eDNA metabarcoding approach is being applied as a cost-effective method for the assessment of species composition of amphibian communities, although its performance compared to traditional approaches is still debated (Cristescu & Hebert, 2018; Ficetola et al., 2019; Moss et al., 2022; Svenningsen et al., 2022).

More recently, the potential of eDNA to estimate the genetic diversity within-species has also been investigated (Elbrecht et al., 2018; Sigsgaard et al., 2020; Andres et al., 2023 and references therein), motivated by the crucial role that genetic diversity plays in the local adaptation and persistence of natural populations in the face of environmental and climatic changes (Höglund, 2009; Hoban et al., 2013). Amphibian species are particularly vulnerable to genetic erosion due to their small effective population sizes and low dispersal rates (Allentoft & O'Brien, 2010). In addition, many amphibian species in the Mediterranean area reproduce in small temporary ponds and streams, which are highly fragmented habitats where land use changes and frequent droughts (Nadin, 2008) favor isolation and reduce gene flow and colonization potential.

Given the vulnerabilities listed above, effective monitoring protocols that aim at protecting wild amphibian species should also include the possibility of gathering population data on genetic diversity and gene flow. However, only a few articles have been published thus far on the application of eDNA approaches for the study of amphibian mitochondrial lineages (Gorički et al., 2017; Wang et al., 2022), and, to our knowledge, only one has aimed at estimating population-level genetic diversity (Zanovello et al., 2023).

Here we 1) developed a family- to genus-specific eDNA metabarcoding assays to estimate both interspecific and intraspecific genetic diversity of Alpine amphibians, and 2) evaluated the efficiency of three different bioinformatic pipelines in filtering non-target DNA from the dataset while retaining amphibian sequence variants.

Most primers developed for eDNA can be divided into two categories: single-species targeting primers, in some cases also designed for capturing interspecific variability (Klymus et al., 2020; Adams et al., 2022; Zanovello et al., 2023), and multi-species primers for metabarcoding studies (e.g., Zhang et al., 2020; Hu et al., 2022). However, as argued by Vences et al. (2012), single markers might have a significant failure rate (5 to 50%) when targeting wide taxonomic ranges. Using the mitochondrial gene Cytochrome B, we designed seven new primer pairs, and their performance and specificity were assessed a) *in silico* against an amphibian reference database that included all species from the target genera;

b) *in vitro* using DNA extracts from tissue samples of most target species; and c) *in situ* using water samples collected at 38 freshwater sites in the Province of Trento, Italy.

The second part of this study was motivated by the need to better understand the efficiency of various pipelines for processing eDNA metabarcoding data, in particular when intraspecific diversity is analyzed. Several tools are available (e.g., Elbrecht et al al., 2018; Mousavi-Derazmahalleh et al., 2021; Wahlberg, 2019; Yoshitake et al., 2021), and a few comparisons among methods do exist (Flück et al., 2022; Straub et al., 2020), but little is known about the impact of parameter choice (Scott et al., 2018; Antich et al., 2021). Here we aimed to provide additional evidence useful for selecting the appropriate bioinformatic approach in further studies.

MATERIALS AND METHODS

Study species

Our study focused on 12 amphibian species with resident breeding populations in the Province of Trento (Italy), which include seven Anurans and five Urodeles (Caldonazzi et al., 2002).

Anurans include *Rana temporaria* (Linneaus, 1758), *Rana dalmatina* (Fitzinger, 1839), *Bombina variegata* (Linneaus, 1758), *Bufo bufo* (Linnaeus, 1758), *Bufotes viridis* (Laurenti, 1768), and the pool frog, a species complex (*Pelophylax* kl. *esculentus* Linnaeus, 1758) that includes *Pelophylax lessonae* (Camerano, 1882) and *Pelophylax ridibundus* (Pallas, 1771) and their hybridogenetic form *P.* kl. *esculentus*. Since *P. lessonae* and the hybridogenetic form are sympatric, almost indistinguishable morphologically (Bovero et al., 2014; Lanza et al. 2009), and traditionally referred to as a complex, in the present paper they are considered as such, following the classification proposed in Caldonazzi et al. (2002). Lastly, here we referred to the treefrog populations in the study area as *Hyla intermedia* (Boulenger, 1882), although some authors have proposed the status of species for this taxon (*H. perrini*; Dufresnes et al., 2018).

The Urodeles found in the Province of Trento include *Salamandra salamandra* (Linnaeus, 1758), *Salamandra atra* (Laurenti, 1768), and *Ichthyosaura alpestris* (Laurenti, 1768). In addition, *Lissotriton vulgaris* (Linnaeus, 1758) occupies the southern part of the Province, while *Triturus carnifex* (Laurenti, 1768) is localized in the area surrounding the 'Laghetti di Marco' (Caldonazzi et al., 2002).

Primer design and adequacy tests

Cytochrome B (hereafter, CytB) was chosen as our 'barcode' region as it presents a good trade-off between identifying conserved regions for primer binding, and the potential to provide information on both species and population genetic diversity, as well as phylogeographic patterns. CytB is also one of the most frequently used genes for amphibian metabarcoding studies, especially those that target both Anuran and Urodeles species (e.g., Cannon et al., 2016; Goldberg et al., 2011; Thomsen et al., 2012). Other commonly used markers such as 12S (Valentini et al., 2016) could have been adequate for species identification as well, but lack the variability needed for an appropriate intraspecific diversity assessment. Moreover, considering the five major vertebrate taxa, CytB has more records in GenBank compared to other widely used markers such as Cytochrome Oxidase subunit I or 12S (van den Burg et al., 2020). Lastly, an online, open-access database is available for CytB curation, with a deployed script for updates (van den Burg et al., 2020).

Seven primer pairs were designed using Primer3Plus (Untergasser et al., 2012) with sequences downloaded from the ACDC Database (van den Burg et al., 2020), except for the forward primer of the pair 'Bufo' which is a modification of the primer Cyt Bufo F developed by Recuero et al. (2012). The resulting primer pairs amplify fragments of variable length, the shortest being 277 base pairs (hereafter, bp) long, and the longest 379 bp; they also target different amphibian families, subfamilies or genera, as reported in Table 1. The relative position of these markers with respect to the CytB gene of one of the target species (*R. temporaria*) is shown in Supplementary Materials, Figure S1.

Table 1. Summary of primer pair characteristics.

MARKER NAME	FRAG. LENGTH (BP)	FAMILY/ SUBFAMILY/ GENUS	TARGET SPECIES	REFERENCE	FORWARD PRIMER	REVERSE PRIMER
BOMB	379	Bombinatoridae	*B. variegata*	This study	AAYGGAGCCTCYTTCTTCTT	AAGTABGGGTGAAATGGRAT
BUFO	356	Bufonidae	*B. bufo,* *B. viridis*	Recuero et al. 2012, modified	ATCTAYCYWCACATTGGACGAG	TTATAGGARTAGTARGCRTGGAA
HYLA	332	Hylidae	*H. intermedia*	This study	AACGGRGCCTCATTYTTYT	GGRTTRGAHGAGCCDGTTTG
PELOP	277	*Pelophylax*	*P. kl. esculentus*	This study	ATCGCMACMGGYYTATWY	AGGTTRGTRATTACWGTRG
RANA	305	*Rana*	*R. temporaria,* *R. dalmatina*	This study	ACTAAYCTYCTCTCMGCCG	GRTTRGCTGGYGTRAARTT
SALA	310	Salamandrinae	*S. salamandra,* *S. atra*	This study	CCVTCAAAYATYTCHTAYTG	AADGAYATTTGTCCTCAWGG
TRIT	325	Pleurodelinae	*I. alpestris,* *L. vulgaris,* *T. carnifex*	This study	TGAGGRCAAATRTCVTTYTG	AAGTTTTCTGGRTCYCCRAG

The primer pairs were tested *in silico* with ecoPCR (Ficetola et al., 2010). Firstly, we used the RScript published by van den Burg et al. (2020) to create a database of CytB sequences for all genera of the target species (namely, *Bombina, Bufo, Bufotes, Hyla, Ichthyosaura, Lissotriton, Pelophylax, Rana, Salamandra* and *Triturus*) available in NCBI (download at 2023/01/18). The FASTA sequences were converted in an ecoPCR database format, and the two available CytB sequences of *H. perrini* (Dufresnes et al., 2018), which currently does not have a NCBI taxid entry, were added with the command *obitaxonomy* using the most recent common ancestor taxid (*Hyla*, thus 8421). An *in silico* PCR was run for each primer pair using default parameters (i.e. allowed number of mismatches equal to zero), and their taxonomic specificity and resolution were measured with a custom script in R.

The primers were then tested *in vitro* on DNA extracted from tissue samples of the target species available at the Fondazione E. Mach from previous studies (*B. variegata, B. viridis, I. alpestris, R. temporaria, R. dalmatina,* and *S. atra*) or collected from museum specimens (*H. intermedia* from the Civic Natural History Museum of Morbegno, and *P. kl. esculentus* from MUSE - Science Museum of Trento) preserved in 70% ethanol, and amplification success was confirmed by screening on a Qiaxcel Advanced System (Qiagen, Germany). For four of the species living in the study area, namely *B. bufo*, *L. vulgaris*, *S. salamandra* and *T. carnifex*, tissue samples were not available, and the test was not possible. Nonetheless, all the primer pairs were tested on at least one target species, as well as on the other species available. The final amplification mixture and PCR program for each primer pair is reported in Table S1. The amplification products were purified following the ExoSAP protocol (Thermo Fisher Scientific, USA) sequenced on an ABI 3130xl Sequencer (Thermo Fisher Scientific, USA), analyzed with Sequencher 5.4.6 (DNA sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA) and assigned to the corresponding taxonomy with BLAST (Altschul et al., 1990) to ensure the correct amplification of the target sequence.

Table 2. Results of primer tests *in silico*, *in vitro* and *in situ*: x: cases in which the marker was positive for the species; *: percentage *in silico* test below 100%; n/a test not possible. For the *in situ* test, numbers correspond to the pipeline that detected the species: 1: MV, 2: OE, 3: OM, otherwise all pipelines detected the species for the specific marker.

	SPECIES & MARKERS	***B. variegata***	***B. bufo***	***B. viridis***	***H. intermedia***	***I. alpestris***	***L. vulgaris***	***P. kl. esculentus***	***R. dalmatina***	***R. temporaria***	***S. atra***	***S. salamandra***	***T. carnifex***
IN SILICO	**Bomb**	x*											
	Bufo		n/a	x*									
	Hyla				x*								
	Pelop							x*		x			
	Rana								x	x			
	Sala						x				x	x	
	Trit					n/a					x	x	x
IN VITRO	**Bomb**	x	n/a				n/a				x	n/a	n/a
	Bufo		n/a	x			n/a					n/a	n/a
	Hyla		n/a	x	x		n/a	x	x	x		n/a	n/a
	Pelop		n/a	x	x		n/a	x	x	x		n/a	n/a

IN SITU									
Trit	Sala	Rana	Pelop	Hyla	Bufo	Bomb	Trit	Sala	Rana
						x	x		
				x 3	x		n/a	n/a	n/a
									x
									x
x	x						x	x	
	x 1, 2						n/a	n/a	n/a
			x				x		x
		x	x				x		x
x	x 2, 3	x	x	x 3		x 2,3	x	x	x
	x						x	x	x
x	x						n/a	n/a	n/a
	x		x 3				n/a	n/a	n/a

For *in situ* testing, water samples were collected during the peak reproductive period for amphibians (March – July, 2021) from 38 wetland sites in the Autonomous Province of Trento (Italy) in the eastern Alps. At each site, two to four spatial replicates were collected, depending on the wetland surface area, to capture as much taxonomic and genetic diversity as possible. Each replicate was sampled manually from just under the water surface using a sterile plastic canister, to avoid stirring up the sediment. The sampling protocol followed Zanovello et al. (2023). Briefly, the water collected in the canister was drawn up with a 100 ml syringe and filtered through two Sterivex GP Filter units (pore size 0.22 µm, Millipore cat. no. SVGPL10RC) until the filters clogged (40 ml to 800 ml). All filters were kept at ambient temperature for transport to the Fondazione E. Mach (FEM) the same day, and stored at -20°C until DNA extraction. All laboratory procedures were performed under BSL2 biological hoods at the Animal, Environmental and Antique DNA Platform at FEM, and followed recommended guidelines for eDNA analyses (Goldberg et al., 2015; Harper et al., 2019). DNA extraction was performed using the DNeasy PowerWater Sterivex Kit (Qiagen, Germany), following the manufacturer's instructions with minor modifications, as reported in Zanovello et al. (2023). The two filters corresponding to the same spatial replicate were processed simultaneously and their extracts were merged into a single tube at the final step of the protocol. DNA extraction involved batches of a maximum of 12 water replicate samples, including one negative control (extraction blank) for each batch. All extracts and extraction blanks were PCR amplified with each primer pair according to the respective amplification mixture and PCR program (see Table S1). The amplification success of the samples was confirmed via screening on a Qiaxcel Advanced System (Qiagen). One PCR was run per replicate, and a second PCR was run only if the first one showed no amplification bands. One negative control (PCR blank) was also included for each PCR reaction. All successful amplification products along with 8 negative controls that showed signs of contamination on the Qiaxcel, and 12 more, randomly chosen, negative controls were then purified with the MinElute PCR Purification Kit (Qiagen) following manufacturer's instructions. Each purified product was sequenced at the FEM Sequencing and Genotyping Platform using paired-end sequencing (2 × 300 bp) on an Illumina Miseq (Illumina, San Diego, CA) with a 30 000 bp coverage.

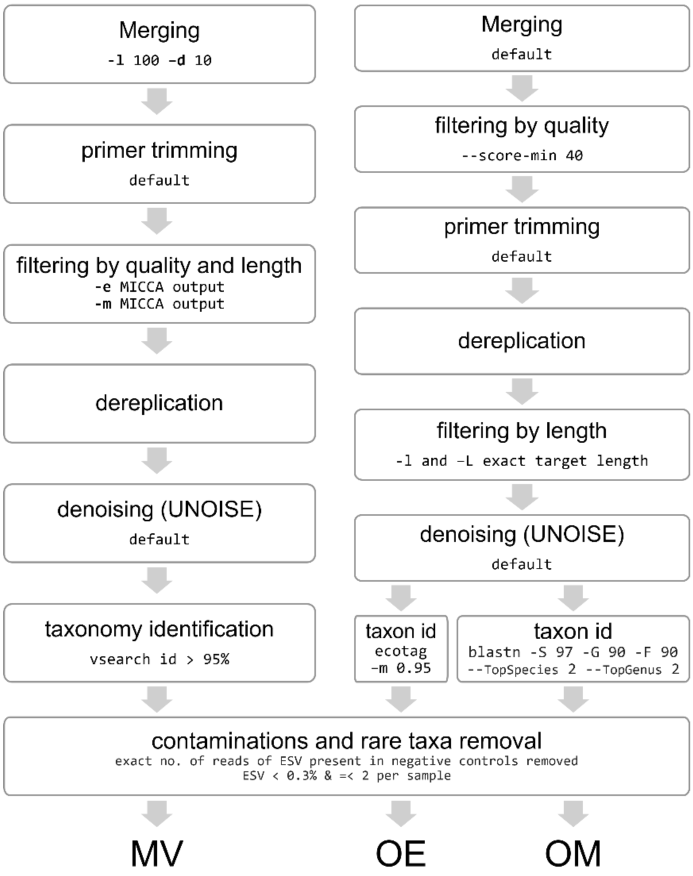

Figure 1. Summary of the bioinformatic steps and parameters for each pipeline: MV: MICCA+VSEARCH, OE: OBITools+ecotag, OM: OBITools+metabinkit.

Bioinformatic workflows design and application

Two bioinformatic tools were applied for processing raw sequence files, MICCA (Albanese et al., 2015) and OBITools (Boyer et al., 2016), and three tools for taxonomic assignment, VSEARCH (Rognes et al., 2016), ecotag (Boyer et al., 2016) or metabinkit (Fonseca & Egeter, 2023), resulting in three different bioinformatic pipelines (MICCA+VSEARCH, OBITools+ecotag and OBITools+metabinkit, hereafter, MV, OE and OM pipelines). A summary of pipeline steps and parameters can be found in Figure 1. Both MICCA and OBITools have previously been used in eDNA metabarcoding studies (e.g., Lopes et al., 2017, Li et al., 2021; Valentini et al., 2016; Zanovello et al., 2023). The VSEARCH-based consensus classifier implemented in MICCA searches the database for each query sequence and retrieves up to a user-defined number of hits above the given identity threshold. Then, VSEARCH assigns to each query the most specific taxonomic label that is associated with at least a user-defined number of the hits. In the case of metabinkit, firstly the query sequences are aligned to the reference database. For each query the alignments are then filtered based on the defined percentage identity thresholds, and the lowest common ancestor is determined for all alignments passing the filters, thus determining the assigned taxon. Ecotag first searches the database for the reference sequence(s) with the highest similarity to the query, then retrieves all other reference sequences whose similarity to the first reference sequence(s) is equal to or greater than the similarity between the first reference and the query. Finally, it assigns the query sequence to the first taxa found in common between the first and second reference sequences, going back to higher taxonomic levels if needed. More details on VSEARCH, ecotag, and metabinkit classifier algorithms can be found in their respective manuals (Rognes et al., 2016, Boyer et al., 2016, Fonseca & Egeter, 2023).

At the merging step of the pipeline MV, sequences were aligned only if overlapping for at least 100 bp and with no more than 10 mismatches within the overlapping region. In the OBITools pipelines (OE and OM) sequences were not merged if the alignment score was below 40. After the primer trimming step, MICCA gives the option to filter the sequences according to a maximum allowed expected error (EE) rate % and minimum length based on the expected target fragment characteristics, while OBITools allows both minimum and maximum expected length for filtering to be set. All pipelines had a denoising step performed with UNOISE3 (Edgar, 2016), an algorithm that preserves intra-cluster variability

(Antich et al., 2021) and included the removal of chimeric fragments based on the default abundance skew (16). The sequences were not clustered to avoid losing information on exact sequence variants (hereafter, ESVs), and thereby potentially true haplotypes (Callahan et al., 2017; Porter & Hajibabaei, 2020; Antich et al., 2021). For taxonomic identification, sequences resulting from MICCA were assigned with VSEARCH using a similarity percentage threshold of 95%. Those from OBITools were classified with ecotag also using the default threshold of 95%, whereas with the package metabinkit a 97% blast identity was used as a threshold for species determination and 90% for genus and family, allowing a range of 2% above and below the threshold to build a consensus. The reference database used for taxonomic assignments corresponded to that used for the *in silico* testing of marker performance. The final step, removal of contaminations based on negative controls and rare taxa, was common to all pipelines and was performed using a custom R script (R Core Team, 2023; see Figure 1 for parameters used). The number of reads and/or ESVs retained after each bioinformatic pipeline processing is shown in Table S2. For each pipeline and each marker, we calculated the proportion of reads and ESVs assigned to the target amphibian species, non-target amphibian species, and unassigned ESVs.

RESULTS AND DISCUSSION

In silico, in vitro *and* in situ *primer evaluation*

Despite the presence of degenerated nucleotides, the *in silico* PCR showed that the majority of the primer pairs designed here amplify, among amphibians of the considered genera, only species from the target taxa, except for the markers Pelop, Sala and Trit. In fact, Pelop appears to bind to several species of the Ranidae family, thus it could in principle be applied in monitoring studies targeting the whole family. The last two markers, Sala and Trit, are both likely to work well for species of the genus *Salamandra* and also amplify some Pleurodelinae species. Since the reference database did not include all European species of amphibians, it is also possible that the primers are useful for detecting additional species.

Regarding their taxonomic resolution, for five out of 12 amphibian species living in the Province of Trento, the respective marker allowed the correct taxonomic assignment of all available sequences, as shown in Table 2. The sequences of an additional species, *L. vulgaris*, were consistently identified by the marker Sala (and not by Trit). The sequences of

R. temporaria were correctly identified by both Pelop and Rana markers, as occurred for the two *Salamandra* species with marker Trit. The sequences of *B. variegata* were correctly classified in 97%, those of *B. viridis* in 94% and *H. intermedia* in 96% of cases. In these cases, we argue that misassignment of the original sequences available in the GenBank database could explain the presence of incorrectly identified sequences (e.g., Bagheri et al., 2020; Schnoes et al., 2009). The genus *Pelophylax* had the lowest score in terms of resolution (*P. lessonae* 67%, *P. ridibundus* 50%). These species might represent a special case, as most of these (and their hybrids) are known to be almost indistinguishable morphologically (Di Nicola et al., 2019), thus possibly leading to taxonomic assignment issues concerning the original specimens that were used for evaluation of the primers. Moreover, it should be noted that for some *Pelophylax* taxa the status of species or subspecies is still debated (e.g., *P. kurtmuelleri*, see Di Nicola et al., 2019). Lastly, for two species (*B. bufo* and *I. alpestris*) the test did not give any result, as the available sequences did not contain the primer binding sites. The results for other species included in the *in silico* test but not present in the study area can be found in Table S3.

For *in vitro* tests, the primer pairs showed 100% amplification success for all the respective target species for which tissue samples were available (as shown in Table 2). Apart from the marker Bufo, the primers also amplified one (in the case of marker Bomb) or more non-target amphibian species. In fact, some degenerated bases were included in the primers as we aimed to target as many species as possible with the same marker. In addition, the PCR mix and programme conditions were very permissive (e.g. allowing up to 55 cycles of PCR reaction, see Table S1), in order to overcome potential barriers to amplification success, such as the presence of PCR inhibitors, as well as the occurrence of degraded DNA fragments, in both museum and field samples. Most of the non-target amphibian sequences we obtained were of overall lower quality (data not shown), plausibly as a result of the PCR conditions used.

Regarding the *in situ* application of the protocol, all markers but one were able to detect at least one of the target species. The only exception was the Hyla marker, which did not detect species of Hylidae in any of the water samples. Unfortunately, no recent traditional survey data was available for species of this Family in our study area for confirmation of this result. However, according to research-level reports from iNaturalist (iNaturalist.org, 2023), *H. intermedia* was confirmed as present in the Province during our sampling period from only three casual observations, one of which referred to one of our sites. Considering the rarity of this species, it is possible that our protocol is not sensitive enough to detect it, an issue already highlighted by other eDNA-based monitoring studies (e.g., Pope et al., 2020). *B. viridis* was also expected in the study area but was not detected by our protocol. In this case, the false negative can be explained by both its relatively limited distribution and the peculiar habitat preferences of the species, that has adapted to living in anthropized areas (Caldonazzi et al., 2012). In fact, the vast majority of iNaturalist reports for *B. viridis* in the Province were from urbanized areas that were not included in our sampling design.

Apart from Bufo and Rana markers, all markers detected non-target amphibian species at least in one of the pipelines used (see Table 2), so combinations of fewer markers could detect all desired species, making the protocol economically efficient. For instance, the primer pair Pelop captured all the species of the family Ranidae known for the study area, detecting one ESV for *R. dalmatina* (compared to one detected with Rana) and eight (MV) to 11 (OE and OM) ESVs of *R. temporaria* (compared to three for MV and 11 for OE and OM, with marker Rana). Therefore we suggest using Pelop for detection of both genera, *Rana* and *Pelophylax*. Similarly, Sala outperformed Trit, which was designed on sequences of Italian Pleurodelinae species, in terms of number of Pleurodelinae taxa detected, identifying all the newts present in the Autonomous Province of Trento. Therefore, since the target regions of markers Sala and Trit overlap almost entirely, for future applications where the target species belong to the family Salamandridae, we recommend using the first primer pair. Lastly, the difference in the length of our markers did not seem to determine differences in the number of target and non-target ESVs detected in our dataset, although this occurrence has been documented in other studies (Andres et al., 2023, and references therein).

Impact of bioinformatic pipelines on detecting target and non-target ESVs from eDNA samples

For each pipeline and each marker, the absolute numbers and proportion of reads and ESVs assigned to the target amphibian species, non-target amphibian species and unclassified taxa are shown in Table 3. Overall, with the exception of marker Bomb, the MV pipeline retained the highest number of reads (Table S2) in all three taxonomic categories. However, the proportion of reads assigned to target amphibians by MV was lower than that of the other two pipelines even if the absolute number was still higher. Regarding the number of identified ESVs, the three pipelines showed similar results for Bomb, Bufo and Sala markers. MV was able to retrieve more target haplotypes for the Pelop marker, while the two OBITools pipelines (OE and OM) outperformed MV in the case of Rana and Trit markers. None of the pipelines identified haplotypes of the target species *H. intermedia* with the Hyla marker, although OM detected five ESVs belonging to other amphibian species. The proportion of ESVs assigned to the target species by MV over the total number of ESV was lower than the other two pipelines, while OE and OM assigned a similar proportion of reads and ESVs to target or non-target amphibian species. In particular, the OM pipeline retained the most non-target amphibian ESVs. This variation in the detection of non-target sequences across pipelines was expected due to the different taxonomic assignment methods used. Notably, the differences in number of reads and ESVs between pipelines is not consistent for all markers. While we applied the default parameters whenever possible, it should be noted that in the MV pipeline the expected error rates allowed at the filtering by quality step varied between markers, corresponding to 0.25% for all markers except Bufo and Sala (0.5%), thus possibly producing differences between markers in the MV dataset.

Table 3. Number of reads and ESVs assigned by the three pipelines to the target amphibian species, non-target amphibian species, and unclassified taxa for each marker. MV: MICCA+VSEARCH, OE: OBITools+ecotag, OM: OBITools+metabinkit.

Marker name	Bioinformatic pipelines											
	MV				OE				OM			
	N. Reads	% Reads	N. ESVs	% ESVs	N. Reads	% Reads	N. ESVs	% ESVs	N. Reads	% Reads	N. ESVs	% ESVs
Bomb												
Target Amphibia	3636	39.53	2	6.06	8100	80.65	2	40	8100	80.65	2	40
Unclassified	5562	60.47	31	93.94	1944	19.35	3	60	1944	19.35	3	60
Bufo												
Target Amphibia	106842	95.56	4	23.53	7837	99.10	3	60	7837	99.10	3	60
Unclassified	4962	4.44	13	76.47	71	0.90	2	40	71	0.90	2	40
Hyla												
Non-target Amphibia									7479	55.38	5	31.25
Unclassified	50688	100	106	100	13505	100	16	100	6026	44.62	11	68.75
Pelop												

Target Amphibia	14374	16.57	5	11.36	4675	12.54	1	4.54	4675	12.54	1	4.54
Non-target Amphibia	57162	65.91	9	20.46	23759	63.75	12	54.54	32270	86.60	18	81.82
Unclassified	15199	17.52	30	68.18	8832	23.70	9	40.91	321	0.86	3	13.63
Rana												
Target Amphibia	38640	60.24	4	1.42	23475	79.58	12	41.38	23475	79.59	12	41.38
Non-target Amphibia									12	0.04	1	3.45
Unclassified	25500	39.76	277	98.58	6022	20.42	17	58.62	6010	20.38	16	55.17
Sala												
Target Amphibia	5733	4.12	3	1.29	1976	4.13	3	3.61	1976	4.13	3	3.61
Non-target Amphibia	47571	34.19	16	6.87	17533	36.64	23	27.71	21657	45.25	31	37.35
Unclassified	85850	61.69	214	91.84	28347	59.23	57	68.68	24233	50.62	49	59.04
Trit												
Target Amphibia	28287	22.35	5	4.42	2832	14.65	13	19.70	2832	14.65	13	19.70
Non-target Amphibia	57177	45.18	8	7.08	7800	40.35	13	19.70	7800	40.35	13	19.70
Unclassified	41072	32.46	100	88.50	8699	45	40	60.60	8699	45	40	60.60

Concerning the difference in terms of ESVs, it should be noted that the extra ESVs retrieved with OBITools could be errors, and not true haplotypes. In order to address this issue, we applied throughout our workflow several of the best practices for identification and removal of erroneous sequences summarized by Andres et al. (2023), such as a sampling design focusing on locations and times of the year where the target species are known to be present, application of a denoising and chimera removal step during raw reads processing, and of a sequence similarity threshold to known (reference) sequences for taxonomic assignment, and lastly removal of low frequencies ESVs. Despite these precautions, undetected chimeric sequences (Edgar et al., 2016) and/or sequencing errors (Nakamura et al., 2011; Schirmer et al., 2015) could still be present in the dataset, leading to false positive errors (Andres et al., 2023). For most of our target species, up-to date field genetic data for the selected gene marker are scarce or not available (for the most recent studies concerning the alpine region see Cornetti et al., 2016; Canestrelli & Nascetti, 2008; Veith et al., 2003; Pichlmüller et al., 2013; Riberon et al., 2002; Canestrelli et al., 2006). Nevertheless, overall, our markers showed patterns of genetic diversity compatible with tissue-based genetic studies. For instance, Cornetti and colleagues (2016) found that *B. variegata* populations have low mitochondrial diversity in the study area, reporting only four haplotypes from nine sampling sites (from 200 individuals in total). As marker Bomb identified two ESVs in water samples from two sites, our results appear in agreement with this previous study. Another traditional study based on Cytochrome Oxidase I (COI) diversity found 12 *R. temporaria* haplotypes in the Province of Trento, only one of which (CA2) was distributed across the Province, while the others showed a clear separation due to the presence of the Adige river (Marchesini et al., 2017). Here, the marker Pelop showed more consistent results across pipelines, as it detected eight *R. temporaria* haplotypes according to pipeline MV, and 11 for both OE and OM, while Rana retrieved only three haplotypes with pipeline MV, but again 11 with OE and OM. The combination of Pelop and MV, in particular, yielded the most similar diversity pattern with respect to the traditional study, with only two CytB haplotypes found in sites located on the opposite sides of the Adige Valley, and the other six that were found in only one side. On the other hand, the 11 haplotypes of OE and OM were each detected in one site only, and therefore no genetic structure emerges from these data. In an effort to further validate our ESVs, the sequences were also compared with all available haplotypes in Genbank. According to this comparison, 16 out of the 56 amphibian (target and non-target) ESVs retrieved by MV were identical to already known haplotypes, as well as 14 out of 79 for OE and 17 out of 88 for OM. Overall, MV could be the most suitable pipeline if the aim is to retrieve the highest number of reads possible, although this comes at the cost of being more computationally demanding as it is less efficient in removing non-target reads. In addition, MV also appears to be the safest choice for decreasing the probability of false positive errors in the dataset, as it had the best rate of Genbank-confirmed over total ESVs, even though the pipeline could also be losing some true genetic information, given its more conservative algorithm for retrieving ESVs.

CONCLUSIONS

The new eDNA workflow presented here proved successful in detecting almost all the target species that were expected in our study area, while at the same time providing information on their mitochondrial genetic diversity. This new marker set combined with MV has the potential to become a standard monitoring tool for the Alpine region and beyond, with the capacity to collect both inter- and intra-specific diversity data in a completely non-invasive framework.

However, the debate about which pipeline would be more efficient for characterizing population genetic diversity is still open. The problem of false positives and negatives in eDNA-based multi-species surveys is known and discussed (e.g., Cristescu & Hebert, 2018), and it is an issue that requires even more attention in intra-specific diversity studies (Elbrecht et al., 2018; Macé et al., 2022; Adams et al., 2022). Additional studies comparing bioinformatic pipelines in their ability to retrieve in eDNA data the correct number and sequence of haplotypes, based possibly also on traditional sampling as controls, are urgently required.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: LZ, GM, MFMS, PB, PP, GB, HCH; acquired the funding: GM, PP, GB, HCH; collected the samples: LZ, GM, SC, GG; performed laboratory analyses: LZ, GM; completed bioinformatic analyses: LZ, MFMS; drafted the manuscript: LZ, MFMS, HCH; read, commented and approved the manuscript: all authors.

DATA ARCHIVING STATEMENT

Sequences available upon request. Correspondence and requests for materials should be addressed to H.C.H. (email: heidi.hauffe@fmach.it).

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

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SUPPLEMENTARY INFORMATION.

Table S1. Summary of PCR program conditions.

	PCR conditions			
Marker names	**MgCl2 (µl)**	**BSA (µl)**	**Vol. DNA (µl)**	**Ann temp (°C)**
Bomb	4	0	2	54
Bufo	8	0	2	56
Hyla	8	0	4	52
Pelop	6	0	4	56
Rana	3	0.5	5	54
Sala	10	0	5	52
Trit	8	0.5	1	60

Each reaction mixture contained 10µl of Promega Flexi Buffer 5X, 1µl each of forward and reverse primer 10 ρmol/µL, 1µl of dNTP's 10mM each, 0.25µl of Promega - GoTaq Hot Start G2 5U/µL, a variable volume of $MgCl_2$ 25mM, BSA 10 mg/mL and template DNA (see Table S1) and H2O up to a volume of 50µl. The PCR programs followed the Promega Taq Kit parameters, except for the number of cycles (50 except for the primers Trit: 55 cycles) and different annealing temperatures for each primer (shown in Table S1). For all primers the programs used an annealing time and elongation time of 30'' (with the exception of Trit: 35'').

Table S2. Number of reads before and after the bioinformatic processing with the three pipelines.

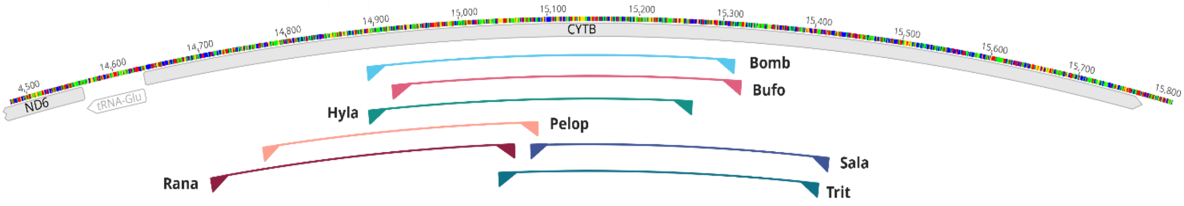
		After bioinformatic steps		
	Raw reads	**MV**	**OE**	**OM**
Total	1734354	588255	165407	165407
Mean reads per sample	57811.82	2178.72	1413.73	1413.73
Standard deviation	28928.28	2527.19	1842.26	1842.26

Table S3. Non-target species resulting from the *in silico* tests with ecoPCR, along with the percentage of sequences that was correctly identified by the marker for each species.

Marker & species	***Percent***
Bomb	
Bombina bombina	100%
Bombina maxima	89%
Bombina pachypus	82%
Bufo	
Bufotes pseudoraddei	100%
Bufotes sitibundus	100%
Hyla	
Hyla arborea	100%
Hyla chinensis	100%
Hyla hallowellii	100%
Hyla meridionalis	95%
Hyla molleri	97%
Hyla orientalis	99%
Hyla sarda	100%
Hyla savignyi	100%
Hyla tsinlingensis	100%
Pelop	
Pelophylax bedriagae	100%
Pelophylax bergeri	33%
Pelophylax cretensis	100%
Pelophylax epeiroticus	100%
Pelophylax kurtmuelleri	70%
Pelophylax perezi	91%
Pelophylax shqipericus	100%
Rana boylii	100%
Rana iberica	100%
Rana sauteri	100%
Rana	
Rana arvalis	100%
Rana chensinensis	84%
Rana dybowskii	83%
Rana johnsi	100%
Rana kukunoris	80%
Rana omeimontis	100%

Rana sauteri	100%
Sala	
Lissotriton boscai	100%
Lissotriton montandoni	100%
Salamandra algira	100%
Salamandra corsica	100%
Salamandra infraimmaculata	100%
Salamandra lanzai	100%
Trit	
Salamandra algira	100%
Salamandra atra	100%
Salamandra corsica	100%
Salamandra infraimmaculata	100%
Salamandra salamandra	100%
Triturus cristatus	100%
Triturus dobrogicus	100%
Triturus karelinii	100%
Triturus macedonicus	100%
Triturus marmoratus	100%
Triturus pygmaeus	100%

Figure S1. Position of each marker in the CytB region reference (from *R. temporaria* mitochondrial genome).



A BEG FOR TOADS

Ralph A. Lewin

See a dried and flattened toad
Crucified upon the road;
From the comfort of his pond,
Sent into the great beyond-
Pressed into unyielding sod-
By an omniprescient God.
Heel or wheel in hurry grim
Permanently cancelled him
In a smudge beside the fence
Of divine expedience.

It another toad emerges
From his green and pleasant verges,
Seeing no alternative-
Let him cross the road, and live!

CHAPTER 4

A POND OF KNOWLEDGE:

APPLYING MULTI-DISCIPLINARY APPROACHES TO AMPHIBIAN CONSERVATION IN THE PROVINCE OF TRENTO

STATUS: IN PREPARATION

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ABSTRACT

Habitat degradation due to intensive agriculture and urbanization is the most important contributing factor to amphibian diversity loss in Europe, and particularly in the Mediterranean region where 49% of the amphibian species are considered threatened by extinction. Although their natural habitats are decreasing, amphibians are known to use artificial water bodies in rural and urban landscapes as reproductive sites, making them potentially critical for the resilience of amphibian communities. This work explores the possibility of using presence data from an environmental DNA-based study on amphibian biodiversity carried out on 37 water bodies in the Province of Trento, including both protected areas and artificial or human-impacted basins, to investigate the relationship between habitat quality, species richness data, anthropogenic impact and climatic/environmental changes. Based on a multivariate correspondence analysis, distinct presence/absence patterns are identified in relation to ecological and landscape variables that are known to significantly influence the structure of amphibian community. This study showed that protected areas are still highly relevant for biodiversity conservation, and particularly so for the rarest species. In addition, farm ponds may be important reproductive sites for amphibians, showing high species richness. Due to time constraints, this chapter presents only an initial exploration based on groupings of sites with similar characteristics, but further analysis are ongoing in order to identify the most useful ecological and landscape variables for environmental quality evaluations, and to develop powerful transdisciplinary tools with applications in endangered species monitoring, environmental and landscape assessment, urban ecology and policy development.

KEYWORDS

Biodiversity, amphibians, protected areas, human pressure, artificial wetlands

INTRODUCTION

According to the IUCN Red List, 41% of the evaluated amphibian species are currently considered ‘threatened’ with extinction (IUCN, 2024). Even within the worldwide biodiversity crisis scenario, amphibian population decline is particularly alarming because several drivers seem to be occurring simultaneously and widely, sometimes affecting populations even inside protected areas (D'Amen et al., 2011; Xu et al., 2022).

Habitat degradation due to intensive agriculture and urbanization has been long recognized as the most important contributing factor to amphibian diversity loss (Whittaker et al., 2013). Changes in land use can lead to local extinctions through extermination of individuals, destruction and fragmentation of their reproductive habitat, and/or creation of barriers limiting access to their reproductive sites or terrestrial refuges (Collins & Storfer, 2003). Amphibians are also particularly sensitive to environmental pollutants because they lack a hard shell protecting their eggs and have highly permeable skin (Blaustein & Kiesecker, 2002). In addition, as a result of wetland distribution patterns, amphibian species tend to be highly structured genetically as metapopulations; therefore, destruction of important source populations can lead to an extensive loss of genetic biodiversity (Whittaker et al., 2013).

Amphibians of the Mediterranean region are particularly at risk, with 49% of the species identified as threatened (Nadin, 2008). In Italy, there is evidence that the intensification of agriculture has contributed substantially to the loss of amphibian species and individuals (D'Amen & Bombi, 2009). Significantly, many amphibian species are adapted to breeding in temporary ponds and streams, habitats that tend to disappear with many land use changes, such as drainage, as well as increasingly frequent droughts associated with climate changes (Caballero-Diaz et al., 2020).

With their natural habitats significantly reduced, amphibians often take advantage of artificial water bodies in rural and urban landscapes, such as reservoirs, fountains and cattle troughs, and farm ponds as reproductive sites (see Buono et al., 2019, and references therein; García-González and García-Vázquez, 2011). Thus, artificial water bodies (even very small ones) as well as case-specific environmental engineering management actions are potentially critical for the resilience of amphibian communities (Brown et al., 2012; Caballero-Diaz et al., 2020; Shoo et al., 2011).

The extent to which urban or agricultural ponds can be successfully used by amphibians as a reproductive habitat is, however, highly debated. It has been reported, for instance, that constructed stormwater wetlands harbored fewer egg masses and larvae than natural wetlands near Edmonton (Alberta, Canada), but young adults exhibited larger body size in reservoirs (Scheffers & Paszkowski, 2016). This last result, however, was best explained by lower survival to metamorphosis, in addition to more stable water temperatures in the stormwater ponds. Moreover, as not all amphibian species have the same preferred habitats, it is not surprising that different species show a variable persistence range in the urbanized landscape (e.g., Hamer and McDonnell, 2010; Hamer et al., 2012; Pereyra et al., 2020). The species most likely to survive and successfully reproduce in city parks or agricultural landscapes are most often generalist species, able to exploit a wide plethora of habitats including those altered by human presence (Ducatez et al., 2018). Nonetheless, artificial ponds could still provide suitable habitats for a variety of common species, especially if both the water body and surrounding landscape characteristics are properly managed (Smallbone et al., 2011; Villasenor et al., 2017). Similar results have been found in rural areas, where certain farming techniques have proved to host numerous amphibian species (e.g., traditional pastoral landscapes, Boissinot et al., 2019).

To date, to our knowledge, all previous studies investigated the connection between amphibian natural dynamics and characteristics of the sites using typical ecological approaches (i.e., generalized linear models, species occupancy models). While amphibian distribution has been used as an assessment tool in urban planning (e.g., Löfvenhaft et al., 2004), conversely, landscape planning workflows have never been applied to amphibian conservation. Here, for the first time, landscape planning methodologies have been used to describe the relationship between amphibian species richness, habitat quality, human activity and climatic/environmental changes. Differently from most ecological approaches, our analysis considered amphibian presence as a factor describing the wetlands, along with all other variables, and all considerations regarding amphibian communities were made *a posteriori* based on the results. In an innovative approach, amphibian presence data from an environmental DNA (eDNA) survey across 37 freshwater sites in the Province of Trento were used together with appropriate ecological and landscape variables to perform a multivariate correspondence analysis with the goal of determining which local and landscape factors concur in shaping the structure of amphibian communities. These preliminary results have been discussed and compared to previous studies, and will be useful to assess each site from an 'ecological health' perspective, thus providing an example of a methodological framework aimed at integrating information from different levels (species presence, ecological and landscape indices) in an easily replicable workflow.

METHODS

Study area

This study was conducted in the Autonomous Province of Trento (Italy), a mountainous region of 6,200 km² located in the southeastern Alps, which encompasses a mosaic of orographic reliefs defined by a very complex water network. More than 60% of the area is covered by forest, with altitudes ranging from 67 m a.s.l. at Lake Garda to 3,764 m a.s.l. at the summit of Cevedale. The region has a temperate climate, with warm summers and mild winters, with a more Mediterranean climate in the south, and a more continental one in the north, characterized by colder winters and cool summers. Likewise, precipitation tends to be more frequent in spring and autumn in the south, and during summer in the north (Caldonazzi et al., 2002). The rainfall is highest in S. Martino di Castrozza (1,500 mm per year) and minimum in the lower Sarca Valley (500 mm per year). The Province as a whole has a population of 542,996 inhabitants (ISTAT, 2023), of which 41.5% live in the six largest municipalities (Trento, Rovereto, Pergine Valsugana, Arco, Riva del Garda and Mori). These municipalities cover about 6.6% of the territory and correspond to the areas with the highest population density (ISTAT, 2022). The biggest urban center is the city of Trento, with approximately 118,277 inhabitants (ISTAT, 2023). 31.4% of the provincial territory are protected areas, parks and networks of reserves such as Natura2000 sites (ISPAT, 2022).

The freshwater sites selected for this study were chosen from a complete database of all water body types in the Province of Trento collated and curated by MUSE, the Science Museum of Trento (Trento, TN). The selection criteria included geographical location, elevation (126 - 2,063 m a. s. l.), and level of human exploitation. Although we attempted to distribute the sites as evenly as possible throughout the Province, low altitude sites are mainly concentrated in the Adige Valley, which represents the largest area at lower altitude in the Province (see Table S1, Supplementary Information). About half of the sites (18 out of 37) were inside protected areas (15 sites) or other areas of low anthropic impact (three), while the remaining 19 water bodies included agricultural reservoirs and ditches (four), artificial basins for irrigation purposes (three), farm ponds freely accessible to livestock (three), and artificial lakes for recreational uses (e.g., sport fishing; eight), while only one site was an artificial pond created to compensate the construction of an irrigation basin. To reduce the possibility of sampling DNA from the same individuals, all sampling sites were located at least 1 km apart, a distance that individuals of most of these species are unlikely to move during the breeding season.

Environmental DNA survey

The dataset of amphibian species present at each study site is based on the environmental DNA (eDNA) survey presented in Zanovello et al. (2024). Briefly, three to five water samples of 300-500 ml were collected from each site during the peak activity period of most amphibian species in the region (March - July, 2021). After filtration and DNA extraction, seven Cytochrome B gene fragments were amplified with PCR (to improve the detection of all species) and sequenced on an Illumina Miseq Platform. The dataset used here is that resulting from the MICCA+ vsearch (MV) bioinformatic pipeline (Zanovello et al., 2024), as it gave the highest proportion of eDNA sequences confirmed as true positives.

For each site, the presence of each species was confirmed if one or more marker sets gave a positive result. Therefore, a species was confirmed as absent from the site only if all seven markers failed to detect it. The eDNA survey detected ten of the 12 amphibian species living in the Province of Trento, including *Bombina variegata* (yellow-bellied toad, Linneaus, 1758), *Bufo bufo* (common toad, Linnaeus, 1758), *Ichthyosaura alpestris* (alpine newt, Laurenti, 1768), *Lissotriton vulgaris* (smooth newt, Linnaeus, 1758), *Pelophylax* synkl *esculentus* (common water frog, Linnaeus, 1758), *Rana dalmatina* (agile frog, Fitzinger, 1839), *Rana temporaria* (European common frog, Linneaus, 1758), *Salamandra atra* (alpine salamander, Laurenti, 1768), *Salamandra salamandra* (fire salamander, Linnaeus, 1758), and *Triturus carnifex* (crested newt, Laurenti, 1768).

To facilitate the subsequent analysis and application of the multidimensional correspondence approach, binary data referred to amphibian species presence/ absence were simplified based on taxonomy, grouping together the species at either Order (Anura and Caudata/ Urodeles) or Family/ Subfamily level. In the latter case, presence/absence of *B. variegata* was assigned to the Bombinatoridae family (firebelly toads), *B. bufo* to Bufonidae (true toads), *P*. kl *esculentus*, *R. dalmatina* and *R. temporaria* to Ranidae (frogs), *I. alpestris*, *L. vulgaris* and *T. carnifex* to Pleurodelinae (newts), and *S. atra* and *S. salamandra* to Salamandrinae (salamanders). All data associated with amphibian community structure at the sites are summarized in Table S2 (Supplementary Information).

Selection and processing of variables

Variables were collected to cover both local and landscape scales. Prior to analysis, for each site, i) amphibian communities were described as presence/absence of each taxon and total number of species. We then estimated ii) ecological variables representing the suitability of the sites for amphibian presence at a local level (hydroperiod, altitude, slope, presence of aquatic vegetation and fish), and iii) at, a landscape level, contextual factors that might directly or indirectly condition amphibian preference for a site (designation, surrounding land use type and forest density, distance to the closest road, water surface temperature, human footprint). The three conceptual categories, with their variables and associated estimates, are presented in Tables S2, S3 and S4 respectively (Supplementary Information).

Numerical values of the variables were measured at each site as follows. Presence/absence and species richness data were collected as described above. Designation, hydroperiod (length of time the wetland holds ponded water), slope at the shoreline, and presence of aquatic vegetation and fish were recorded during eDNA sampling, while altitude, aspect, and overall slope were calculated in GRASS GIS. The Vegetation Productivity Indicator (VPI) was calculated from the 2018 Copernicus Global Land Monitoring data (Sannier et al., 1998), and Rao's quadratic entropy (RaoQ) index values were calculated following Rocchini et al. (2017) for the year of sampling (2021). Land use type was defined by Corine Classes, and the proportion of land covered by each Class, in both 500 m and 1000 m buffer zones surrounding each pond, were calculated using the GRASS GIS addons v.rast.bufferstar (GRASS Development Team, 2023). The subsequent analyses were applied considering both 500 m and 1000 m buffer zones, in light of the fact that some amphibian species are known to be more mobile than others (e.g., Ponsero and Joly (1998) reported migrations of 50 to 300 m for *R. dalmatina*, while Kovar et al. (2009) noted migration distances longer than 2 km for *B. bufo* and *R. temporaria*), as already considered by other authors (e.g., Bounas et al., 2020). Forest density was assigned to each site with a raster estimated from 2018 Sentinel satellite data, with a resolution of 10 m. Distances from the closest roads were measured considering both distance to gravel roads and distance from paved roads, using OpenStreetMap data for the road network and GRASS GIS network modules. The 2009 Human Footprint index was extracted for each site based on data from the 2018 Release.

Lastly, the Land Surface Temperature was measured at the beginning of each season of the sampling year and as average of the season of sampling, following Metz et al. (2014).

Before data analysis with the software ADDATI, all continuous variables were converted to categories in order to obtain equally frequent classes, a necessary step to facilitate the subsequent analysis and to apply the multidimensional correspondence approach. The specific range of values assigned to each class is reported in Table S5 (Supplementary Information). For the same reason, these variables were further evaluated, in terms of direct or indirect impact on the overall environmental quality evaluation, as either 'active' or 'supplementary' variables (see Table S6, Supplementary Information). In this light, all data of amphibian taxa presence and species richness were considered 'active' variables. All other parameters that have already been shown to have an impact on amphibian communities, or that are linked to overall quality of the habitat, were classified as 'active': hydroperiod (Guderyahn et al., 2016, Konowalik et al., 2020), slope at the shoreline (Clevenot et al., 2018) and of the site (Marchesini et al., 2018), presence of aquatic vegetation and fish (Guderyahn et al., 2016, Hamer & Parris, 2011, Bounas et al., 2020), altitude (Caldonazzi et al., 2002), VPI (Chester & Robson, 2013). Aspect of the site, along with the integer values of RaoQ, instead, were considered 'supplementary'. Lastly, general designation of the site, forest density and distance to the closest road were considered 'active' (Fuyuki et al., 2014; Guderyahn et al., 2016; Holzer, 2014; Pillsbury & Miller, 2008), while specific designation of the site, all Corine Class data, the human footprint and data of Land Surface Temperature are presented here as 'supplementary'.

Data analysis

The normal distribution of the number of species per site data was checked with a Shapiro-Wilk test using Past v. 4.16c (Hammer et al., 2001), which showed that these data did not have a normal distribution (W: 0.89, p= 0.002). Therefore, a Mann-Whitney test was performed in Past v. 4.16c to test for differences in the number of species between these two categories.

The software ADDATI v. 6.0 (Griguolo, 2008), a package for exploratory data analysis mostly used for territorial applications (e.g., Ceccato et al., 2007; Mattia et al., 2012; see Griguolo & Palermo, 1984 and Palermo, 1983 for theoretical-methodological insights and applications in urban and regional studies) was used here to perform a non-hierarchical clustering aimed at verifying whether the presence of amphibian species varies with characteristics of the sites and at establishing whether the 'ecological health' of a site correlates to physical and biophysical characteristics. The input file for the analysis consisted of a recoded matrix, including 37 vectors (one for each site) per 36 categorical variables (see Tables S6 and S7, Supplementary Information). The overall matrix was then factorized using multiple correspondence and cluster analysis in a two-step workflow. First, statistically significant factors representing the structural relationships among input variables are isolated using chi-square metrics with a correspondence factor analysis (Acorr; Griguolo, 2008). These factors, thus summarizing information of the most important variables, confer specific 'factorial scores' to each of the sites utilized as input in the following step (Griguolo, 2008). Here, the first nine factors represented over 80% of total inertia and were imported in the next step. Secondly, all sites are clustered into groups with a specific descriptive profile using a non-hierarchical classification (Nonger) based on the method of 'dynamic clouds and shape recognition' (Diday, 1971; Figure 1). This classification is thus based on an optimization function that maximizes the internal variance of each class against the external variance among the classes. The non-hierarchical classification was conducted here using four exploratory partitions (see Griguolo, 2008).

The software also assigned a relative weight value to each cluster, and to each component used for clustering, allowing the identification of the most important variables for further analysis. In particular, for subsequent discussion the variables were considered relevant when having a relative weight > 50% inside their cluster, and a ratio between the frequency of each variable in the cluster and its overall frequency over 1.20.

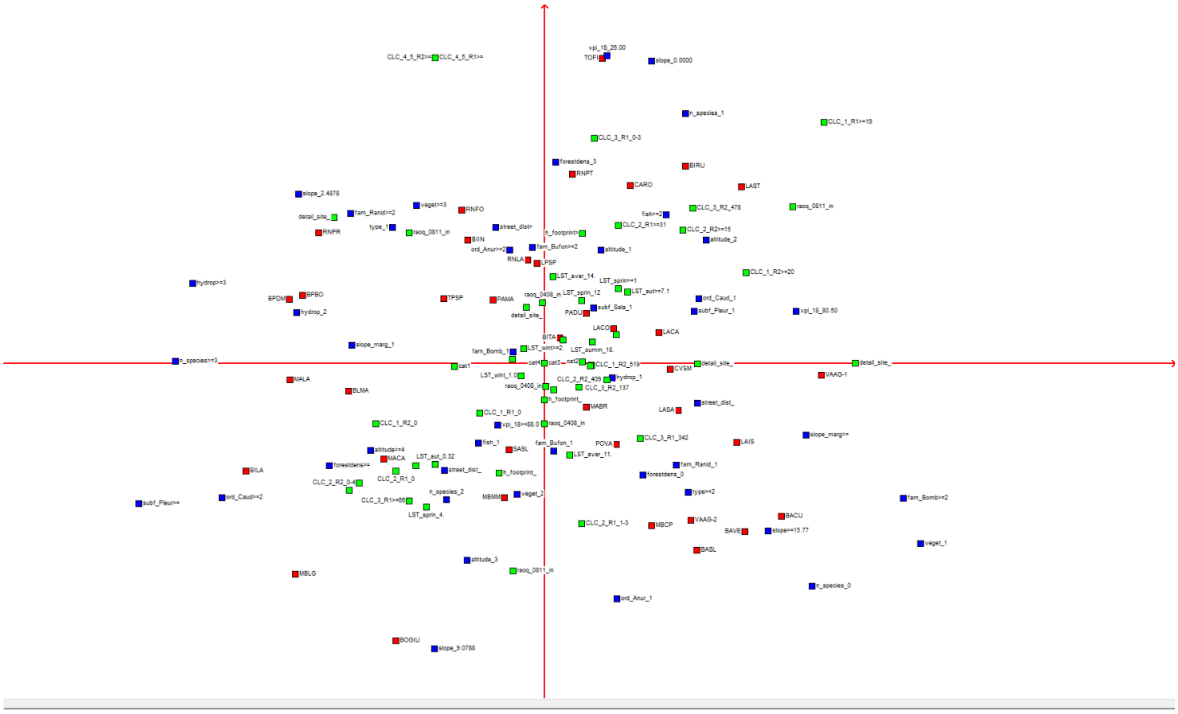

Figure 1. Factorial plan resulting from ADDATI, representing the positioning of sites with respect to the categorical variables. Sites are represented in red; active variables in blue; supplementary variables in green.

RESULTS

A total of 10 amphibian species were identified in the 37 wetland sites (Table S2, Supplementary Information). Of these, the three most common were *B. bufo* (15 sites; 40.54%), *R. temporaria* (14 sites; 37.82%) and *I. alpestris* (11 sites; 29.73%), while the rarest ones were found in only one site (*L. vulgaris, R. dalmatina, S. atra, T. carnifex*). The remaining species were detected respectively in three (*B. variegata*) or four sites (*P.* kl *esculentus*, *S. salamandra*). The total number of species detected at a pond varied between zero and four (mean of 1.48, standard deviation 1.1), although the median was one species, as shown in Figure 2.

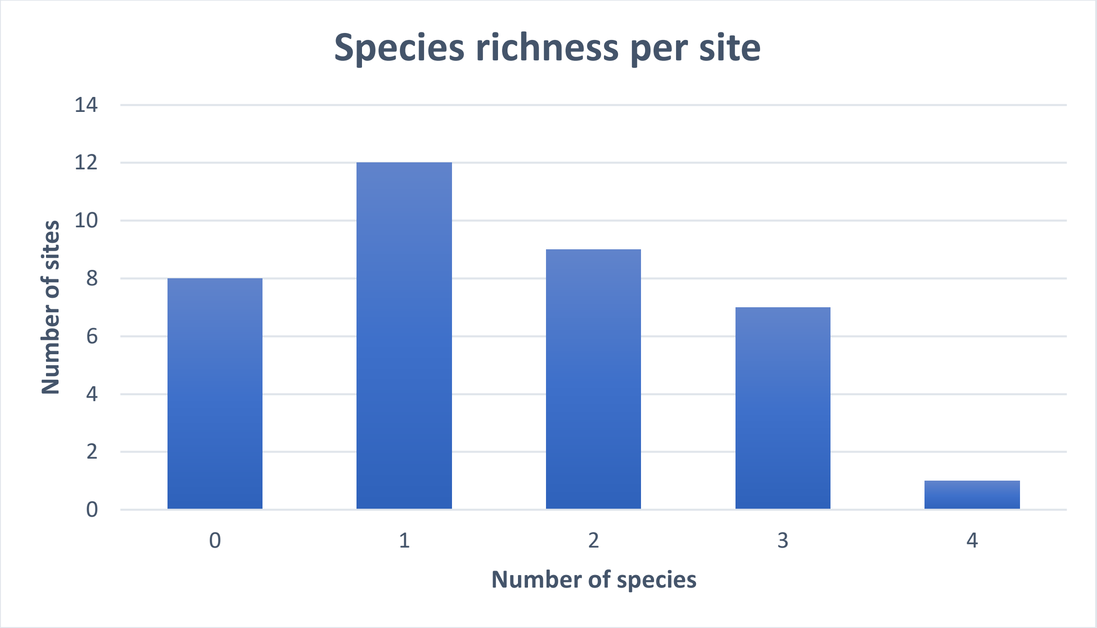

Figure 2. Number of wetland sites for each number of species detected with the eDNA survey, out of a total of 37 wetlands in the Province of Trento, Italy.

Considering the division of sites based on designation, in 16 of the 18 protected or less impacted areas at least one amphibian species was detected (88.89%, with a mean of 1.67 species per site, standard deviation, sd ± 1.00). At least one species was identified in almost all protected areas (13 out of 14, 92.85%), with a mean of 1.92 species per site (sd ± 0.96). Moreover, three of the four less common species (*L. vulgaris, R. dalmatina* and *T. carnifex*) were found only inside a protected area. On the other hand, in only 13 of the 19 artificial or heavily impacted sites at least one species was detected (68.42%), with a mean of 1.31 species per site and sd ±1.17. In particular, the three artificial irrigation basins did not host any amphibians, while the farm ponds showed the highest richness (mean of 2.25 species per site, sd ±1.48). The agricultural reservoirs and ditches had the second higher number of species (mean 1.75, sd ±0.83) for artificial/impacted habitats, with artificial lakes third (mean 1.12, sd ±0.78). Overall, the number of species per site found in the two macro-groupings of sites based on their designation (protected/less impacted or artificial/impacted) did not show statistically significant differences (Mann-Whitney z: 1.04, p= 0.29).

The sites were clustered by ADDATI into four clusters, which consisted respectively of eight, 11, 16 and two sites (Table S7, Supplementary Information). The relative weight inside their cluster of each variable, as well as the ratio between the frequency of each variable in the cluster and its overall frequency, among other details, are shown in Table S7 (Supplementary Information).

The first cluster grouped together all sites where amphibians were not detected. Other variables that play a role in forming this cluster were permanent hydroperiod (shared by 100% of the sites), steep shoreline slopes (62.5%), overall slope $\geq$15.77° (50%), eastern exposure (87.5%), and low Rao's Q diversity (62.5%). On a landscape scale, this cluster included artificial/impacted sites (75%), with a relatively small distance to the nearest road (between 4.71 and 17.15 km, 62.5%), a small proportion of the area occupied by Corine Class 3 (forest and seminatural areas) within the 500 m buffer (50%), as well as relatively high temperatures in winter and during the sampling season (50% of cases).

The second cluster grouped together most sites with three or more species (63.3% of the cases), and almost all sites where at least one Urodele was detected (90.9%). More specifically, these sites shared a lack of *B. bufo* (72.7%), but a consistent presence of Ranidae (63.6%) as well as of Pleurodelinae (81.8%) species. They were also characterized by gentle shorelines (all sites), abundant aquatic vegetation (63.6% of the sites), lack of fish (90.9%), high elevation (54.5%), and moderate to high Rao's Q diversity values (54.5%). The most relevant landscape factors for this clustering included designation as natural or less impacted site (72.7%), position inside protected areas (63.6%), the complete absence of both Corine Class 1 (urban areas) and 2 (agricultural areas) surfaces with the 500 m buffer (respectively 90.9% and 54.5%), as well as within the 1000 m buffer (72.7% and 63.6%, respectively), and the widespread presence of Corine Class 3 (forest and seminatural areas) within both buffers (63.6% and 72.7%, respectively). Low spring, summer and autumn temperatures were also common factors for this cluster (54.5% each).

The third cluster consisted of sites where one species was detected (56.2%), including Anurans (present in all sites), but not Urodeles (93.8%). In detail, these sites shared the presence of Bufonidae (81.2%) and Ranidae (50%) and the absence of Pleurodelinae (93.8%). Considering the local variables, this cluster was characterized by steep shorelines (half of the cases), and presence of fish (56.2%), and on a landscape level by moderately high temperatures in spring (between 12.35 and 15.5°C, 50% of the cases).

The last cluster grouped together only two sites, in which either one or two species were detected, and where, in particular, *B. variegata* was always present while a Salamandrinae species was present in half of the cases. In addition, all sites were characterized by the absence of Bufonidae, Ranidae and Pleurodelinae. These two sites were also clearly defined by a permanent hydroperiod, steep shorelines, absent or almost absent aquatic vegetation, absence of fish, low to moderate altitude (300- 900 m a.s.l.), overall slope>= 15.77°, moderate values of VPI, moderate Rao'Q diversity at the beginning of the season and moderate or high at the end. From a landscape perspective, the sites shared an artificial origin, both being, in particular, agricultural reservoirs and ditches with an eastern exposure. Both were surrounded by agricultural lands (CC2) and not by forests (CC3) within the 500 m buffer. Within the 1000 m buffer their landscape was characterized by moderate (in one case) to high urbanization (in the other), again agricultural lands, and absence or scarce presence of forests. They also shared moderate values of the Human Footprint index, relatively high temperatures in spring, summer, and autumn, moderate or high temperatures in winter (50% and 50%, respectively), but moderate mean temperatures during the sampling season.

DISCUSSION

The aim of the work presented here was to provide updated information on the presence and distribution of amphibian species in the Province of Trento and to investigate the local and landscape variables potentially influencing these species, although as mentioned in the Introduction, these are preliminary results.

Overall, the relative frequency of detection and distribution of each amphibian species is consistent with available data for the study area (Caldonazzi et al., 2002). In particular, the three most common species (*B. bufo, R. temporaria*, and *I. alpestris*) are known to be widespread in the Province of Trento, while the four less represented in this dataset are also particularly rare in the Province (especially *S. atra* and the two newt species, *L. vulgaris* and *T. carnifex*).

The protected areas and less impacted sites showed a higher proportion of positive sites (sites where at least one amphibian species was detected). The protected areas included in this study, in particular, showed the highest proportion of sites hosting at least one species, while also constituting the only sites in which three of the four rarest species were found. These results reinforce the importance of protected areas in the context of amphibian species conservation, despite the possible negative effects of climate changes in these areas (e.g., Whitfield et al., 2007; D'Amen et al., 2011; Xu et al., 2022). Nevertheless, when considering the total number of species per site, no significant differences were found between the two categories of protected areas or natural sites and artificial or heavily impacted sites. This result may be related to the small sample size, but it may also suggest that the artificial or heavily impacted water body type could still support amphibian conservation by providing additional breeding sites for these species, as already highlighted by previous studies (e.g., Brand & Snodgrass, 2010; Scheffers & Paszkowski, 2013). However, the extent to which these sites can contribute to amphibian populations persistence appears to vary widely according mostly to the site designation. For instance, farm ponds appear to be the richest sites in terms of number of species and could therefore benefit from appropriate protection measures. To inform management decisions, the most relevant local and landscape variables defined in our analysis and their possible impact on amphibian species presence are discussed below.

Concerning the first cluster, that grouped together the sites where the eDNA survey did not detect any amphibians, the analysis found that all sites had a permanent hydroperiod. This result can be explained by the fact that a long hydroperiod is also associated with presence of fish, which have a negative impact on amphibian communities through predation of adults or larvae (Hamer & Parris, 2011; Snodgrass et al., 2001). However, it should be noted that a too short hydroperiod has also been shown to negatively affect species richness due to the need of constant water presence for eggs and larvae development (e.g., Babbitt, 2005; Brannelly et al., 2019; Konowalik et al., 2020; Snodgrass et al., 2001). For this reason, the wetland hydroperiod effect could be directly linked to other unidentified factors that could be revealed only with a more in-depth analysis. Steep shoreline slopes, instead, have frequently been associated with absence of amphibians, which confirmed the soundness of this result (e.g., Klimaszewski et al., 2016; Shulse et al., 2010). Similarly, the overall slope of the site has also been reported as a factor negatively influencing amphibian presence (e.g., Guderyahn et al., 2016; Ribeiro et al., 2018). Most of the remaining factors seem to characterize the surrounding landscape as poor from a natural biodiversity perspective (low Rao's Q diversity, small proportion of the 500 m buffer occupied by forests), and highly impacted by human presence (most of the sites are artificial and relatively close to the nearest street). Forest cover, in particular, has been shown to positively affect amphibian communities (Guderyahn et al., 2016; Holzer, 2014; Ribeiro et al., 2018), as is the case for high habitat heterogeneity in the surrounding landscape (Li et al., 2018), while urbanization appears to have the opposite effect (Pillsbury & Miller, 2008). About half of the sites shared relatively high temperatures in winter and during the sampling season. As the sites of this cluster are distributed between approximately 250 and 1,250 m a.s.l., this condition could be due to high exposure of the water bodies to the sun (considering the scarce presence of forests) rather than to altitude effects. Lastly, to date there are no studies addressing the possible influence of exposure of wetlands on amphibian species richness, and it is thus impossible to confirm if the eastern exposure of these sites is indeed an explanatory variable.

The second cluster is characterized mainly by the presence of newt (Pleurodelinae), and frog (Ranidae) species. Of the local variables characterizing these sites, gently sloping shorelines, as well as aquatic vegetation and lack of fish, have already been reported to promote amphibian presence (e.g., Klimaszewski et al., 2016; Shulse et al., 2010; Vehkaoja et al., 2023). Newts appear to be particularly affected by the presence of fish (Denoël et al., 2008; Orizaola & Braña, 2006), and they are known to use aquatic vegetation both for egg deposition and as refuges from predators (Marco et al., 2001; Orizaola & Braña, 2003). The location of most sites of this cluster at higher altitudes might be merely a consequence of the dominant species' habitat preferences (*I. alpestris* and *R. temporaria*), but further analysis is needed to rule out the possibility of a climate-related shift to higher altitudes of the amphibian communities (e.g., Canestrelli et al., 2017). At the landscape level, most of these sites are characterized by high natural diversity, expressed by the Rao's Q metric as well as by the absence or limited presence of urban and agricultural areas and concomitant presence of forests, and fall within the categories of protected areas or other low-impact sites. All these factors could plausibly explain the relatively high amphibian diversity of these sites (Guderyahn et al., 2016; Holzer, 2014; Li et al., 2018; Pillsbury & Miller, 2008; Ribeiro et al., 2018). As the low spring, summer and autumn temperatures might be related to altitude of these sites, further analysis is due in order to better understand the influence of this factor on amphibian diversity.

The third cluster included mostly sites where only one species was detected, with a particularly high presence of Anurans and lack of Urodeles (especially newts). Considering the local and landscape variables, this cluster was undoubtedly the least well-defined one. It must be noted, however, that this is also the biggest cluster, including 16 sites. Nevertheless, about half of the sites have steep shorelines and presence of fish, as well as moderately high temperatures in spring. As discussed above, the first two factors are usually associated with lower species richness, which is confirmed by the fact that in most of these sites the eDNA survey detected only one species. The latter factor has also been proven to greatly affect amphibian presence and reproductive activity (e.g., Broadman, 2007; Cayuela et a., 2016).

The last cluster was also the smallest, including only two sites in which one or two species were detected. This cluster was characterized by the presence of *B. variegata*, a species that is relatively rare in the Province (Caldonazzi et al., 2002). These two sites are characterized by permanent water, steep shorelines, scarce aquatic vegetation, absence of fish and steeply sloping surrounding landscape. In this case, the low to moderate species richness found at the sites could be explained by the complex interplay of factors that have shown respectively a positive (permanent hydroperiod, Brannelly et al., 2019; absence of fish, Shulse et al., 2010) or negative (steep shorelines, Klimaszewski et al., 2016; lack of aquatic vegetation, Shulse et al., 2010; steep surroundings, Ribeiro et al., 2018) impact on amphibian diversity. Both sites also shared a low to moderate altitude, moderate values of VPI, a moderate Rao'Q diversity at the beginning of the season and moderate or high at the end, despite being surrounded mostly by agricultural lands and secondly by urbanized areas. In fact, the sites were both agricultural reservoirs with an eastern exposure, and shared moderate values of the Human Footprint index as well as relatively high temperatures across the year, but moderate mean temperatures during the sampling season.

Overall, the non-hierarchical clustering analysis applied here for the first time to an amphibian conservation study, highlighted the possible relationships between natural dynamics (i.e., amphibian species presence and richness), anthropic activities and climatic stressors. Importantly, the analysis pointed out some of the ecological and landscape variables that could be used for environmental quality evaluations. To better understand the relative importance of these variables in explaining amphibian diversity, a series of regression tests will be carried out to test the influence of each variable, as well as autocorrelation testes to isolate variables potentially correlating with each other. The results of the regression tests, in particular, will provide information that could then be easily integrated into planning future monitoring frameworks, with particular attention to land use changes evaluation, management and governance practices. More specifically, these tests will highlight which factors need to be monitored and/or protected in order to improve, or preserve, the ecological health of alpine wetlands to maintain or improve amphibian diversity. Finally, this workflow also has methodological relevance, as it has the potential to become a powerful transdisciplinary tool both in terms of result (replicability) and process (operability).

AUTHOR CONTRIBUTIONS

Conceptualization: LZ, GP, MG, HCH, GB, DM; Data curation; LZ, MG, GP, LD; Formal analysis: LZ, GP; Funding acquisition: HCH, GB, PP; Methodology: LZ, MG, GP; Supervision: HCH, GB, DM, PP, LR; Writing - original draft: LZ, GP; Writing - review & editing: all authors.

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SUPPLEMENTARY INFORMATION.

Table S1. Details of the 37 wetlands of the Province of Trento considered in this study.

ID_SITE	MUNICIPALITY	LOCALITY	LATITUDE	LONGITUDE
BACU	Cunevo	Artificial basin	46.28668	11.02392
BASL	Sfruz	Artificial basin ‘7 larici’	46.34745	11.1319
BAVE	Vervò	Artificial basin	46.31171	11.10557
BIIN	Levico	Biotope ‘Inghiaie’	45.998	11.3094
BILA	Pinè	Biotope ‘Laghestel’	46.11229	11.22397
BIRU	Mezzolombardo	Biotope ‘La Rupe’	46.19113	11.09696
BITA	Nomi e Volano	Biotope ’Taio’	45.92506	11.0797
BLMA	Lizzana	Biotope ‘Lavini di Marco’	45.85427	11.01437
BOGIU	Tiarno di Sotto	‘Bocca Giumella’	45.91679	10.65286
BPBO	Avio	Biotope ‘Palù di Borghetto’	45.69086	10.92322
BPDM	Valda	Biotope ‘Prati di Monte’	46.22355	11.24303
CARO	Roverè della Luna	Agricultural ditch	46.25215	11.18113
CVSM	San Michele all'Adige	Fondazione Mach ditch/tank	46.18904	11.13595
LACA	Trento	‘Lago delle Cannelle’	46.01871	11.13717
LACO	Predaia	‘Lago di Coredo’	46.35466	11.1043
LAIS	Mezzolombardo	‘Laghetto Ischia’	46.22792	11.09513
LASA	Arco	Località ‘Laghel’	45.9311	10.88349
LAST	Novaledo	‘Lago Stefy’	46.02642	11.37765
LPSP	Moena	‘Laghetto Passo San Pellegrino’	46.37705	11.78505
MABR	Trento	Malga Brigolina	46.05586	11.05868
MACA	Drena	Malga Campo	45.93799	10.96979
MALA	Lavarone	Malga Laghetto	45.95726	11.2967
MBCP	Brentonico	Monte Baldo - Colme di Pravecchio	45.77723	10.93362
MBLG	Albiano	Biotope ‘Monte Barco - le Grave’	46.12168	11.16801
MBMM	Brentonico	Monte Baldo - Malga Mortigola	45.80694	10.92562
PADU	Bleggio Superiore	‘Passo del Duron’	46.03435	10.79467
PAMA	Molina di Fiemme	Lago delle Buse- Passo Manghen	46.17761	11.45391
POVA	Valda	Valda	46.21971	11.25678
RNFO	Grigno	Natural Reserve ‘Fontanazzo’	46.01418	11.60734
RNLA	Tiarno di Sopra	Natural Reserve ‘Lago d'Ampola’	45.87029	10.65
RNPR	Roncegno	Natural Reserve ‘Paludi di Roncegno’	46.04951	11.42526
RNPT	Tuenno	Natural Reserve ‘Palù di Tuenno’	46.34076	11.03014
SASL	Sfruz	artificial wetland ‘7 larici’	46.34669	11.13291
TOFI	Fiavè	Natural Reserve ‘Fiavè’	45.99146	10.83161
TPSP	Moena	Torbiera Passo San Pellegrino	46.3768	11.7658
VAAG-1	Giovo	Verla - tank 1	46.15401	11.15355

VAAG-2	Cembra Lisgnago	Cembra - tank 2	46.16499	11.21161

ID_site shows the identification code for each of the 37 sites considered here; municipality and local name of the wetland are also shown, along with latitude and longitude coordinates (in D.D°).

Table S2. Amphibian taxa detected at each of the 37 wetlands surveyed in the Province of Trento (first part).

ID_site	**n_sp**	**o_Anur**	**o_Caud**	**f_Bomb**	**f_Bufo**	**f_Ran**	**sf_Pleur**	**sf_Sala**
BACU	0	0	0	0	0	0	0	0
BASL	0	0	0	0	0	0	0	0
BAVE	0	0	0	0	0	0	0	0
BIIN	2	1	0	0	1	1	0	0
BILA	3	1	1	0	0	1	1	1
BIRU	1	1	0	0	1	0	0	0
BITA	0	0	0	0	0	0	0	0
BLMA	3	0	1	0	0	0	1	0
BOGIU	2	0	1	0	0	0	1	1
BPBO	3	1	1	0	0	1	1	0
BPDM	3	1	1	0	1	1	1	0
CARO	1	1	0	0	0	1	0	0
CVSM	3	1	0	1	1	1	0	0
LACA	0	0	0	0	0	0	0	0
LACO	1	1	0	0	1	0	0	0
LAIS	0	0	0	0	0	0	0	0
LASA	1	1	0	0	1	0	0	0
LAST	1	1	0	0	1	0	0	0
LPSP	2	1	0	0	1	1	0	0
MABR	2	1	0	0	1	0	0	0
MACA	3	1	1	0	1	1	1	0
MALA	4	1	1	0	0	1	1	1
MBCP	0	0	0	0	0	0	0	0
MBLG	2	0	1	0	0	0	1	1
MBMM	2	1	1	0	1	0	1	0
PADU	1	1	0	0	1	0	0	0
PAMA	1	0	1	0	0	0	0	0
POVA	0	0	0	0	0	0	0	0
RNFO	2	1	0	0	1	1	0	0
RNLA	1	1	0	0	0	1	0	0
RNPR	3	1	1	0	1	1	1	0
RNPT	1	1	0	0	1	0	0	0
SASL	2	1	0	0	1	1	0	0
TOFI	1	1	0	0	0	1	0	0
TPSP	1	1	0	0	0	1	0	0
VAAG-1	1	1	0	1	0	0	0	0
VAAG-2	2	1	1	1	0	0	0	1

ID_site shows the identification code for each site (see Table S1); n_sp = number of species per sites, all other columns report data in terms of absence (0) or presence (1) of each taxon, where o_Anur refers to Anurans, o_Caud = Caudata, f_Bomb = Bombinatoridae, f_Bufo = Bufonidae, f_Ran = Ranidae, sf_Pleur = Pelurodelinae, sf_Sala = Salamandrinae.

Table S2. Amphibian taxa detected at each of the 37 wetlands surveyed in the Province of Trento (second part).

ID_site	Bv	Bb	Ia	Lv	Pe	Rd	Rt	Sa	Ss	Tc
BACU	0	0	0	0	0	0	0	0	0	0
BASL	0	0	0	0	0	0	0	0	0	0
BAVE	0	0	0	0	0	0	0	0	0	0
BIIN	0	1	0	0	0	0	1	0	0	0
BILA	0	0	1	0	0	0	1	0	1	0
BIRU	0	0	0	0	1	0	0	0	0	0
BITA	0	0	0	0	0	0	0	0	0	0
BLMA	0	0	1	1	0	0	0	0	0	1
BOGIU	0	0	1	0	0	0	0	0	1	0
BPBO	0	0	1	0	1	1	0	0	0	0
BPDM	0	1	1	0	0	0	1	0	0	0
CARO	0	0	0	0	1	0	0	0	0	0
CVSM	1	1	0	0	0	0	1	0	0	0
LACA	0	0	0	0	0	0	0	0	0	0
LACO	0	1	0	0	0	0	0	0	0	0
LAIS	0	0	0	0	0	0	0	0	0	0
LASA	0	1	0	0	0	0	0	0	0	0
LAST	0	1	0	0	0	0	0	0	0	0
LPSP	0	1	0	0	0	0	1	0	0	0
MABR	0	1	0	0	0	0	1	0	0	0
MACA	0	1	1	0	0	0	1	0	0	0
MALA	0	0	1	0	1	0	1	1	0	0
MBCP	0	0	0	0	0	0	0	0	0	0
MBLG	0	0	1	0	0	0	0	0	1	0
MBMM	0	1	1	0	0	0	0	0	0	0
PADU	0	1	0	0	0	0	0	0	0	0
PAMA	0	0	1	0	0	0	0	0	0	0
POVA	0	0	0	0	0	0	0	0	0	0
RNFO	0	1	0	0	0	0	1	0	0	0
RNLA	0	0	0	0	0	0	1	0	0	0
RNPR	0	1	1	0	0	0	1	0	0	0
RNPT	0	1	0	0	0	0	0	0	0	0
SASL	0	1	0	0	0	0	1	0	0	0
TOFI	0	0	0	0	0	0	1	0	0	0
TPSP	0	0	0	0	0	0	1	0	0	0
VAAG-1	1	0	0	0	0	0	0	0	0	0
VAAG-2	1	0	0	0	0	0	0	0	1	0

ID_site shows the identification code for each site (see Table S1); all other columns report data in terms of absence (0) or presence (1), where Bv = *B. variegata*, Bb = *B.bufo*, Ia = *I. alpestris*, Lv= *L.*

vulgaris, Pe = *P.* kl. *esculentus*, Rd = *R. dalmatina*, Rt = *R. temporaria*, Sa = *S. atra*, Ss = *S. salamandra*, Tc = *T. carnifex*.

Table S3. Local variables data measured for each of the 37 wetlands from the Province of Trento, Italy.

ID_SITE	hydrop	slope	veget	fish	altitude	Aspect	Slope	VPI	raoq_0408	raoq_0811
BACU	0	2	0	0	697.31	4.40	23.88	88	14	4
BASL	0	2	0	0	1178.91	278.64	13.50	95	17	12
BAVE	0	2	0	0	872.28	235.55	10.83	95	20	21
BIIN	0	0	2	0	438.94	79.51	2.49	88	21	15
BILA	1	0	2	0	922.61	169.00	11.73	95	18	15
BIRU	0	2	2	1	211.10	205.38	1.59	84.5	26	35
BITA	0	0	2	0	173.89	221.61	3.57	79.5	22	32
BLMA	2	0	1	0	174.23	329.92	1.93	88	18	19
BOGIU	0	0	1	0	1466.77	231.80	11.76	95	22	6
BPBO	2	0	1	1	126.07	341.67	3.37	88	19	20
BPDM	0	0	2	0	1381.47	180.94	7.88	95	18	13
CARO	0	2	2	1	203.65	184.60	1.58	88	24	30
CVSM	0	2	1	1	207.57	170.35	16.66	95	22	26
LACA	0	0	1	1	240.40	63.39	0.20	59.5	16	13
LACO	0	0	1	1	878.00	0.00	0.00	95	35	23
LAIS	0	2	1	1	249.95	219.81	19.98	95	13	18
LASA	0	2	0	0	208.00	11.36	11.86	95	14	13
LAST	0	2	0	1	423.40	248.25	1.94	26	24	31
LPSP	0	0	2	1	1893.85	86.99	0.28	95	12	16
MABR	0	2	2	0	993.12	114.81	15.78	95	18	7
MACA	0	0	1	0	1372.70	176.88	12.30	95	26	5
MALA	0	0	2	0	1188.63	214.68	4.83	95	28	10
MBCP	0	0	1	0	1257.27	46.29	23.98	95	22	13
MBLG	1	0	2	0	883.66	315.55	11.06	95	12	8
MBMM	0	2	1	1	1162.79	2.56	16.68	95	14	8
PADU	0	2	1	1	1034.26	78.00	5.10	95	12	6
PAMA	0	0	2	0	2063.39	102.67	5.42	80.5	7	15
POVA	0	2	2	1	1250.12	276.14	18.89	95	11	6
RNFO	0	0	2	0	258.71	234.87	4.61	95	18	16
RNLA	1	0	2	0	730.07	327.24	26.39	95	26	15
RNPR	0	0	2	0	404.57	331.09	4.42	64	19	17
RNPT	0	0	2	0	671.65	94.27	0.59	95	16	22
SASL	0	0	1	0	1168.27	2.10	9.27	95	18	12
TOFI	1	1	2	1	649.06	26.59	0.78	79.5	19	15
TPSP	2	0	2	0	1804.59	255.47	9.08	95	26	17
VAAG-1	0	2	0	0	464.65	202.50	32.56	87.5	17	21
VAAG-2	0	2	0	0	491.64	300.02	30.93	87.5	15	15

ID_site shows the identification code for each of the 37 sites considered here (see Table S1); hydroperiod (hydrop) is classified as 1= permanent, 2= vary across the site, 3= temporary; slope refers to the shorelines (0= shallow, 1= vary across the site, 2= steep); aquatic vegetation (veget) considers both floating and rooted plants (1= absent, 2= scarce, 3= abundant); fish are either absent

(0) or present (1); altitude, overall Aspect and Slope of the site are presented as numerical values; VPI as a percentile ranking of the current NDVI value against its historical range of variability (https://land.copernicus.eu/global/products/vpi), while values of Rao's Q at beginning (raoq_0408) and end (raoq_0811) of the sampling season are shown as integer.

Table S4. Context/ Landscape variables data from each of the 37 wetlands considered in the Province of Trento, Italy (first part).

ID_site	type	detail	CLC1_R1	CLC2_R1	CLC3_R1	CLC4_5_R1	CLC1_R2	CLC2_R2	CLC3_R2	CLC4_5_R2
BACU	2	5	0	32841	752354	0	229663	708326	2203210	0
BASL	2	5	0	0	785206	0	0	151013	2990177	0
BAVE	2	5	0	447026	342948	0	64775	2057547	1211157	0
BIIN	1	1	0	763154	22051	0	60228	3546367	317368	0
BILA	1	1	0	115218	669972	0	170838	293291	2677065	0
BIRU	1	1	172236	476963	350956	0	400855	1587947	1488214	0
BITA	1	1	120504	552716	0	111970	448322	2066030	375651	251199
BLMA	1	1	4038	432914	348244	0	656509	1002038	1482629	0
BOGIU	2	6	0	0	785178	0	0	0	3141144	0
BPBO	1	1	0	522398	260676	2105	0	1513246	1374241	253667
BPDM	1	1	0	0	785213	0	0	0	3141199	0
CARO	2	3	422811	356535	5858	0	1031900	1318879	790437	0
CVSM	2	3	243047	229271	312811	0	689460	1298612	1153044	0
LACA	2	4	2795	198869	584737	0	421413	1561014	1339918	0
LACO	2	4	0	563130	503656	0	25730	1575917	2118872	0
LAIS	2	4	0	377553	407647	0	91943	1307596	1741637	0
LASA	2	4	0	192054	593139	0	57492	1582074	1964413	0
LAST	2	4	381649	389324	40195	0	824474	1676071	1010512	0
LPSP	2	4	0	0	785198	0	0	0	3141213	0
MABR	1	1	0	193886	591318	0	105783	551402	2634700	0
MACA	2	6	0	269345	515844	0	0	410000	2731193	0
MALA	2	6	0	0	785196	0	0	0	3141169	0
MBCP	2	6	9495	314136	461516	0	207168	1749438	1184535	0
MBLG	1	1	0	0	785218	0	0	217641	2923566	0
MBMM	2	4	0	308836	476362	0	0	1172141	1969045	0
PADU	1	2	0	0	785200	0	0	5934	3135251	0
PAMA	1	2	0	0	785190	0	0	0	3141163	0
POVA	1	2	0	0	785190	0	0	118333	3022841	0
RNFO	1	1	0	765420	245362	0	0	2158436	1563295	0
RNLA	1	1	0	313484	471704	0	0	514680	2626494	0
RNPR	1	1	0	584562	325548	0	58332	2552186	1130818	0
RNPT	1	1	194679	590521	0	0	548781	2587634	4781	0
SASL	2	4	0	0	785195	0	0	150743	2990440	0
TOFI	1	1	0	805538	58037	272512	519	2224944	1318394	445048
TPSP	1	2	0	0	785188	0	0	0	3141165	0
VAAG-1	2	3	233269	726820	105536	0	380074	2004031	1160498	0
VAAG-2	2	3	0	460867	325995	0	31292	1777579	1554217	0

ID_site shows the identification code for each of the 37 sites considered here (see Table S1); type refers to the two macro-categories of protected areas/ less impacted sites (1) and artificial/ heavily impacted sites (2); detail show the designation of each site (1= protected area, 2= natural, but not protected area, 3= agricultural ditch or tank, 4= artificial lake, 5= irrigation basin, 6= farm pond); columns from CLC1_R1 to CLC4_5_R1 refer to the area of land classified as, respectively, Corine Class 1 (CLC1- urban areas), Corine Class 2 (CLC2- agricultural areas), Corine Class 3 (CLC3- forests and seminatural areas) and Corine Classes 4 and 5 (CLC4_5- wetlands and water bodies) in a buffer of 500 m, while columns from CLC1_R2 to CLC4_5_R2 show the same variables but considering a buffer of 1000 m.

Table S4. Context/ Landscape variables data from each of the 37 wetlands considered in the Province of Trento, Italy (second part).

ID_site	fordens	street	hf	LST_sprin	LST_summ	LST_aut	LST_wint	LST_aver
BACU	0	8.03	28	13.29	19.50	5.64	0.87	13.82
BASL	0	78.96	9	11.56	18.31	4.89	0.86	12.33
BAVE	0	9.79	10	13.97	20.09	5.90	1.03	14.49
BIIN	54	17.67	29	15.51	21.29	6.35	-0.08	15.81
BILA	91	9.35	26	13.76	19.95	6.22	1.62	14.16
BIRU	51	14.67	30	15.96	22.28	7.84	1.70	16.64
BITA	86	15.20	47	17.20	22.81	8.30	2.72	17.77
BLMA	89	61.57	37	17.35	23.76	8.50	2.71	18.05
BOGIU	0	47.67	9	11.23	18.52	5.28	1.18	17.86
BPBO	65	53.76	30	16.54	22.49	8.88	4.98	17.12
BPDM	61	17.22	11	10.85	17.67	5.01	2.33	17.57
CARO	48	88.78	26	16.52	22.48	8.38	1.47	17.28
CVSM	0	4.71	36	16.51	22.56	7.77	1.43	17.26
LACA	65	20.05	37	16.61	22.59	7.95	2.88	17.24
LACO	0	38.16	25	13.37	19.68	5.62	0.64	13.94
LAIS	38	9.41	38	16.26	21.75	8.51	3.25	16.95
LASA	0	29.48	33	16.15	22.71	9.81	5.21	17.02
LAST	62	86.51	32	15.31	20.86	7.05	3.57	15.66
LPSP	0	199.11	23	4.79	14.14	0.32	-4.55	14.31
MABR	0	49.92	29	13.36	19.95	5.49	0.41	13.65
MACA	0	7.02	9	11.64	18.79	4.90	0.51	11.95
MALA	58	137.10	13	11.48	18.18	5.05	2.06	17.80
MBCP	0	6.69	10	11.87	18.97	5.10	1.02	12.19
MBLG	91	28.24	15	13.51	20.02	6.57	2.98	14.17
MBMM	74	30.08	12	11.54	18.69	5.64	1.29	12.25
PADU	76	5.13	13	12.35	18.79	5.61	0.92	12.81
PAMA	52	905.74	12	5.78	14.10	0.80	-3.40	14.45
POVA	70	24.65	18	11.42	18.06	5.65	3.53	18.00
RNFO	67	276.58	12	14.12	19.98	6.20	2.46	14.96
RNLA	0	59.50	19	12.65	19.30	7.16	4.15	13.70
RNPR	86	170.34	37	15.58	21.02	7.20	2.74	16.01
RNPT	68	69,60	34	14,70	20,18	5,84	0,17	15,07
SASL	0	21,46	9	11,45	18,23	4,93	1,14	12,26
TOFI	0	11,84	10	13,51	19,75	6,37	1,69	14,08
TPSP	0	69,20	17	5,49	14,42	0,91	-3,31	14,51
VAAG-1	43	5,15	24	15,96	21,82	7,46	1,97	16,59
VAAG-2	0	72,09	21	15,76	21,44	7,86	3,93	16,39

ID_site shows the identification code for each of the 37 sites considered here (see Table S1); fordens (forest density) shows the level of tree cover density in a range (percentage); street is the distance to the nearest street (m); hf (human footprint) is and index to express cumulative human pressure on the environment; columns from LST_sprin to LST_aver show the Land Surface Temperature measured respectively at beginning of spring, summer, autumn, winter of 2021 and as average of the sampling season (°C).

Table S5. Transformation of continuous variables in categories for the correspondence analysis, along with ranges for each new category.

VARIABLE NAME	RANGE FOR EACH NEW CATEGORY
n_sp	Equi "1" "n_species 0" "2" "n_species 1" "3" "n_species 2" "4" "n_species>=3"

Aspect	IF (A25>=315 OR A25<=45) val= 1 (Nord)
	IF (A25>45 OR A25<135) val= 2 (est)
	IF (A25<=235 OR A25>=135) val= 3 (sud)
	IF (A25>235 OR A25<315) val= 4 (ovest)

Slope	Equi "1" "slope 0.00000000-2.48788140" "2" "slope 2.48788141-9.07881330" "3" "slope 9.07881331-15.77394965" "4" "slope>=15.77394966"
VPI	User defined "1" "vpi_18 26.00-80.49" "2" "vpi_18 80.50-87.99" "3" "vpi_18>=88.00"
raoq_0408	User defined "1" "raoq_0408_int 7-13" "2" "raoq_0408_int 14-20" "3" "raoq_0408_int>=21"
raoq_0811	User defined "1" "raoq_0811_int 4-13" "2" "raoq_0811_int 14-20" "3" "raoq_0811_int>=21"
CLC_1_R1	User defined "1" "CLC_1_R1 0" "2" "CLC_1_R1 1-194616" "3" "CLC_1_R1>=194617"
CLC_2_R1	Equi "1" "CLC_2_R1 0" "2" "CLC_2_R1 1-313416" "3" "CLC_2_R1>=313417"
CLC_3_R1	Equi "1" "CLC_3_R1 0-342894" "2" "CLC_3_R1 342895-669924" "3" "CLC_3_R1>=669925"
CLC_4_5_R1	User defined "1" "CLC_4_5_R1 0" "2" "CLC_4_5_R1>=2105"
CLC_1_R2	Definite dall'utente "1" "CLC_1_R2 0" "2" "CLC_1_R2 519-207040" "3" "CLC_1_R2>=207041"
CLC_2_R2	Equi "1" "CLC_2_R2 0-409332" "2" "CLC_2_R2 409333-1581996" "3" "CLC_2_R2>=1581997"
CLC_3_R2	Equi

	"1" "CLC_3_R2 4781-1373793" "2" "CLC_3_R2 1373794-2676928" "3" "CLC_3_R2>=2676929"
CLC_4_5_R2	User defined "1" "CLC_4_5_R2 0" "2" "CLC_4_5_R2>=251199"
fordens	User defined "1" "forestdens 0" "2" "forestdens 38-69" "3" "forestdens>=70"
street	Equi "1" "street_dist 4.711013350-17.147720598" "2" "street_dist 17.147720599-59.324379962" "3" "street_dist>=59.324379963"
hf	Equi "1" "h_footprint 9-12" "2" "h_footprint 13-28" "3" "h_footprint>=29"
LST_sprin	Equi "1" "LST_sprin 4.7876344090-12.3530509301" "2" "LST_sprin 12.3530509302-15.5099157642" "3" "LST_sprin>=15.5099157643"
LST_summ	Equi "1" "LST_summ 14.101914890-18.972871080" "2" "LST_summ 18.972871081-21.020140534" "3" "LST_summ>=21.020140535"
LST_aut	Equi "1" "LST_aut 0.3223595510-5.6136448414" "2" "LST_aut 5.6136448415-7.1609708234" "3" "LST_aut>=7.1609708235"
LST_wint	Equi "1" "LST_wint -4.5472727270-1.0247253087" "2" "LST_wint 1.0247253088-2.4631009837" "3" "LST_wint>=2.4631009838"
LST_aver	Equi "1" "LST_aver 11.950635730-14.166051375" "2" "LST_aver 14.166051376-16.642534724" "3" "LST_aver>=16.642534725"

Table S6. Classification of all 36 variables in active or supplementary based on the direct or indirect impact on habitat quality.

VARIABLES	ACTIVE	SUPPLEMENTARY
n_species	x	
o_Anura	x	
o_Caudata	x	
f_Bomb	x	
f_Bufo	x	
f_Ran	x	
subf_Pleur	x	
subf_Sala	x	
hydrop	x	
slope	x	
veget	x	
fish	x	
altitude	x	
Aspect		x
Slope	x	
VPI	x	
raoq_0408		x
raoq_0811		x
type	x	
detail		x
CLC1_R1		x
CLC2_R1		x
CLC3_R1		x
CLC4_5_R1		x
CLC1_R2		x
CLC2_R2		x
CLC3_R2		x
CLC4_5_R2		x
fordens	x	
street	x	
hf		x
LST_sprin		x
LST_summ		x
LST_aut		x
LST_wint		x
LST_aver		x

Table S7. ADDATI analysis output, with details on the four clusters of wetland sites and their respective profiles based on the considered categorical variables.

```
DISTRIBUZIONE DELLE UNITA' NELLA PARTIZIONE CON 4 CLASSI
-----------------------------------------
CLASSE        1     2     3     4   TOT
-----------------------------------------
UNITA'        8    11    16     2    37
PESO(%)     21.6  29.7  43.2   5.4 100.0
-----------------------------------------
              ******************************************
              *  DESCRIZIONE DETTAGLIATA DELLE CLASSI  *
              ******************************************

***************
*  CLASSE  1  *
***************
UNITA': 8 - PESO:  21.62%
UNITA' ASSEGNATE ALLA CLASSE:
U04 U05 U08 U14 U15 U24 U30 U34
UNITA' PIU' VICINA AL CENTRO DELLA CLASSE (d2 =  0.3859) : U30
UNITA' PIU' LONTANA DAL CENTRO DELLA CLASSE (d2 =  1.0069) : U05
RAGGIO DELLA CLASSE : 0.76261
DISTANZA DEL CENTRO DI CLASSE DAL CENTRO GLOBALE :  0.75350
***************
*  CLASSE  2  *
***************
UNITA': 11 - PESO:  29.73%
UNITA' ASSEGNATE ALLA CLASSE:
U06 U07 U16 U26 U27 U29 U31 U32 U35 U36 U37
UNITA' PIU' VICINA AL CENTRO DELLA CLASSE (d2 =  0.5251) : U29
UNITA' PIU' LONTANA DAL CENTRO DELLA CLASSE (d2 =  1.6020) : U26
RAGGIO DELLA CLASSE : 1.05384
DISTANZA DEL CENTRO DI CLASSE DAL CENTRO GLOBALE :  0.69341
```

```
***************
*  CLASSE  3  *
***************
UNITA': 16 - PESO:  43.24%
UNITA' ASSEGNATE ALLA CLASSE:
U01 U02 U03 U09 U10 U11 U17 U18 U19 U20 U21 U22 U23 U25 U28 U33
UNITA' PIU' VICINA AL CENTRO DELLA CLASSE (d2 =  0.4271) : U03
UNITA' PIU' LONTANA DAL CENTRO DELLA CLASSE (d2 =  3.2383) : U18
RAGGIO DELLA CLASSE : 0.98768
DISTANZA DEL CENTRO DI CLASSE DAL CENTRO GLOBALE :  0.42551
***************
*  CLASSE  4  *
***************
UNITA': 2 - PESO:   5.41%
UNITA' ASSEGNATE ALLA CLASSE:
U12 U13
UNITA' PIU' VICINA AL CENTRO DELLA CLASSE (d2 =  0.2614) : U13
UNITA' PIU' LONTANA DAL CENTRO DELLA CLASSE (d2 =  0.2614) : U12
RAGGIO DELLA CLASSE : 0.51127
DISTANZA DEL CENTRO DI CLASSE DAL CENTRO GLOBALE :  1.42446

INERZIA INTERNA (INTRACLASSE) :  0.89189
INERZIA ESTERNA (INTERCLASSE) :  0.45368
INERZIA TOTALE                :  1.34557
QUOTA DI INERZIA SPIEGATA (INERZIA ESTERNA/INERZIA TOTALE): 0.33716

          ************************************
          *  PROFILI DELLE 4 CLASSI STABILI  *
          ************************************
  I simboli  + e -  adottati per descrivere il profilo di classe
  vanno interpretati come segue.
  Il riferimento e` al RAPPORTO tra la frequenza di ciascuna
  variabile nella classe e la sua frequenza globale.
  Con le soglie attuali, tale rapporto risulta cosi rappresentato:
```

```
 ----        - -        ~ ~        + +        ++++   valore
---------->|<---------->|<---------->|<---------->|<---------- del
      0.50       0.80       1.20       2.00        rapporto R

CLASS   NUM   PESO  n_spec n_spec n_spec n_spec  ord_A  ord_A  ord_C  ord_C  fam_B  fam_B fam_Bu
fam_Bu
                         ies 0  ies 1  ies 2 ies>=3  nur 1  nur 2  aud 1  aud 2  omb 1  omb 2  fon 1  fon 2


  1      8   21.6   100.0    0.0    0.0    0.0  100.0    0.0  100.0    0.0  100.0    0.0  100.0    0.0
                  ++++    ~~~    ~~~    ~~~   ++++    ~~~    ++     ~~~    ~~~    ~~~    ++     ~~~
  2     11   29.7     0.0   18.2   18.2   63.6   36.4   63.6    9.1   90.9  100.0    0.0   72.7   27.3
                   ~~~    --     --    ++++    ~~~    ~~~   ----   ++++    ~~~    ~~~    ++     --
  3     16   43.2     0.0   56.2   37.5    6.2    0.0  100.0   93.8    6.2   93.8    6.2   18.8   81.2
                   ~~~    ++     ++    ----    ~~~    ++     ++    ----    ~~~    --    ----    ++
  4      2    5.4     0.0   50.0   50.0    0.0    0.0  100.0   50.0   50.0    0.0  100.0  100.0    0.0
                   ~~~    ++    ++++    ~~~    ~~~    ++     --     ++     ~~~   ++++    ++     ~~~
PROFILO
GLOBALE   37  100.0    21.6   32.4   24.3   21.6   32.4   67.6   67.6   32.4   91.9    8.1   56.8   43.2

 CLASS   NUM   PESO  fam_Ra fam_Ra subf_P subf_P subf_S subf_S  hydr   hydr   hydr   slop   slop   slop
                         nid 1  nid 2 leur 1 leur 2  ala 1  ala 2   op 1   op 2   op 2    e 1    e 2    e 2


  1      8   21.6   100.0    0.0  100.0    0.0  100.0    0.0  100.0    0.0    0.0   37.5    0.0   62.5
                   ++     ~~~    ++     ~~~    ~~~    ~~~    ++     ~~~    ~~~    --     ~~~    ++
  2     11   29.7    36.4   63.6   18.2   81.8   63.6   36.4   54.5   18.2   27.3  100.0    0.0    0.0
                   --     ++    ----   ++++    --    ++++    --     ++    ++++    ++     ~~~    ~~~
  3     16   43.2    50.0   50.0   93.8    6.2  100.0    0.0   87.5   12.5    0.0   43.8    6.2   50.0
                   ~~~    ++     ++    ----    ~~~    ~~~    ~~~    ~~~    ~~~    --    ++++    ++
  4      2    5.4   100.0    0.0  100.0    0.0   50.0   50.0  100.0    0.0    0.0    0.0    0.0  100.0
                   ++     ~~~    ++     ~~~    --    ++++    ++     ~~~    ~~~    ~~~    ~~~   ++++
PROFILO
GLOBALE   37  100.0    59.5   40.5   73.0   27.0   86.5   13.5   81.1   10.8    8.1   56.8    2.7   40.5
```

```
 CLASS    NUM    PESO     vege   vege   vege fish 1 fish 2  altit  altit  altit  altit   slop   slop   slop
                            t 1    t 2    t 2          ude 1  ude 2  ude 3  ude 4    e 1    e 2    e 3


  1      8   21.6    37.5   37.5   25.0   62.5   37.5   37.5   25.0   12.5   25.0   12.5   12.5   25.0
                 ++    ~~~    --    ~~~    ~~~    ++    ~~~    --    ~~~    --    --    ~~~
  2     11   29.7     0.0   36.4   63.6   90.9    9.1   18.2    9.1   18.2   54.5    9.1   45.5   45.5
                 ~~~    ~~~    ++    ++   ----    --   ----   ~~~   ++++  ----    ++    ++
  3     16   43.2    12.5   31.2   56.2   43.8   56.2   31.2   31.2   31.2    6.2   43.8   18.8   12.5
                 --    ~~~    ~~~    --    ++    ~~~    ~~~    ++   ----    ++    --    --
  4      2    5.4   100.0    0.0    0.0  100.0    0.0    0.0  100.0    0.0    0.0    0.0    0.0    0.0
                 ++++   ~~~    ~~~    ++    ~~~    ~~~   ++++   ~~~    ~~~    ~~~    ~~~    ~~~
PROFILO
GLOBALE    37   100.0    18.9   32.4   48.6   64.9   35.1   27.0   27.0   21.6   24.3   24.3   24.3   24.3


 CLASS    NUM    PESO     slop vpi  1 vpi  2  vpi 3 type 1 type 3 forest forest forest   stre   stre   stre
                           e 4                           dens 1 dens 2 dens 3   et 1   et 2   et 3


  1      8   21.6    50.0   25.0    0.0   75.0   25.0   75.0   50.0   25.0   25.0   62.5   25.0   12.5
                 ++    ++    ~~~    ~~~    --    ++    ~~~    --    ~~~    ++    --   ----
  2     11   29.7     0.0    9.1    9.1   81.8   72.7   27.3   27.3   36.4   36.4   18.2   36.4   45.5
                 ~~~    --    ~~~    ~~~    ++    --    --    ~~~    ++    --    ~~~    ++
  3     16   43.2    25.0   12.5    6.2   81.2   50.0   50.0   50.0   37.5   12.5   25.0   37.5   37.5
                 ~~~    ~~~    --    ~~~    ~~~    ~~~    ~~~    ~~~    --    --    ~~~    ~~~
  4      2    5.4   100.0    0.0  100.0    0.0    0.0  100.0   50.0   50.0    0.0   50.0    0.0   50.0
                 ++++   ~~~   ++++   ~~~    ~~~    ++    ~~~    ++    ~~~    ++    ~~~    ++
PROFILO
GLOBALE    37   100.0    27.0   13.5   10.8   75.7   48.6   51.4   43.2   35.1   21.6   32.4   32.4   35.1


 CLASS    NUM    PESO     Aspe   Aspe   Aspe   Aspe raoq_0 raoq_0 raoq_0 raoq_0 raoq_0 raoq_0 detail detail
                            ct 1   ct 2   ct 3   ct 4  408_1  408_2  408_3  811_1  811_2  811_3 _site  _site


  1      8   21.6    12.5   87.5    0.0    0.0   25.0   50.0   25.0   62.5   12.5   25.0   12.5   12.5
                 ----   ++    ~~~    ~~~    ++    ~~~    --    ++   ----   ~~~   ----   ~~~
  2     11   29.7    36.4   63.6    0.0    0.0   18.2   45.5   36.4   45.5   54.5    0.0   63.6   18.2
```

```
                ++    ~~~   ~~~   ~~~   ~~~   ~~~   ~~~   ~~~   ++    ~~~   ++    ++

  3     16    43.2    37.5   62.5    0.0    0.0   12.5   43.8   43.8   31.2   31.2   37.5   43.8    6.2

                ++    ~~~   ~~~   ~~~   --    ~~~   ++    --    ~~~   ++    ~~~   --

  4      2     5.4     0.0  100.0    0.0    0.0    0.0  100.0    0.0    0.0   50.0   50.0    0.0    0.0

                ~~~   ++    ~~~   ~~~   ~~~   ++++  ~~~   ~~~   ++   ++++   ~~~   ~~~

PROFILO

GLOBALE   37   100.0    29.7   70.3    0.0    0.0   16.2   48.6   35.1   40.5   35.1   24.3   40.5   10.8


 CLASS    NUM    PESO   detail detail detail detail  CLC_1  CLC_1  CLC_1  CLC_2  CLC_2  CLC_2  CLC_3  CLC_3

                         _site  _site  _site  _site   _R1 1  _R1 2  _R1 3  _R1 1  _R1 2  _R1 3  _R1 1  _R1 2


  1      8    21.6     0.0   25.0   37.5   12.5   62.5   37.5    0.0   25.0   25.0   50.0   12.5   50.0

                ~~~   ~~~   ++++   ++    ~~~   ++++   ~~~   ~~~   ~~~   ~~~   ----   ++

  2     11    29.7     0.0    0.0    0.0   18.2   90.9    9.1    0.0   54.5   18.2   27.3   18.2   18.2

                ~~~   ~~~   ~~~   ++++   ++    --    ~~~   ++    ~~~   --    --    --

  3     16    43.2    12.5   37.5    0.0    0.0   68.8    6.2   25.0   18.8   25.0   56.2   43.8   37.5

                ~~~   ++    ~~~   ~~~   ~~~   ----   ++    --    ~~~   ~~~   ++    ~~~

  4      2     5.4   100.0    0.0    0.0    0.0   50.0    0.0   50.0    0.0    0.0  100.0  100.0    0.0

               ++++   ~~~   ~~~   ~~~   --    ~~~   ++++   ~~~   ~~~   ++++  ++++   ~~~

PROFILO

GLOBALE   37   100.0    10.8   21.6    8.1    8.1   73.0   13.5   13.5   29.7   21.6   48.6   32.4   32.4


 CLASS    NUM    PESO    CLC_3 CLC_4_ CLC_4_  CLC_1  CLC_1  CLC_1  CLC_2  CLC_2  CLC_2  CLC_3  CLC_3
CLC_3

                         _R1 3 5_R1 1 5_R1 2  _R2 1  _R2 2  _R2 3  _R2 1  _R2 2  _R2 3  _R2 1  _R2 2  _R2 3


  1      8    21.6    37.5   87.5   12.5   25.0   25.0   50.0   25.0   37.5   37.5   50.0   25.0   25.0

                ~~~   ~~~   ++    --    ~~~   ++    --    ~~~   ~~~   ++    --    --

  2     11    29.7    63.6   90.9    9.1   72.7   18.2    9.1   63.6   27.3    9.1    9.1   18.2   72.7

                ++    ~~~   ~~~   ++    --    ----   ++    ~~~   ----  ----   --   ++++

  3     16    43.2    18.8   93.8    6.2   37.5   31.2   31.2   18.8   37.5   43.8   37.5   43.8   18.8

                --    ~~~   --    ~~~   ~~~   ~~~   --    ~~~   ++    ~~~   ++    --

  4      2     5.4     0.0  100.0    0.0    0.0   50.0   50.0    0.0    0.0  100.0   50.0   50.0    0.0

                ~~~   ~~~   ~~~   ~~~   ++    ++    ~~~   ~~~   ++++   ++    ++    ~~~
```

```
PROFILO

GLOBALE    37   100.0    35.1   91.9    8.1   43.2   27.0   29.7   32.4   32.4   35.1   32.4   32.4   35.1


 CLASS    NUM    PESO   CLC_4_ CLC_4_ h_foot h_foot h_foot LST_sp LST_sp LST_sp  LST_s  LST_s  LST_s
LST_a

                              5_R2 1 5_R2 2 print  print  print   rin 1  rin 2  rin 3  umm 1  umm 2  umm 3   ut 1


   1       8    21.6    87.5   12.5   37.5   25.0   37.5   37.5   25.0   37.5   25.0   37.5   37.5   25.0

                    ~~~    ++     ++     --     ~~~    ~~~    --     ~~~    --     ~~~    ~~~    --

   2      11    29.7    90.9    9.1   36.4   36.4   27.3   54.5   18.2   27.3   54.5   18.2   27.3   54.5

                    ~~~    ~~~    ++     ~~~    --     ++     --     --     ++     --     --     ++

   3      16    43.2    93.8    6.2   25.0   31.2   43.8   18.8   50.0   31.2   25.0   43.8   31.2   25.0

                    ~~~    --     ~~~    ~~~    ++     --     ++     ~~~    --     ++     ~~~    --

   4       2     5.4   100.0    0.0    0.0  100.0    0.0    0.0    0.0  100.0    0.0    0.0  100.0    0.0

                    ~~~    ~~~    ~~~   ++++    ~~~    ~~~    ~~~   ++++    ~~~    ~~~   ++++    ~~~

PROFILO

GLOBALE    37   100.0    91.9    8.1   29.7   35.1   35.1   32.4   32.4   35.1   32.4   32.4   35.1   32.4


 CLASS    NUM    PESO    LST_a  LST_a  LST_w  LST_w  LST_w  LST_a  LST_a  LST_a

                              ut 2   ut 3  int 1  int 2  int 3  ver 1  ver 2  ver 3


   1       8    21.6    37.5   37.5   37.5   12.5   50.0   37.5   12.5   50.0

                    ~~~    ~~~    ~~~   ----    ++     ~~~   ----    ++

   2      11    29.7    18.2   27.3   27.3   36.4   36.4   18.2   36.4   45.5

                    --     --     ~~~    ~~~    ~~~    --     ~~~    ++

   3      16    43.2    43.8   31.2   37.5   37.5   25.0   43.8   31.2   25.0

                    ++     ~~~    ~~~    ~~~    --     ++     ~~~    --

   4       2     5.4     0.0  100.0    0.0   50.0   50.0    0.0  100.0    0.0

                    ~~~   ++++    ~~~    ++     ++     ~~~   ++++    ~~~

PROFILO

GLOBALE    37   100.0    32.4   35.1   32.4   32.4   35.1   32.4   32.4   35.1
```

THE MONSTER

Jill Bart

An ugly toad
squats in the corner
of my room.   Twice I swept
him out with a stiff broom.   Blood
spatters his sallow, warty skin.   My
blood? from mosquitos he sucks in? He
watches me, racing heart thumping his side.
I see he too loves life and suddenly I care-
I, this toad’s nightmare.

CHAPTER 5

MORE THAN MEETS THE EYE:

UNRAVELING PATTERNS OF SKIN MICROBIOTA DIVERSITY OF AN OPPORTUNISTIC AMPHIBIAN SPECIES IN DIFFERENT HABITATS

STATUS: IN PREPARATION

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ABSTRACT

With amphibians still holding the record of most threatened taxon among vertebrates, can artificial ponds provide additional habitats for their survival in a fast-changing world? Here we chose *Bombina variegata*, a small anuran that colonizes both natural and artificial water bodies, to investigate the composition of both bacterial and fungal skin communities, as well as the association of these microbiotas with biotic and abiotic variables such as water microbiota, temperature, pH and dissolved oxygen. We sampled populations from 14 sites in the Province of Trento (Italy), including natural pools along the Avisio River, ephemeral ponds near apple orchards, irrigation tanks in vineyards, and higher-altitude farm ponds. Both alpha and beta diversity indices calculated from *B. variegata* skin swabs were found to be statistically different across habitats, although different patterns were found for bacterial and fungal diversity. Beta diversity indices in particular showed close clustering of water and skin communities for each habitat and sampling site. We also found a strong association between skin and water microbial communities in terms of relative abundance of bacterial and fungal taxa shared between the two microbial communities. Water pH, temperature and dissolved oxygen proved to be statistically significant factors affecting both bacterial and fungal diversity, but, again, with different patterns between alpha and beta diversity. Overall, this comprehensive study on bacterial and fungal skin microbiotas highlighted the existence of complex patterns that will require further research to be entirely understood.

INTRODUCTION

As amphibians continue to register population declines worldwide due to human-driven habitat modifications and emerging diseases, their skin microbiota has stirred major interest as both a possible means of adaptation to the changing environment and a barrier against pathogens (for reviews see Jiménez & Sommer, 2017; Bernardo-Cravo et al., 2020; Woodhams et al., 2023). A substantial body of research has been produced in the last ten years, ranging from the simple characterization of bacterial taxa found on amphibian skin (e.g., Hernández-Gómez et al., 2017) to the assessment of microbiota variation between species and/or life stages (e.g., Bird et al., 2018; Hartmann et al., 2023). Unsurprisingly, several studies have focused on the interaction between amphibian skin microbiota and potential pathogens, especially the deadly chytrid fungus *Batrachochytrium dendrobatidis* (Longcore, Pessier & Nichols, 1999; hereafter, Bd). Such studies have shown how bacterial skin communities can influence the resistance of amphibian wild populations to this pathogen (e.g., Bates et al., 2018; Woodhams et al., 2018), and conversely, the impact of the fungal pathogen infection on skin microbiota (e.g., Walke et al., 2015; Chen et al., 2022).

However, only a few articles have been published thus far on the characterization of amphibian skin mycobiota and its potential defensive role against pathogens (e.g., Poonlaphdecha & Ribas, 2016, although this article targets only yeasts; Kearns et al., 2017).

Another aspect that has been the subject of several articles is the impact of extrinsic factors such as overall habitat quality on amphibian skin microbiota variations (see Hernández-Gómez & Hua, 2023 for a recent review). In particular, it has been shown that the use of pesticides for agricultural purposes could affect bacterial and fungal communities hosted by amphibian skin (see McCoy & Peralta, 2018, and references therein). Recently, the strong association between habitat, skin microbiota composition and host pathogen resistance has also been highlighted (Assis et al., 2020; Basanta et al., 2022). However, the relative importance of specific abiotic variables like water temperature and pH to the observed variations in microbiota composition is only very recently becoming a research focus (Varela et al., 2018; Assis et al., 2020; Martínez-Ugalde et al., 2022; Chen et al., 2023). Finally, environmental microbiota has been shown to have an influence on amphibian skin microbiota composition, as it constitutes a primary source of bacteria and fungi for maintaining skin microbiota (e.g., Loudon et al., 2020; Zhu et al., 2022; Leonhardt et al., 2023; Ramírez-Barahona et al., 2023).

Considering the current lack of amphibian skin studies including both bacteria and fungi, here we decided to characterize the richness and composition of both communities living on the skin of *Bombina variegata* (Linneaus, 1758). So far, to our knowledge, skin microbiota diversity of this species has never been investigated, although there are a few studies (Bataille et al., 2016, 2017; Sabino-Pinto et al., 2016) on the congeneric *B. orientalis* (Boulenger, 1890). We also explored the associations between these microbiotas with biotic and abiotic parameters of both natural and artificial habitats, including the microbiota of the wetland itself. Therefore, four habitat categories were defined by degree of human impact to test for differences in bacterial and fungal diversity across habitats. Lastly, the bacterial sequences found in our samples were compared to a 16S sequence database of antifungal bacterial taxa (Woodhams et al., 2015), to identify any strain with Bd-inhibitory activity.

MATERIALS AND METHODS

Study species and study area

Despite being widely distributed across central and southern Europe, the yellow-bellied toad *B. variegata*, is comparatively rare in the study area, the Province of Trento, with most populations in decline (Caldonazzi et al., 2002). This species is most frequently found at low elevations, although adults have been noted up to 1,900 m a.s.l. (Bressi & Barbieri in Sindaco et al., 2006). *B. variegata* was chosen as a target species due to its conservation status in the Province as well as its potential to colonize a plethora of wetlands, including lakes, swamps, rivers, cisterns and even ephemeral water bodies such as tyres filled with rainwater. Populations can be found in deciduous, coniferous and mixed forests, in bushlands, prairies and alluvial plains, but the typical breeding habitat is sunny temporary pools close to woodlands. This species can even tolerate low levels of water pollution (Di Cerbo & Bressi in Lanza et al., 2007). A recent study on *B. variegata* genetic diversity found that gene flow between the small populations of our study area is limited, leading to genetic structuring (Cornetti et al., 2016), although such structuring appears to be moderate compared to northern populations (Hantzschmann et al., 2021).

Regarding its conservation status, *B. variegata* is included in the Convention on the Conservation of European Wildlife and Natural Habitats, Appendix II (Council of Europe, 1979), as well as in Annex II and IV of the European Habitats Directive (Council Directive 92/43/EEC). Although numerous cases of local population decline have been reported (e.g., for Italy, Barbieri et al., 2004; D'Amen & Bombi, 2009), this species is classified as Least Concern (LC) in the IUCN Red List. The evaluation considered its wide distribution, adaptability to a wide range of habitats, presumably large populations, and that its decline appears relatively slow with respect to species included in more threatened categories (IUCN, 2022). It should be noted, however, that this species is reported as Vulnerable (VU) in the Red List of the Province of Trento, because of its small declining populations (Caldonazzi et al., 2002). The main causes for *B. variegata* population decline include habitat loss due to urbanization, road construction and industrial development, and discharge of pollutants into wetlands (Temple & Cox, 2009).

The Autonomous Province of Trento (Italy) is a mountainous region located in the eastern Alps. To date, no studies on Bd presence and virulence have been carried out in the provincial territory, and no amphibian mass mortality events have been reported.

Skin swabs, and water samples and parameters collection

Handling of the animals for research purposes outlined here was authorized by the Italian Ministry of the Environment in derogation to DPR 357/97 on April 6th, 2021. Overall we sampled *B. variegata* individuals from 14 populations in the Province of Trento (Caldonazzi et al., 2002), including two natural pools along the Avisio River (historical lowland habitat of this species in the study area, hereafter corresponding to habitat 'fluvial'- FLU), four ephemeral ponds near apple orchards in the Piana Rotaliana (habitat 'valley bottom ecotone'- VBE), four irrigation tanks in vineyards in Val di Cembra ('agricultural' area- AGR), and four high elevation farm ponds on Monte Baldo, Monte Bondone and the Altopiano della Vigolana ('pasture'- PAS; see also Table S1, Supplementary Information).

A mean of 10 (minimum of six for only one site, and a maximum of 15) adult individuals per site were sampled between April and July 2022 (Table S1, Supplementary Information). During handling, sterile gloves were worn and changed between individuals. All toads were captured by hand or using a dip-net, then rinsed with sterile water to rinse off transient bacteria. Each animal was then swabbed with GenoTube Livestock Swabs (Thermo Fisher Scientific, USA) using gentle strokes on their ventral and axillary areas for about 20s. Since each animal's ventral pattern is unique, we also took individual photos in order to be able to recognize the individuals in the framework of long-time monitoring programs. Two water samples of about 200- 500 ml were collected per site and filtered with Sterivex GP Filter units (pore size 0.22 µm, Millipore cat. no. SVGPL10RC) following the protocol in Zanovello et al. (2023). Water temperature and dissolved oxygen were measured with a Mettler Toledo oximeter model Seven2Go DO, and pH with a Mettler Toledo pH meter model Seven2Go pH/Ion meter S8. Site elevation was measured a posteriori using Google Earth (Google Earth Pro version 7.3.6.9345, 2022). All samples (146 swabs and 28 filters) were transported at about 25° C to the laboratory the same day of sampling, and stored at -20°C until DNA extraction.

Laboratory workflow

All laboratory procedures were carried out under BSL2 biological hoods at the Animal, Environmental and Antique DNA Platform at FEM, and the handling of environmental samples followed the recommended guidelines for eDNA studies (Harper et al., 2019). DNA extraction from the skin swabs was performed using the DNeasy Blood & Tissue Kit (Qiagen, Germany) following manufacturer's instructions for extraction from tissue samples. The lysis step was carried out overnight to ensure extraction of all bacteria and fungi. Filter samples were extracted with the DNeasy PowerWater Sterivex Kit (Qiagen, Germany), following the manufacturer's instructions. Each extraction batch consisted of up to 23 samples for swabs and 11 for filters, plus one negative control (extraction blank).

The bacterial V3-V4 fragment of 16S rRNA gene was amplified with the primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACNVGGGTWTCTAATCC) (Klindworth et al., 2013; Apprill et al., 2015) anchored with Illumina forward and reverse overhang adapters (https://support.illumina.com/documents/documentation/). The reaction mixture consisted of 12.5µl of 2x KAPA HiFi HS ReadyMix, forward and reverse primer to a final concentration of 0.32µM, and 3µl of template DNA. The PCR program followed the KAPA Kit protocol, including 31 cycles and an annealing temperature of 55°C. For fungal metataxonomy, we amplified the ITS1 region with primers ITS1-ITS5-F (GGAAGTAAAAGTCGTAACAAG) and ITS1-ITS2-R (GCTGCGTTCTTCATCGATGC) (Gardes & Bruns, 1993) anchored with Illumina forward and reverse overhang adapters (as above). PCR reactions were carried out in a reaction mixture consisting of 10µl of Promega Flexi Buffer 5X, forward and reverse primer to a final concentration of 0.2µM, 1µl of dNTP's 10mM each, 0.25µl of Promega - GoTaq HS G2 5U/µL, 5µl of $MgCl_2$ 25mM, 5µl of template DNA and H_2O up to a volume of 50µl. The PCR program followed the Promega Taq Kit parameters, with 40 cycles and an annealing temperature of 55°C. Each PCR reaction consisted of up to 91 samples, plus three extraction blanks and two to three PCR blanks (reaction mixture only). All amplification products were then purified with the CleanNGS Kit CNGS-0050 (CleanNA, The Netherlands) following manufacturers' instructions, and sequenced at the FEM Sequencing and Genotyping Platform with paired-end sequencing (2 × 300 base pairs, bp) on an Illumina Miseq (Illumina, San Diego, CA). The sequencing depth per sample was set at 100 000X for bacteria and 30 000X for fungi.

Bioinformatics and statistical analysis

Raw reads processing and taxonomic assignment were performed with the R package DADA2 V1.30.0 (Callahan et al., 2016a, 2016b) using default parameters, except for the following modifications: for the 16S bioinformatic workflow, at the filterAndTrim step the option trimLeft was set as c(17,21) and truncLen as c(280,250). The database used for taxonomic assignment was silva_nr99_v138.1 (Quast et al., 2013). The initial raw reads were 21,407,900, and after bioinformatic processing 10,318,149. For the ITS1 bioinformatic workflow, the trimming step with cutadapt was repeated after changing the orientation of primers to ensure that all primer sequences were removed. Taxonomic assignment was performed with the UNITE database version 9.0 (UNITE general FASTA release for eukaryotes, released on 18.07.2023; Abarenkov et a., 2023), and only fungal taxa were kept for further analysis. The initial dataset consisted of 8,481,680 raw reads, and after bioinformatic processing 3,683,426. At the end of both workflows, all data was converted into a phyloseq object (McMurdie & Holmes, 2013) for further analysis. The prevalence-based method of the R package *decontam* (v1.20.0; Davis et al., 2017) was applied to clean the samples of contaminating taxa present in the negative controls. The number of retrieved taxa (amplicon sequence variants, ASVs) at the end of all bioinformatic steps was 50,984 for bacteria and 7,343 for fungi.

The following statistical analyses were performed with the R packages: *microeco* v1.3.0 (Liu et al., 2021) and *vegan* v2.6.4 (Oksanen et al., 2022). Plots were generated with the R package *ggplot2* (Wickham, 2016) and formatted using GIMP v2.10.18 (The GIMP Development Team, 2019).

To estimate alpha diversity indices (Faith PD, Chao1, Shannon diversity, Inverse Simpson), bacterial and fungal libraries were rarefied to 21,598 and 2,200 reads per sample, respectively. Rarefaction resulted in the loss of one bacterial and six fungal libraries, which were removed from the dataset due to low sequencing depth. Differences in alpha diversity estimates across habitats were tested using nonparametric Dunn's Kruskal-Wallis Multiple Comparisons test with Holm p-value adjustment and using a significance level of 0.05. The number of ASVs shared between water and skin microbiota within and between habitats was estimated using the *microeco* function "trans_venn" following the merging of all libraries according to the considered variable (e.g., habitat, habitat and sample type). The impact of water temperature, dissolved oxygen and pH on alpha diversity estimates was investigated using linear mixed models (lmer) with the R package *lme4* (Bates et al., 2015). Alpha diversity estimates were transformed using the R package *bestNormalize* as follows: Box-Cox transformation was applied to Chao1 and InvSimpson, arcsin transformation to Faith's PD, while Ordered Quantile (ORQ) normalization (orderNorm) was applied to Shannon. Shannon diversity estimates of bacterial and fungal communities found on skin and water samples were also tested as explanatory variables in further analysis.

To estimate beta-diversity indices (Bray-Curtis and Jaccard), bacterial and fungal libraries were first normalized using a scaling with ranked subsampling (SRS, Beule & Karlovsky, 2020). Unconstrained ordination based on Bray-Curtis and Jaccard dissimilarity estimates was performed using non-metric multidimensional scaling (NMDS) with the *vegan* metaMDS function. Beta-diversity estimates were then compared across habitats using ANOSIM (Clarke, 1993) and permutational MANOVA (PERMANOVA, Anderson, 2008) statistical tests with 999 permutations. Multivariate homogeneity of group dispersions was investigated using the *vegan* betadisper function (Anderson, 2006) implemented in the R package *microeco*. Distance-based redundancy analysis (dbRDA, R package *microeco*) was used to investigate if differences in microbiota composition are associated with variation in water temperature, dissolved oxygen, pH, Shannon diversity estimates of skin and water microbial communities. Autocorrelation among environmental variables was calculated using the Spearman correlation coefficient with the *microeco* function cal_autocor. Dissolved oxygen was found to positively correlate with water temperature (Spearman ρ = 0.450) and pH (Spearman ρ = 0.541), and therefore excluded from the model. Similarly, the Shannon diversity estimate of fungal communities in water samples was excluded from the model as it showed a significant correlation with the Shannon diversity measure of bacteria in the water (Spearman ρ = 0.622). The correlation between both bacterial and fungal beta diversity indices with abiotic variables as well as bacterial and fungal alpha diversity (Shannon) of skin and water were tested with a Mantel test in R.

Differential abundance testing was carried out using non-normalized data with the package *ALDEx2* in R (via ALDEx2_t function implemented in *microeco*) (Fernandes et al., 2013). P-values were corrected for false discovery rate using the Benjamini-Hochberg correction (significance cutoff of FDR corrected p-values: 0.05). Heatmaps were generated using the web tool ClustVis (Metsalu & Vilo, 2015) considering bacterial and fungal ASV counts normalized using relative proportions (TSS) multiplied by median sequencing depth.

The presence and distribution of bacteria with putative antifungal activity in skin and water samples was investigated by mapping the bacterial ASVs found in skin and water samples onto the sequence database provided by Woodhams et al. (2015). Briefly, only sequences spanning the entire V3-V4 16S rRNA gene region annotated as *B. dendrobatidis* inhibitory bacteria (BD-inhibitory) were included in the analysis (n: 649 sequences). All ASV sequences from skin and water samples were matched to the corresponding BD-inhibitory sequences trimmed to the V3-V4 16S rRNA gene portion using the closed_reference clustering strategy implemented in MICCA (Albanese et al., 2015). The sequence identity threshold at this step was set to 1.0 (100% identity between query and reference sequence).

RESULTS

The main Phyla detected in skin and water samples from each sampling site and habitat are represented in Figure 1, together with their relative distributions, for both bacteria and fungi. The most common bacterial phyla were Proteobacteria, Bacteroidota and Verrucomicrobiota, while the most common fungal taxon in skin swabs was Ascomycota, followed by Basidiomycota. Differently from skin samples, the majority of fungal taxa found in the water samples could not be classified even at the Phylum taxonomic level (Figure 1B). Here, and to our knowledge, for the first time in the study area, 28 ASVs identified as *B. dendrobatidis* were found in ten swab samples, four from the FLU habitat, three from PAS, two VBE and one AGR.

Despite the lack of taxonomic information for the fungal communities in water samples, we found a clear association between skin and water microbial communities, as shown by the relative abundance of bacterial and fungal ASVs shared between the two microbial communities (Figure S1, Supporting Information). In fact, despite being numerically low in terms of absolute numbers (e.g., only 50 bacterial ASVs were shared by skin and water samples from all four habitats, Figure S1A, B, Supporting Information) ASVs shared by skin and water communities both within and across habitats were detected with high frequency (Figure S1C, D).

Figure 1. Taxonomic classification to Phylum and relative abundance of taxa of *B. variegata* skin (B) and water (W) microbiota. Upper panel: Bacteria; lower panel: Fungi. Upper fields (1_FLU etc.) refer to habitat types; codes below (AMB-1_1 etc.) to sampled sites. Main phyla are reported in the legend on the right.

The alpha diversity estimates calculated from *B. variegata* skin swabs (shown in Figure 2) varied between habitats, with individuals from FLU and AGR showing significantly lower bacterial Faith's phylogenetic diversity and Chao1 richness estimates than the other two habitats. However, Shannon diversity index highlighted significant differences in bacterial diversity estimates across all four habitats, with FLU being characterized by significantly lower diversity than AGR (Dunn's Kruskal-Wallis Multiple Comparisons, $p<0.05$; Figure 2, Table S2). We found that fungal richness (i.e., Chao1 and Shannon diversity) also varied significantly between habitats, although with a different pattern. In fact, in this case our results indicate a clear division between the highly diversified habitat VBE and the two less diversified habitats AGR and PAS (Dunn's Kruskal-Wallis Multiple Comparisons, $p<0.05$; Figure 2, Table S2), with FLU skin-associated mycobiota lying between these extremes. Interestingly, significant differences in both bacterial and fungal alpha diversity estimates were also observed across sampling sites of the same habitat (Dunn's Kruskal-Wallis Multiple Comparisons, p<0.05; Figure S2).

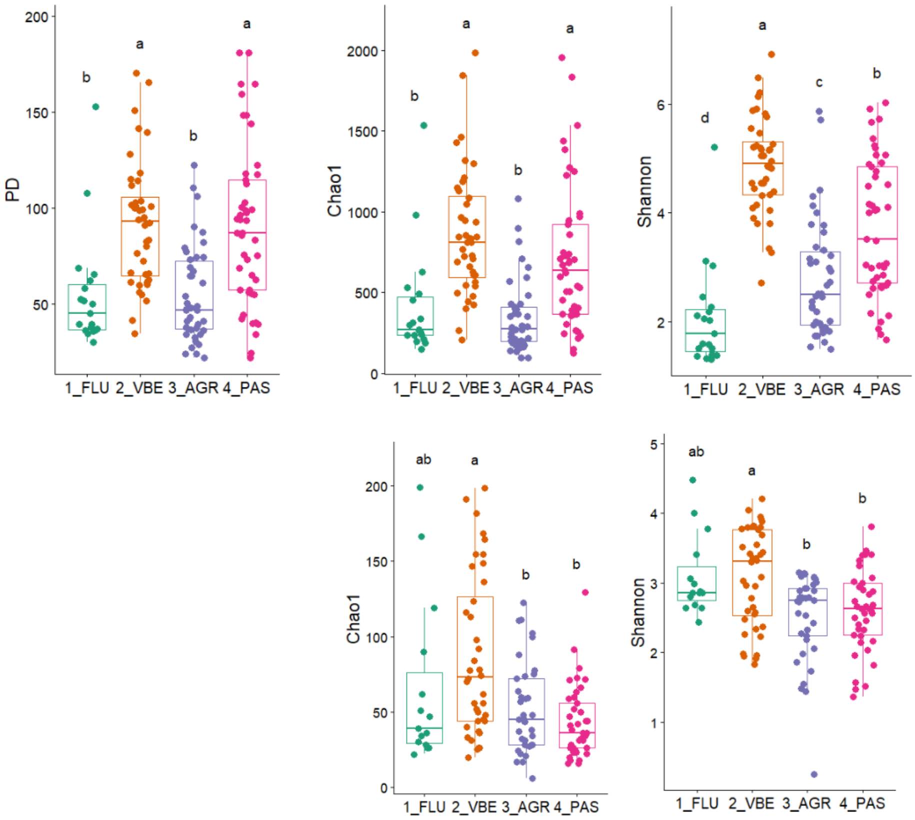

Figure 2. Alpha diversity estimates of *B. variegata* skin microbiota across the four habitats (FLU, VBE, AGR, PAS). Upper panel: Bacteria; lower panel: Fungi.

The composition of these skin microbial communities, as measured by Bray-Curtis and Jaccard dissimilarity indices, also varied greatly between habitats, with skin samples collected on different habitats showing significant differences in the amount of dispersion around centroids (Beta dispersions for Bray-Curtis F: 40.50, p-value ≤ 0.001; for Jaccard F: 6.2695, p-value ≤ 0.001). Nevertheless, ordination of skin samples according to beta diversity estimates highlighted that samples corresponding to the same habitat cluster more closely together (ANOSIM Bray-Curtis R: 0.248, p-value ≤ 0.001; Jaccard R: 0.601, p-value ≤ 0.001), especially if only presence/absence data were considered (Jaccard dissimilarity PERMANOVA R2: 0.075, F: 10.63, p-value ≤ 0.001; Figure 3B). The clustering is less evident for the plots based on Bray-Curtis distances, especially for bacterial communities (PERMANOVA R2: 0.183, F: 3.85, p-value ≤ 0.001; Figure 3A). Instead, the fungal communities were more distinctly divided into clusters corresponding to the four habitat types. However, again, separation between habitats was more pronounced when ecological dissimilarity between communities was estimated using the Jaccard index (ANOSIM R: 0.5204, p-value ≤ 0.001; PERMANOVA R2: 0.074, F: 3.450, p-value ≤ 0.001), suggesting that the presence of common taxa, rather than their abundance, might be the driver of observed variability (as shown in Figure 3C,D, and Table S4, Supporting Information). Of note, the close clustering of water and skin communities for each habitat and sampling site (Figure S3) indicates that bacterial and fungal communities found on skin samples are characterized by a marked compositional similarity with the corresponding water samples (PERMANOVA habitat:sample type interaction term p-value ≤ 0.001 for both Bray-Curtis and Jaccard indices).

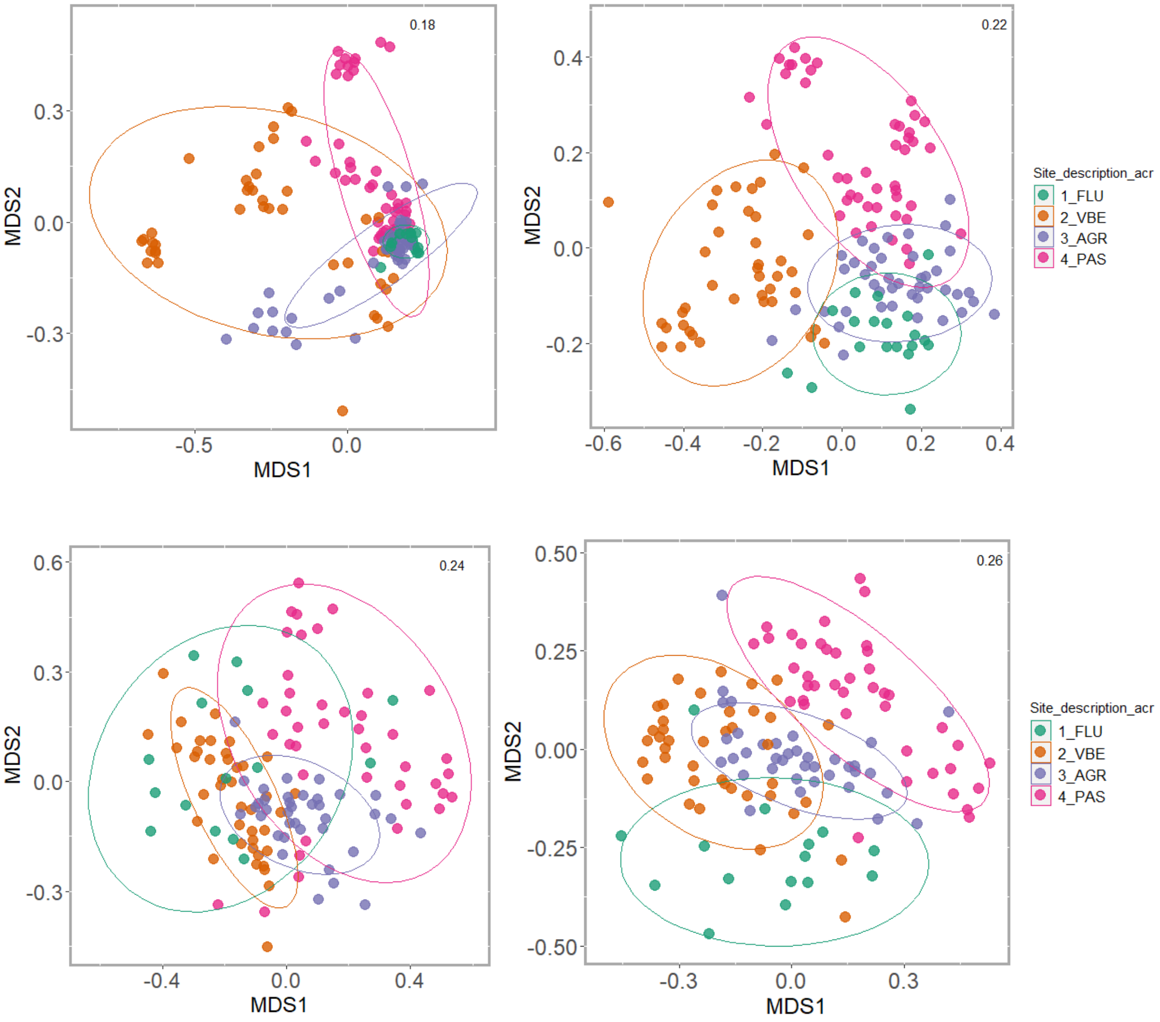

Figure 3. Beta diversity estimates of *B. variegata* skin microbiota across the four habitats (FLU, VBE, AGR, PAS). Upper panel: Bacteria; lower panel: Fungi. Left: Bray-Curtis; Right: Jaccard.

The comparison of skin bacterial communities at ASVs level identified 17 bacterial taxa showing significant differences in their abundance across habitats (Figure 4). The clustering of skin samples according to abundance of these differential ASVs highlighted a clear separation between VBE and the other habitats (Figure 4A) and, to a lower extent, the separate grouping of FLU samples from the other two habitats (i.e., AGR and PAS, Figure 4B). In detail, ten, five and three taxa found in FLU samples showed significant differences in terms of abundances compared to the same taxa in, respectively, VBE, PAS and AGR samples (Figure 4A). Most differential taxa are commonly found in water samples and belong to the following taxonomic classes: Gammaproteobacteria (N: 6), Bacteroidia (N: 4), Alphaproteobacteria (N: 3), Gracilibacteria (N: 2), Planctomycetes (N: 1) and Verrucomicrobiae (N: 1). According to their taxonomic classification, some differential ASVs belonged to the same bacterial genera of species of known anti-Bd activity (e.g., Aeromonas, Chryseobacterium) or, instead, potentially pathogenic (e.g., Aeromonas). However, none of the 63 ASVs from our study that matched a known Bd-inhibitory bacterial taxa (according to Woodhams et al., 2015) were found to be differentially present or abundant across habitats (Figure S4).

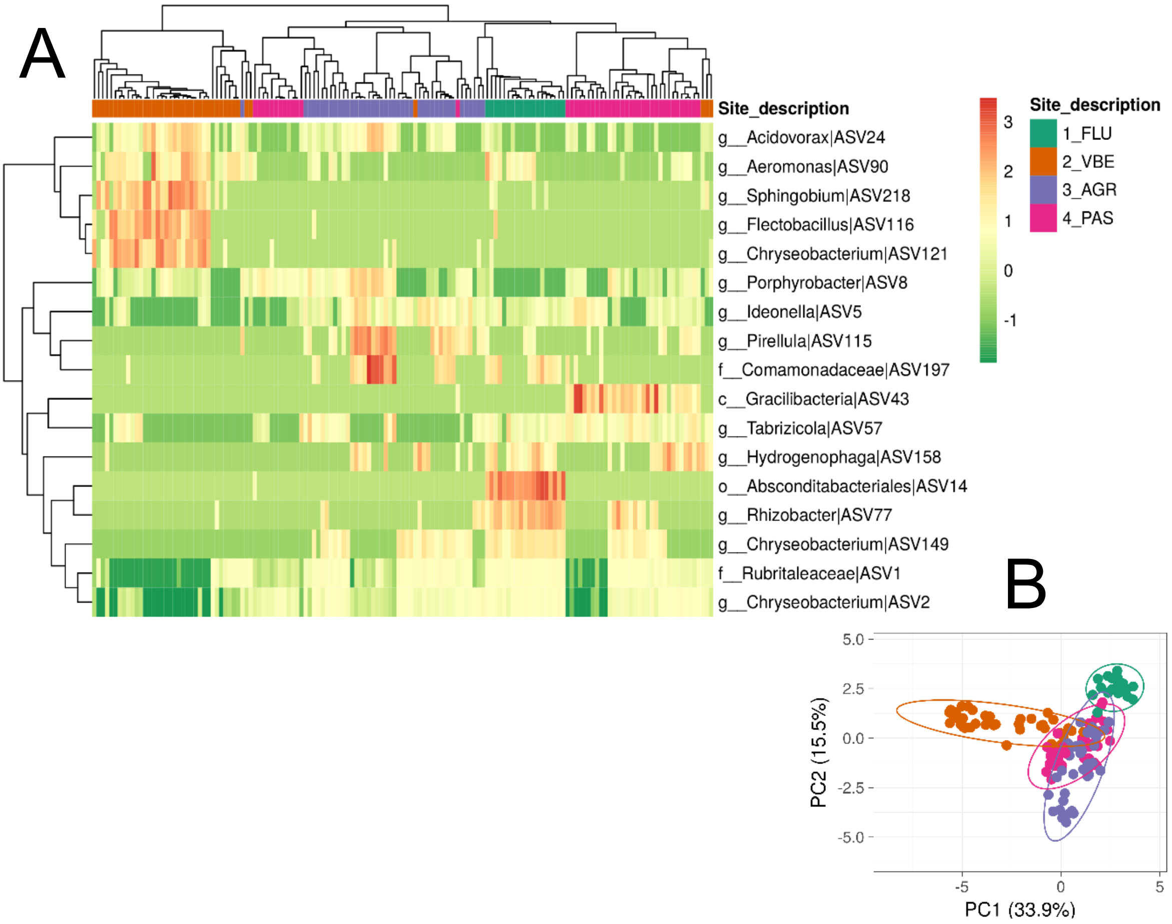

Figure 4. Heatmap (A) and PCA (B) of the 17 bacterial taxa of *B. variegata* skin with significant differences in abundance across habitats (FLU, VBE, AGR, PAS). To generate both Heatmap and PCA, original values ASV abundance were ln(x + 1)-transformed. In (A) rows were centered; unit variance scaling was applied to rows; both rows and columns were clustered using correlation distance and average linkage (74 rows, 147 columns). For the PCA, unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. X and Y axis show principal components 1 and 2 that explain 33.9% and 15.5% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. N = 147 data points.

Association between skin bacterial diversity (i.e. PD, Chao1, Shannon) and the following explanatory variables: temperature, pH, dissolved oxygen and skin fungal diversity and water bacterial diversity was investigated using linear mixed models (Table S5). Within these models, we found that skin bacterial PD displays statistically significant and positive associations with the estimated skin fungal diversity (diversity index: Shannon; beta = 0.92, 95% CI [0.59, 1.24], t(140) = 5.63, p < .001; Std. beta = 0.41, 95% CI [0.27, 0.56]) and dissolved oxygen (beta = 0.27, 95% CI [0.05, 0.50], t(140) = 2.41, p = 0.017; Std. beta = 0.35, 95% CI [0.06, 0.63]). Additionally, we found that water pH has a statistically significant and negative effect (beta = -3.30, 95% CI [-5.39, -1.21], t(140) = -3.12, p =0.002; Std. beta = -0.60, 95% CI [-0.97, -0.22]) on skin bacterial PD. Significant associations were also found between skin bacterial Chao1 (as response variable) and skin fungal diversity (diversity index: Shannon; beta = 1.67, 95% CI [1.08, 2.26], t(140) = 5.57, p < .001; Std. beta = 0.40, 95% CI [0.26, 0.54]), as well as dissolved oxygen (beta = 0.43, 95% CI [9.13e-03, 0.85], t(140) = 2.02, p = 0.045; Std. beta = 0.29, 95% CI [6.10e-03, 0.57]) and pH (beta = -5.89, 95% CI [-9.90, -1.88], t(140) = -2.90, p = 0.004; Std. beta = -0.56, 95% CI [-0.94, -0.18]) (Table S6). Furthermore, we found that skin fungal diversity (diversity index: Shannon), but not water pH and dissolved oxygen, has a statistically significant effect (beta = 1.23, 95% CI [0.68, 1.79], t(142) = 4.40, p < .001; Std. beta = 0.28, 95% CI [0.16, 0.41]) on skin bacterial diversity estimated as Shannon index. Within the same models, water temperature and water bacterial diversity did not play a statistically significant effect on skin bacterial alpha diversity (i.e. PD, Chao1, Shannon).

The Mantel tests highlighted, instead, that temperature differences had the strongest correlation with the observed differences in bacterial (Bray-Curtis R=0.53, p= 0.002 and Jaccard R=0.43, p= 0.001) and fungal beta diversity estimates (Bray-Curtis R=0.31, p= 0.002 and Jaccard R=0.30, p= 0.002) (Table S6, Supplementary Information). Conversely, weaker and/or not significant correlations were found between beta diversity estimates and pH, dissolved oxygen, alpha diversity estimates (see Table S6 and Figure S5).

A total of 28 ASVs identified as *B. dendrobatidis* were found in four swab samples from the FLU habitat, three from PAS, two VBE and one AGR. However, none of the sampled individuals had notable symptoms of pathogen infection. Overall, 63 ASVs from our *B. variegata* skin dataset found a match with the available database of bacterial taxa with anti-Bd activity. However, the relative abundances of these Bd-inhibitory bacterial taxa did not show a correlation with the putative *B. dendrobatidis* ASVs detected in our skin swabs (Figure S4, Supplementary Information).

DISCUSSION

This study aimed to characterize diversity of microbial communities living on the skin of *B. variegata,* a species whose microbiota has not been studied so far, considering populations living in a variety of habitats, including artificial and/or impacted by human activities wetlands. This study is also one of the first focusing on both bacterial and fungal diversity, providing relevant insights into this relatively unexplored component of wild populations biodiversity. In fact, we highlighted complex, and sometimes opposing, trends, which will require more studies to be fully understood. These first, promising, results also support the hypothesis that human activities contribute in shaping amphibian skin microbiota, as significant differences were found between habitat types.

Regarding the main bacterial Phyla detected, Proteobacteria, Bacteroidota and Verrucomicrobiota were found also on the skin of the congeneric *B. orientalis,* another opportunistic species, by Sabino-Pinto et al. (2016), although the relative abundance of Verrucomicrobiota in our study was generally higher (despite some differences among habitats). Overall, our samples show higher diversity with respect to previous studies on *B. orientalis,* even when only considering the Phylum level (Bataille et al., 2016; Sabino-Pinto et al., 2016). Considering less related host species, the three bacterial Phyla mentioned above have been reported among the most prevalent taxa by other studies on amphibians (e.g., Belasen et al., 2021; García-Sánchez et al., 2022; Chen et al., 2023; Wei et al., 2023; Bates et al., 2023). The high prevalence of Ascomycota taxa is in agreement with a previous study on skin mycobiota of several anuran species (Kearns et al., 2017), while the presence of Basidiomycota species has already been reported for anurans by Poonlaphdecha and Ribas (2016). Both taxa were found also by Medina and colleagues (2019). Another recent study, however, did not mention either of these fungal Phyla among the most abundant ones (García-Sánchez et al., 2022). It is thus plausible that fungal diversity shows high variability across geographical areas and/or species (Medina et al., 2019). Interestingly, the majority of fungal taxa found in the water samples remains unfortunately unidentified. In fact, so far most studies addressing wetlands fungal diversity selectively targeted plant-associated mycorrhizal or phyllospheric fungi (e.g., Bohrer et al., 2004; Turner et al., 2000; Young-Hyun et al., 2015; Zhan et al., 2021). Therefore, there is currently a lack of information on freshwater fungal species not associated with plants (Gandhi et al., 2018; Onufrak et al., 2020). Considering the evident influence of fungal water communities on those living on this species' skin, we suggest further exploring this line of research, in order to deepen our understanding of how environmental and skin mycobiota interact with one another.

We found a relatively low number of ASVs shared between skin and water, although present with a high relative abundance in the samples. Similar numbers have been reported, for other amphibian species, by other authors (e.g., Leonhardt et al., 2023), although in some cases a direct comparison is hindered by the use of operational taxonomic units (OTUs) instead of ASVs (Bates et al., 2018; Hernández-Gómez et al., 2018). However, other studies found instead higher proportions of shared ASV/OTUs, which led the authors to suggest that these trends could be influenced by the host species or habitat (e.g., Bataille et al., 2016; Loudon et al., 2020). We found a rather high number of unique ASVs in skin samples, which was highlighted also by Bird et al. (2018) and Martínez-Ugalde et al. (2022). Nonetheless, the overall *B. variegata* skin microbial community appears to be largely made of the few, but highly abundant, ASVs that are also found in the environment, thus supporting the influence of habitat conditions on skin microbiota.

Moreover, our results showed that both alpha and beta diversity estimates for skin microbiota were significantly different between populations living in distinct habitats, three of which (VBE, AGR, PAS) are impacted by human activities, confirming the results for other amphibian species. In fact, several studies have demonstrated that amphibian populations from different habitats present divergent skin microbiota (e.g., *Thoropa thaofora*: Belasen et al., 2021; *Anaxyrus boreas*: Basanta et al., 2022). Land use type, agricultural antimicrobials, and even proximity to roads have been shown to shape microbiota diversity in several amphibian species (Hughey et al., 2017; Hernández-Gómez et al., 2020; Jiménez et al., 2020; Wuerthner et al., 2022). The unexpected similarity in alpha diversity indices between FLU and AGR, instead, could be explained by the geographical closeness of the sites (as reported also by Weitzman et al., 2023), all in the Avisio Valley despite being at different elevations.

Beta diversity estimates of the fungal dataset showed an evident division into clusters corresponding to the considered habitats (Figure 3). García-Sánchez and colleagues (2023) also reported that fungal skin community composition appears to be more strongly influenced by climatic conditions than that of bacterial communities, which they found more dependent on host phylogenetic relatedness. Another study highlighted the high heterogeneity of fungal communities in their dataset, although the relative importance of phylogenetic relatedness, geographical distance and habitat conditions in shaping these communities was not clarified (Medina et al., 2019). Overall, our results support the hypothesis of a strong effect of environmental conditions on amphibian skin mycobiota.

In the present study, water pH, temperature and dissolved oxygen appear to be significant factors affecting both bacterial and fungal diversity of *B. variegata*, although with different patterns between alpha and beta diversity. The influence of these variables has only been described for bacterial microbiota composition thus far. Martínez-Ugalde et al. (2022) found that temperature and pH strongly impacted microbial variations, while dissolved oxygen had a smaller, but still statistically significant, influence. Another study highlighted a negative correlation between microbiota diversity and both temperature and pH (Chen et al., 2023). Varela et al. (2018) also confirmed the negative correlation of pH and bacterial skin communities' diversity. The role of all three factors (temperature, pH and dissolved oxygen) had previously been demonstrated also by Assis et al. (2020), although the authors did not distinguish the effect of the single variables. Based on our results, fungal skin communities appear to be particularly influenced by water temperature and, secondly, dissolved oxygen, while also showing a positive correlation with both fungal and bacterial diversity in the water.

For the first time in the study area, we report that ASVs identified as *B. dendrobatidis* were found in ten swab samples. As four of them were sampled from the FLU habitat, three from PAS, two VBE and one AGR, there was no clear pattern associated with habitat or any other variable. However, none of the sampled individuals appeared to be affected by the pathogen presence. Nonetheless, this result, if confirmed with further tests, is highly relevant for amphibian conservation in the study area as the protocol applied here has the potential of providing information on the spread of this pathogen.

We found a relatively low number of ASVs matching the inhibitory bacteria from Woodhams et al. (2015), which can be explained considering that most of the reference sequences of the inhibitory bacteria were obtained from taxa native to other continents (e.g., America, New Zealand) and only 89 were from Europe (Switzerland). This underrepresentation of bacteria could underestimate the true number of protective ASVs, and many inhibitory taxa could be missed from this analysis. Although several studies have reported a negative effect of human-related disturbances (i.e., roads, agriculture, invasive vegetation, and pollutants) on the relative abundance of Bd-inhibitory bacteria on the skin of amphibians (see Hernández-Gómez & Hua, 2023, and references therein), no clear pattern emerged in our samples.

The bacterial and fungal skin communities of *B. variegata* showed highly complex, and sometimes opposing, patterns when considering the four habitat types involved in this study. Therefore, although important differences have emerged between habitats, a clear definition of ‘healthy’ populations is still missing. Nevertheless, as both microbial communities have shown a strong association with environmental microbial communities, we suggest that conservation strategies for *B. variegata*, and amphibians more in general, take into account aquatic/environmental microbiota and the impact of anthropogenic activities in these environments. To achieve this, we urge taking into account both bacterial and fungal communities in future studies, as well as their reciprocal interactions with environmental variables and each other.

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DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: LZ, MG, PP, GB, HCH; acquired the funding: MG, PP, GB, HCH; collected the samples: LZ, MG, SC, GG, ILP; performed laboratory analyses: LZ, MG; completed bioinformatic and statistical analyses: LZ, GG; drafted the manuscript: LZ, GG, HCH; read, commented and approved the manuscript: all authors.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests. LZ was supported by a PhD scholarship cofunded by the University of Ferrara and MUSE - Science Museum Trento. HCH and MG were partially funded by the Ledro Alps and Judicaria UNESCO Biosphere Reserve (Acqua e Vita project). The authors would like to thank the Fondazione E. Mach for the use of laboratory facilities.

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SUPPLEMENTARY INFORMATION

Table S1. General description of habitats in the Province of Trento considered in this study and sample size for skin and water.

Habitat	Code	Location	N. skin samples	N. water samples	Altitude	pH	Diss. oxygen	Temp.
FLU	AMB-1_1	Pozzolago, Lona Lases	10	2	411	7.76	7.86	18.6
FLU	AMB-1_2	Località Falesia di Prà, Segonzano	9	2	499	8.73	8.73	20.9
VBE	AMB-2_1	San Michele all'Adige,	10	2	210	7.52	0.44	13.2
VBE	AMB-2_2	Maso delle Part- Maso Inon, Mezzolombardo	15	2	211	7.15	5.78	18.1
VBE	AMB-2_3	Zambana	6	2	199	8.29	7.88	31.1
VBE	AMB-2_4	Mezzolombardo	10	2	209	7.23	1.94	19.8
AGR	AMB-3_1	Verla vasca 1, Giovo	10	2	471	8.45	8.1	22
AGR	AMB-3_2	Verla vasca 2, Giovo	11	2	470	9.15	10.61	22.7
AGR	AMB-3_3	Cembra vasche 1 e 3, Lisignago	10	2	486	8.06	5.78	25.7
AGR	AMB-3_4	Lisignagno vasca 1	11	2	499	6.83	0.05	22.8
PAS	AMB-4_2	Monte Baldo Postemon, Brentonico	11	2	1466	7.32	2.42	23.5
PAS	AMB-4_3	Postemon, Monte Baldo, Brentonico	12	2	1379	7.35	3.28	19.4
PAS	AMB-4_4	Malga Campei, Monte Baldo, Brentonico	11	2	1306	7.21	1.01	19.2
PAS	AMB-4_5	Malga Palazzo, Besenello	11	2	1533	6.96	13.72	29.1

Habitat = codes referred to each habitat type; Code = identification codes for each site; Location = Municipality/ local name of the site location; N. skin samples = number of *B. variegata* skin samples collected; N. water samples = number of water samples collected; other variables showed are Altitude of the site, and water pH, dissolved oxygen (Diss. oxygen), and temperature (Temp.).

Table S2. Summary of Dunn's Kruskal-Wallis Multiple Comparisons. Statistical tests were performed using habitat and sampling site as explanatory variables.

Target microbiota	Diversity index	Group	Comparison	Z	P.unadj	P.adj	Significance
Bacteria	PD	2_VBE	2_VBE - 4_PAS	0.6253747	5.32E-01	1.00E+00	ns
	PD	2_VBE	2_VBE - 3_AGR	4.8990177	9.63E-07	5.78E-06	***
	PD	2_VBE	2_VBE - 1_FLU	-4.0993872	4.14E-05	1.66E-04	***
	PD	4_PAS	4_PAS - 3_AGR	-4.4112579	1.03E-05	5.14E-05	***
	PD	4_PAS	4_PAS - 1_FLU	-3.678043	2.35E-04	7.05E-04	***
	PD	3_AGR	3_AGR - 1_FLU	-0.2164913	8.29E-01	8.29E-01	ns
	Chao1	2_VBE	2_VBE - 4_PAS	1.9411099	5.22E-02	1.04E-01	ns
	Chao1	2_VBE	2_VBE - 3_AGR	6.3807383	1.76E-10	1.06E-09	***
	Chao1	2_VBE	2_VBE - 1_FLU	-4.4356149	9.18E-06	3.67E-05	***
	Chao1	4_PAS	4_PAS - 3_AGR	-4.6043904	4.14E-06	2.07E-05	***
	Chao1	4_PAS	4_PAS - 1_FLU	-2.975408	2.93E-03	8.78E-03	**
	Chao1	3_AGR	3_AGR - 1_FLU	0.6286823	5.30E-01	5.30E-01	ns
	Shannon	2_VBE	2_VBE - 4_PAS	3.4850509	4.92E-04	1.48E-03	**
	Shannon	2_VBE	2_VBE - 3_AGR	6.4633039	1.02E-10	5.12E-10	***
	Shannon	2_VBE	2_VBE - 1_FLU	-7.1124831	1.14E-12	6.84E-12	***
	Shannon	4_PAS	4_PAS - 3_AGR	-3.1256323	1.77E-03	3.55E-03	**
	Shannon	4_PAS	4_PAS - 1_FLU	-4.4751915	7.63E-06	3.05E-05	***
	Shannon	3_AGR	3_AGR - 1_FLU	-2.0029736	4.52E-02	4.52E-02	*
Fungi	Chao1	2_VBE	2_VBE_-_3_AGR	2.90657823	3.65E-03	0.01827	*
	Chao1	2_VBE	2_VBE_-_1_FLU	-1.86197572	6.26E-02	0.25043	ns
	Chao1	2_VBE	2_VBE_-_4_PAS	4.25488306	2.09E-05	0.00013	***
	Chao1	3_AGR	3_AGR_-_1_FLU	0.32419064	7.46E-01	0.74579	ns
	Chao1	3_AGR	3_AGR_-_4_PAS	1.24640751	2.13E-01	0.42523	ns
	Chao1	1_FLU	1_FLU_-_4_PAS	1.26545956	2.06E-01	0.61712	ns
	Shannon	2_VBE	2_VBE_-_1_FLU	-0.06927829	9.45E-01	1	ns
	Shannon	2_VBE	2_VBE_-_3_AGR	3.17050578	1.52E-03	0.00761	**
	Shannon	2_VBE	2_VBE_-_4_PAS	3.19315574	1.41E-03	0.00844	**
	Shannon	1_FLU	1_FLU_-_3_AGR	2.29406256	2.18E-02	0.08715	ns
	Shannon	1_FLU	1_FLU_-_4_PAS	2.28220265	2.25E-02	0.06743	ns
	Shannon	3_AGR	3_AGR_-_4_PAS	-0.059669	9.52E-01	0.95242	ns
	InvSimpson	2_VBE	2_VBE_-_1_FLU	0.70063922	4.84E-01	0.96706	ns
	InvSimpson	2_VBE	2_VBE_-_3_AGR	2.96250548	3.05E-03	0.01831	*
	InvSimpson	2_VBE	2_VBE_-_4_PAS	2.84159606	4.49E-03	0.01796	*
	InvSimpson	1_FLU	1_FLU_-_3_AGR	2.9006091	3.72E-03	0.01862	*
	InvSimpson	1_FLU	1_FLU_-_4_PAS	2.79577515	5.18E-03	0.01553	*
	InvSimpson	3_AGR	3_AGR_-_4_PAS	-0.19499921	8.45E-01	0.84539	ns

Tables S3. Results of PERMANOVA on beta diversity of both Bacteria and Fungi, considering skin and water samples.

Target microbiota	Sample type	Dissimilarity index	Variable	Df	SumOfSqs	R2	F	Pr(>F)	
Bacteria	Skin samples	Bray-Curtis	Habitat	3	9.293	0.18339	10.63	0.001	***
			Residual	142	41.381	0.81661			
			Total	145	50.674	1			
		Jaccard	Habitat	3	5.112	0.07517	3.8474	0.001	***
			Residual	142	62.89	0.92483			
			Total	145	68.002	1			
	Skin and water samples	Bray-Curtis	Habitat	3	9.233	0.1378	10.0559	0.001	***
			Sample type	1	4.064	0.06065	13.2773	0.001	***
			Habitat:Sample type	3	2.901	0.04329	3.1591	0.001	***
			Residual	166	50.804	0.75826			
			Total	173	67.001	1			
		Jaccard	Habitat	3	5.536	0.06784	4.1916	0.001	***
			Sample type	1	0.937	0.01148	2.1284	0.001	***
			Habitat:Sample type	3	2.048	0.0251	1.5506	0.001	***
			Residual	166	73.084	0.89558			
			Total	173	81.605	1			
Fungi	Skin samples	Bray-Curtis	Habitat	3	6.494	0.12559	6.176	0.001	***
			Residual	129	45.212	0.87441			
			Total	132	51.706	1			
		Jaccard	Habitat	3	4.411	0.07428	3.4503	0.001	***
			Residual	129	54.969	0.92572			
			Total	132	59.379	1			
	Skin and water samples	Bray-Curtis	Habitat	3	7.52	0.10828	7.0878	0.001	***
			Sample type	1	2.45	0.03527	6.9258	0.001	***
			Habitat:Sample type	3	2.54	0.03657	2.3935	0.001	***
			Residual	161	56.943	0.81988			
			Total	168	69.453	1			
		Jaccard	Habitat	3	5.366	0.07012	4.2156	0.001	***
			Sample type	1	1	0.01307	2.3576	0.001	***
			Habitat:Sample type	3	1.851	0.02419	1.4542	0.001	***
			Residual	161	68.312	0.89262			
			Total	168	76.53	1			

Table S4. Results of pairwise ADONIS2 on beta diversity of both Bacteria and Fungi, considering habitat type.

Target microbiota	Dissimilarity index	Groups	R2	F	p.value	p.adjusted	Significance
Bacteria	Bray	1_FLU_vs_3_AGR	0.0890147	5.765041	0.001	0.001	***
		2_VBE_vs_1_FLU	0.23094606	17.117038	0.001	0.001	***
		2_VBE_vs_3_AGR	0.13889633	12.904028	0.001	0.001	***
		2_VBE_vs_4_PAS	0.10447067	9.682615	0.001	0.001	***
		4_PAS_vs_1_FLU	0.14424227	10.450412	0.001	0.001	***
		4_PAS_vs_3_AGR	0.08058555	7.450146	0.001	0.001	***
	Jaccard	1_FLU_vs_3_AGR	0.0457373	2.827839	0.001	0.001	***
		2_VBE_vs_1_FLU	0.05034614	3.021869	0.001	0.001	***
		2_VBE_vs_3_AGR	0.05005939	4.215791	0.001	0.001	***
		2_VBE_vs_4_PAS	0.05152401	4.508805	0.001	0.001	***
		4_PAS_vs_1_FLU	0.04983432	3.251778	0.001	0.001	***
		4_PAS_vs_3_AGR	0.05092821	4.561191	0.001	0.001	***
Fungi	Bray	2_VBE_vs_3_AGR	0.072169	5.833686	0.001	0.001	***
		2_VBE_vs_1_FLU	0.05329657	2.983741	0.001	0.001	***
		2_VBE_vs_4_PAS	0.10009136	8.786689	0.001	0.001	***
		3_AGR_vs_1_FLU	0.09263758	5.104773	0.001	0.001	***
		3_AGR_vs_4_PAS	0.09831396	8.286544	0.001	0.001	***
		1_FLU_vs_4_PAS	0.0710644	4.131048	0.001	0.001	***
	Jaccard	2_VBE_vs_3_AGR	0.04215922	3.301114	0.001	0.001	***
		2_VBE_vs_1_FLU	0.04051868	2.238178	0.001	0.001	***
		2_VBE_vs_4_PAS	0.05451323	4.554845	0.001	0.001	***
		3_AGR_vs_1_FLU	0.04942069	2.599504	0.001	0.001	***
		3_AGR_vs_4_PAS	0.05469676	4.397482	0.001	0.001	***
		1_FLU_vs_4_PAS	0.04857599	2.757029	0.001	0.001	***

Table S5. Results from linear mixed models estimating the effect of skin fungal diversity, water pH and dissolved oxygen (set as explanatory variables) on skin alpha diversity estimates (response variables). Models included the sampling site as random effect. 95% Confidence Intervals (95% CIs) and p-values used to define the statistical significance were computed using a Wald t-distribution approximation.

Response variable	Explanatory variable	Estimate	95% CI	t-score	Pr(>|t|)	Significance
Skin bacterial diversity (PD)	Skin fungal diversity (Shannon)	0.917	0.597. 1.236	5.629	9.58E-08	***
	water pH	-3.298	-5.371. -1.226	-3.119	6.53E-03	**
	water dissolved O2 (mg/L)	0.275	0.052. 0.498	2.414	1.98E-02	*
	Intercept	7.066	5.076. 9.055	6.96	3.00E-06	***
Skin bacterial diversity (Chao1)	Skin fungal diversity (Shannon)	1.669	1.081. 2.256	5.569	1.26E-07	***
	water pH	-5.89	-9.970. -1.910	-2.901	1.01E-02	*
	water dissolved O2 (mg/L)	0.432	0.013. 0.851	2.02	4.88E-02	*
	Intercept	11.191	7..371. 15.012	5.741	2.56E-05	***
Skin bacterial diversity (Shannon)	Skin fungal diversity (Shannon)	1.235	0.685. 1.785	4.398	2.16E-05	***
	Intercept	-1.203	-2.265. -0.276	-3.363	1.35E-03	**
Skin fungal diversity (Chao1)	Skin bacterial diversity (Shannon)	0.939***	0.578. 1.301	5.087	1.41E-06	***
	Intercept	3.735***	3.319. 4.150	17.611	<2.0e-16	***
Skin fungal diversity (Shannon)	Skin bacterial diversity (Shannon)	0.803***	0.420. 1.185	4.117	7.34E-05	***
	Intercept	2.082***	1.648. 2.516	9.409	2.49E-12	***

Table S6. Mantel tests with Spearman correlation coefficients on beta diversity of both Bacteria and Fungi, using temperature (TC), pH, dissolved oxygen (O2.mg.L.), water and skin diversity as explanatory variables.

Target microbiota	Dissimilarity index	Variable	Correlation	p.value	p.adjusted	Significance
Bacteria	Bray-Curtis	TC	0.53	0.001	0.002	**
		pH	0.11	0.004	0.005	**
		O2.mg.L.	0.25	0.001	0.002	**
		Water_mean_Shannon (bacteria)	0.22	0.001	0.002	**
		Water_mean_Shannon (fungi)	-0.02	0.656	0.656	
		Skin_Shannon (fungi)	0.20	0.001	0.002	**
	Jaccard	TC	0.43	0.001	0.001	**
		pH	0.13	0.001	0.001	**
		O2.mg.L.	0.20	0.001	0.001	**
		Water_mean_Shannon (bacteria)	0.15	0.001	0.001	**
		Water_mean_Shannon (fungi)	0.09	0.006	0.006	**
		Skin_Shannon (fungi)	0.17	0.001	0.001	**
Fungi	Bray-Curtis	TC	0.31	0.001	0.002	**
		pH	0.00	0.508	0.508	
		O2.mg.L.	0.20	0.001	0.002	**
		Water_mean_Shannon (fungi)	0.07	0.032	0.038	*
		Water_mean_Shannon (bacteria)	0.13	0.001	0.002	**
		Skin_Shannon (bacteria)	0.05	0.015	0.023	*
	Jaccard	TC	0.30	0.001	0.002	**
		pH	0.08	0.009	0.009	**
		O2.mg.L.	0.27	0.001	0.002	**
		Water_mean_Shannon (fungi)	0.12	0.002	0.002	**
		Water_mean_Shannon (bacteria)	0.13	0.001	0.002	**
		Skin_Shannon (bacteria)	0.13	0.001	0.002	**

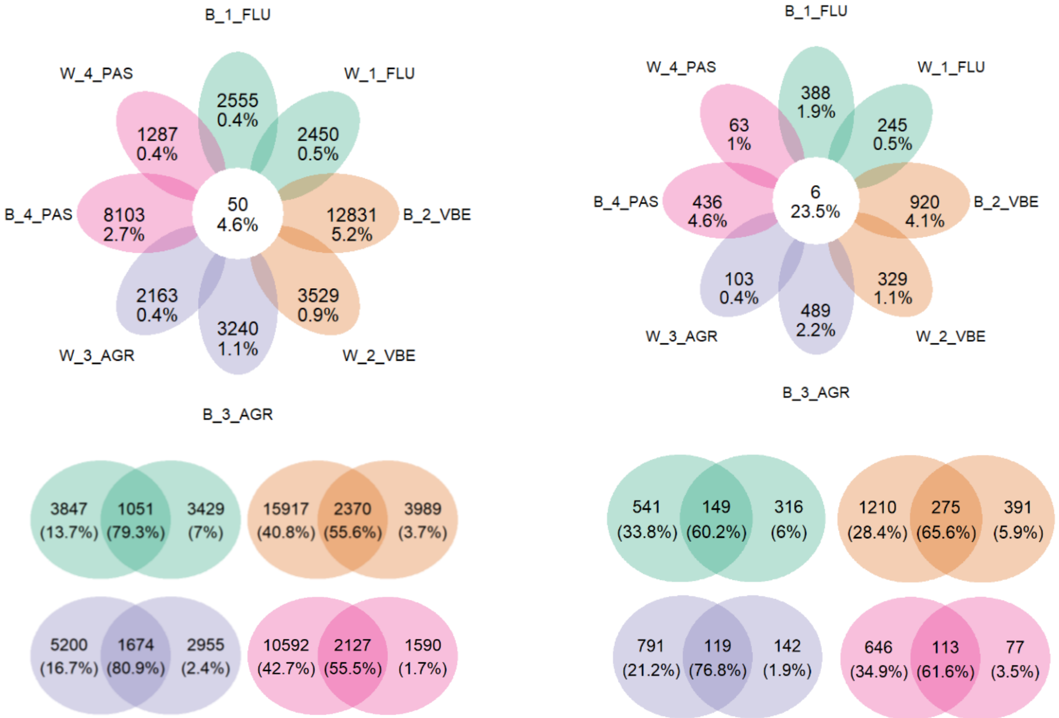

Figure S1. Venn diagrams representing private and shared amplicon sequence variants across habitats and sample types (as both absolute numbers and percentages). Left panels: Bacteria; right panels: Fungi. Codes are composed of either W (water) or B (skin) followed by the habitat type (1_FLU, 2_VBE, etc.).

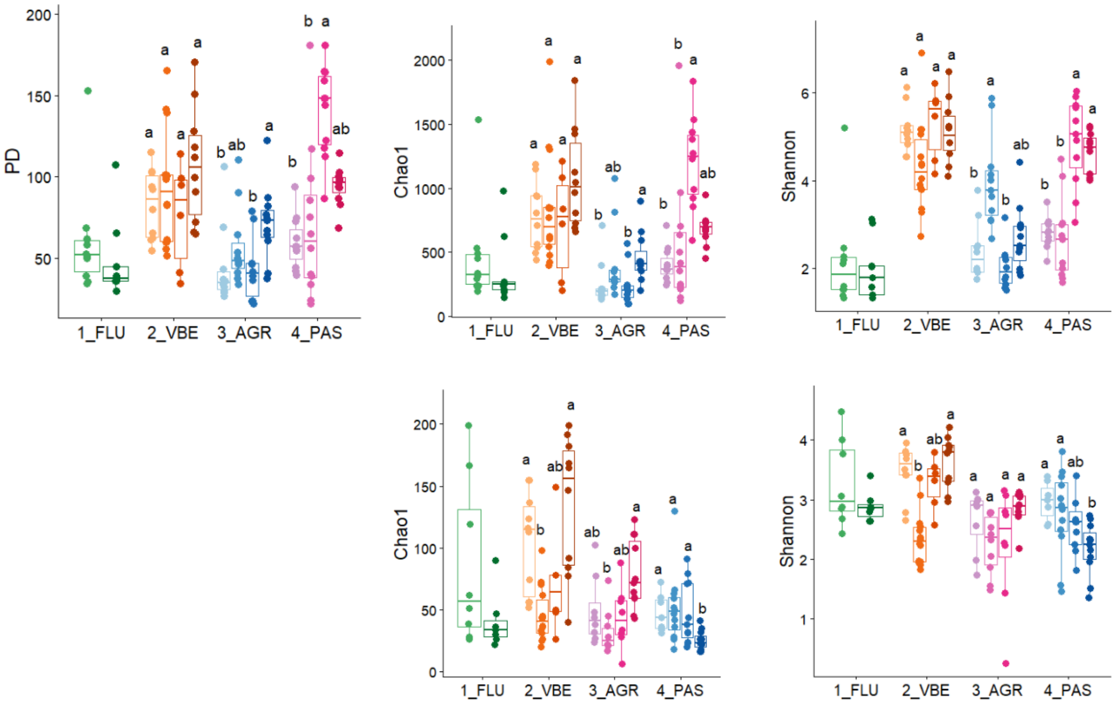

Figure S2. Alpha diversity estimates of *Bombina variegata* skin microbiota, per site. Upper panel: Bacteria; lower panel: Fungi. Codes at the bottom correspond to habitat type.

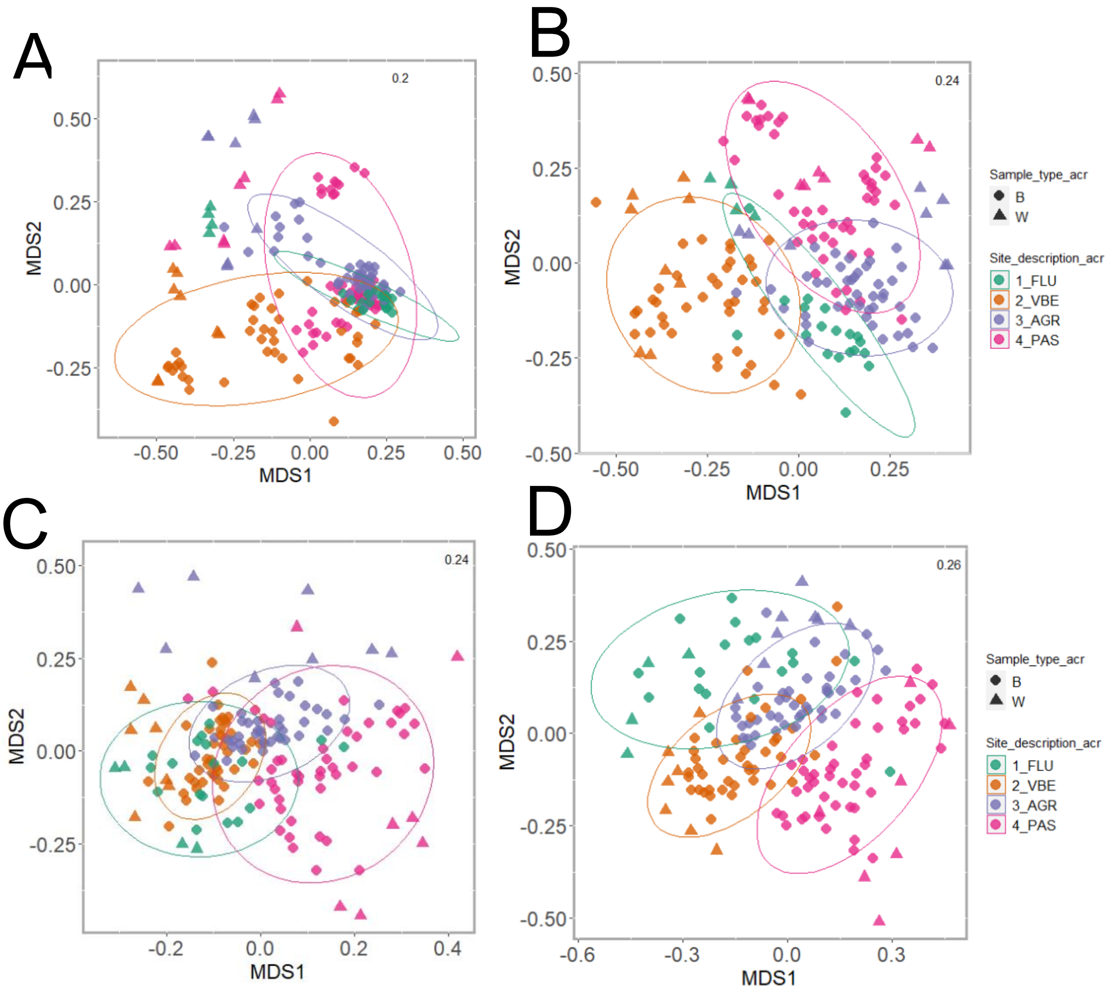

Figure S3. Beta diversity estimates of microbiota across *Bombina variegata* skin and water samples. Upper panel: Bacteria; lower panel: Fungi. Left: Bray-Curtis; Right: Jaccard.

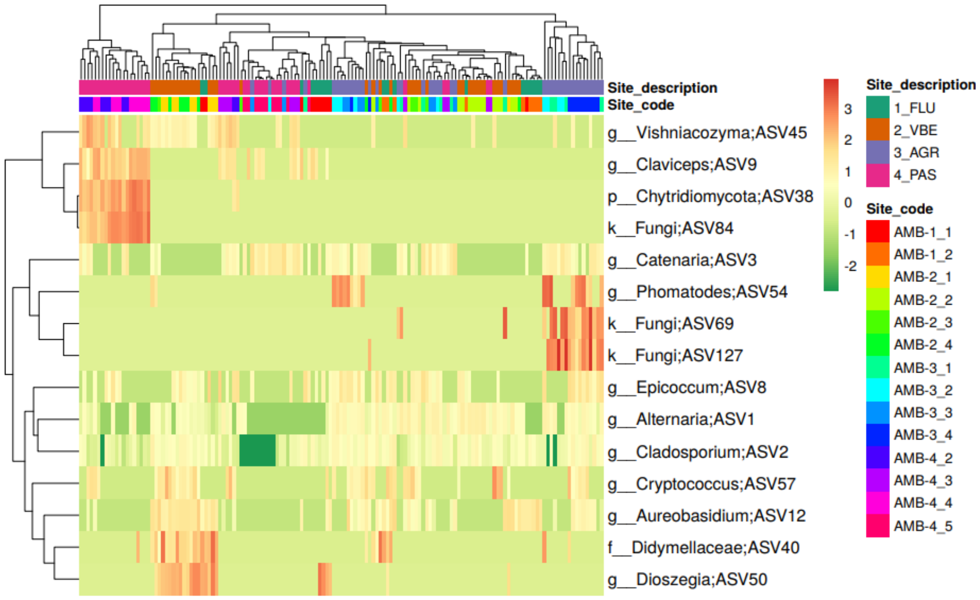

Figure S4. Fungal taxa with significant differences in their abundance across habitats. Original values were ln(x + 1)-transformed. Rows were centered; unit variance scaling was applied to rows. Both rows and columns were clustered using correlation distance and average linkage. 15 rows, 147 columns.

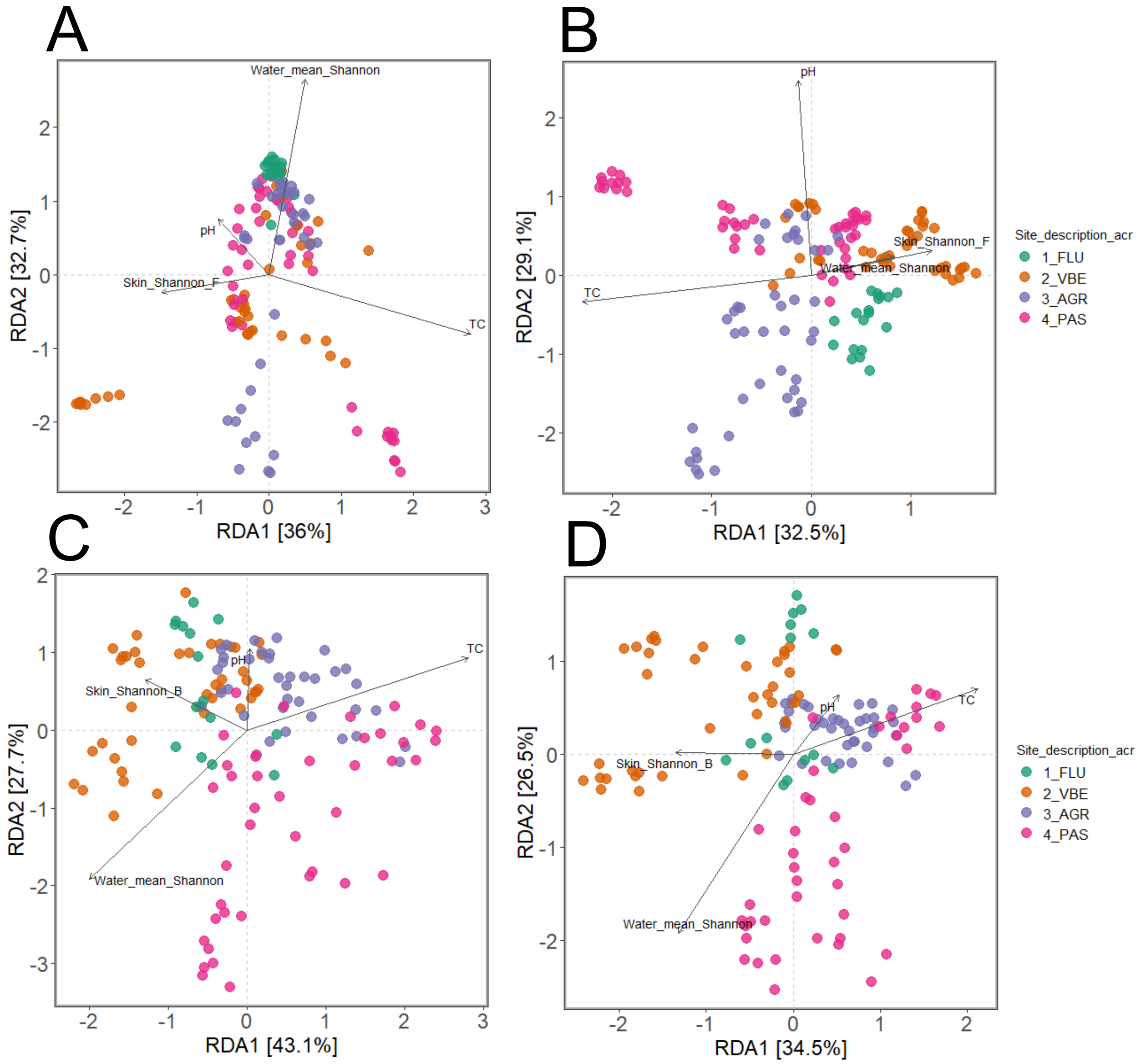

Figure S5. dbRDA of *Bombina variegata* skin microbiota, considering temperature (TC), pH, water (Water_mean_Shannon) and skin alpha (Skin_Shannon) diversity. Upper panel: Bacteria; lower panel: Fungi. Left: Bray-Curtis; Right: Jaccard.

KANSAS CASSANDRAS

Cynthia Pederson

If frogs are found
on high ground,
expect wet.
But if they head
for the riverbed,
that foretells
of dry spells.

If you want it to pour,
flip a poor frog over-
on its back,
that'll do it.

And you know,
if you first spy a toad
come spring,
it'll bring a good summer.
But if you sight a snake instead,
beware:
a bad summer's coming.

Listen to the way toads talk;
they know a lot about rain
and things like that.

CHAPTER 6

GENERAL CONCLUSIONS

6.1 SUMMARY OF MAIN RESULTS

In view of the alarming decline of amphibian populations, as well as the recent renewed interest in the conservation of their habitats in the context of sustainable landscape planning, this thesis aimed to provide new non-invasive protocols to characterize amphibian species and genetic biodiversity, using amphibian communities of the Province of Trento across protected areas as well as artificial and human-influenced sites as a case study. This data will be used to identify landscape drivers of amphibian richness, in an effort to provide useful insights for amphibian conservation beyond this case study.

In the second chapter, I developed an eDNA-based protocol for detecting a single model species (*R. temporaria*) across seasons (and consequently, life stages). On the basis of these results, I found that the optimal time of year in terms of probability of detection corresponds to spring-summer, when this species (like most European amphibians) emerges from hibernation to reach nearby water bodies for reproduction. Moreover, I demonstrated that eDNA-based approaches can be used to obtain reliable genetic diversity data of populations (i.e., number of haplotypes, haplotype and nucleotide diversity), a field that is to date largely unexplored. The comparison with tissue-based data, in fact, showed a strong correlation between indices calculated with the two approaches. Therefore, overall eDNA filtered from water has the potential to provide both species and population genetic data, and could be a reliable alternative to observational data for estimating species richness (which is time-consuming) and invasive sampling (up to now relying on skin biopsies), to estimate standard measures of genetic diversity in amphibian species.

In the third chapter, I aimed at expanding the eDNA method presented in Chapter 2 to all amphibian species of PAT (potentially including also most European species), while also improving the overall protocol in order to reach the best trade-off between taxonomic resolution and genetic diversity sensitivity. I also tested several bioinformatic pipelines for raw data processing. The seven-marker approach proved the applicability of eDNA methods to retrieve presence data of most target species in addition to their genetic diversity. Interestingly, some markers also amplified non-target amphibian species, introducing the possibility of further decreasing the number of markers. However, two of the rarest species (*H. intermedia* and *B. viridis*) were not detected, possibly due to their absence from the sampled wetlands. The comparison of three different bioinformatic pipelines showed that one, in particular (combining the software MICCA with VSEARCH) might be the most appropriate for minimizing the probability of false positives, while at the same time, retrieving the highest number of reads. Nonetheless, this study highlighted the existence, to date, of technical challenges regarding possible false positive and false negative errors of the eDNA approach.

In the fourth chapter, I used a novel multidisciplinary approach combining biological and urban planning data to investigate the complex interplay of amphibian species richness and presence data, habitat quality and human presence, as well as climate changes. The preliminary results presented here showed that this workflow, based on landscape planning theory, can in fact be applied to wildlife conservation, providing critical support for the recognition of protected wetlands as important sites for the survival of amphibians in a fast-changing environment. Moreover, this approach highlighted the relevance of farm ponds as additional reproductive sites for amphibians, thus placing them at the intersection of wildlife conservation and human socioeconomic activities. This consideration is particularly significant for future landscape development plans that aim at maximizing sustainability.

In the fifth chapter, I targeted *B. variegata* populations living in a variety of habitats, both natural and human-impacted (i.e., natural pools along the Avisio River, ephemeral ponds near apple orchards, irrigation tanks in vineyards, farm ponds), as models to study patterns of biodiversity in bacterial and fungal skin communities. This study showed that bacterial and fungal diversity vary significantly in relation to habitat, pH, dissolved oxygen and temperature, but with different patterns according to the diversity index examined (alpha or beta diversity indices). These first, promising results, support the hypothesis that human activities contribute in shaping amphibian skin microbiota, and therefore possibly amphibians' immune health and resistance to pathogens, which are likely to increase with climate warming and the deliberate or involuntary spread of invasive species.

6.2 IMPLICATIONS FOR AMPHIBIAN CONSERVATION

Concerning amphibian conservation, this doctoral thesis contributes to the ongoing research efforts aimed at developing rapid and efficient monitoring tools that do not harm the target species. In fact, eDNA sampling from water does not require disturbing the animals in any way. The non-invasive nature of these methods is particularly important in the case of amphibians, as they are experiencing a steep worldwide decline, and some species tend to have relatively small and isolated populations, which could be comparatively more affected by disturbing or harming individuals. In the study area, for example, this is the case for *B. variegata*, *L. vulgaris*, *T. carnifex*, and the subspecies *S. a. aurorae*. In addition, since the eDNA approach does not require handling of individuals, the chance of transmitting potential pathogens between animals and/or populations is minimized. This advantage is especially virtuous for the case of amphibians, as a major cause of their decline is in fact the spread of pathogens such as *Batrachochytrium dendrobatidis* and *B. salamandrivorans*.

The eDNA protocol presented and applied here also represents a first significant stepping stone on the path of developing a unified framework, aimed at measuring and monitoring amphibian diversity over time, taking into account two levels simultaneously: species diversity and genetic diversity. Since the latter is as fundamental as number of individuals to ensuring survival and adaptability of wild species, the inclusion of genetic data in routine monitoring programmes is bound to deliver a truly comprehensive picture of the conservation status of these species. In particular, this approach could help retrieve genetic diversity data for all species for which molecular studies are still lacking in the alpine region (i.e., *R. dalmatina*, *B. bufo*, *B. viridis*, *H. intermedia*, *I. alpestris*). As the markers presented here were designed to include more European species of the same Families or Genera of the target species, this protocol has also the potential to be tested and possibly adopted in other Italian regions and even countries, therefore providing the ground for the trans-national species monitoring and connectivity of protected areas management intended by several European Directives and international treaties. In fact, it has since been successfully applied for monitoring projects both in Italy and abroad.

These tools are useful not only for long-term monitoring programs (which are helpful for understanding populations trends but might be inefficient in the light of fast environmental changes), but also for informing policymakers and urban and landscape planners. For instance, the eDNA multi-species protocol could be used for quickly assessing amphibian biodiversity in urban pools or agricultural ditches. Since amphibians are already recognized as indicators of ecological health, these data could then be used as proxy for evaluation of these water bodies' relevance in terms of biodiversity. The study of skin microbiota will then provide additional information on the health of amphibian populations, if fine-scale evaluations are needed. In this sense, the three disciplines dialoguing in this thesis proved the importance of multidisciplinary in both supporting each other's conclusions and highlighting the gaps still existing. For example, on the one hand, in the case study presented in Chapter 4 a software well-known in, and largely applied to, landscape planning projects has been undoubtedly useful for identifying the common characteristics of wetlands hosting the highest amphibian species richness. On the other hand, as mentioned above, biological data such as amphibian species have the potential to be integrated into landscape planning workflows as a means of representing wildlife biodiversity in development plans. Similarly, the existing law provides a huge support to amphibian conservation, but could still be improved, especially at the local scale, when informed of specific necessities (i.e., particular water bodies might benefit from more protection, see section 6.3).

In the case of PAT, this thesis has shown that amphibians are currently present in most of the considered water bodies, although amphibians were absent from some artificial sites (see also section 6.3). Two of the 12 amphibian species known to be present in the area were not detected during my thesis (*H. intermedia* and *B. viridis*), although it remains to be confirmed if the reason is an actual decline in their already small populations, or rather some technical limitations of the eDNA protocol used here (i.e., the sampling period chosen might not have been the ideal for all species, including these). In the study area, this thesis also provided the first ever, genetic diversity data obtained from eDNA samples, contributing to the study of amphibian diversity at all levels. Lastly, the study of *B. variegata* skin microbial communities constitutes the first of its kind in the Province of Trento and one of the firsts in Italy. By characterizing both bacterial and fungal diversity, moreover, this research highlighted complex, and sometimes opposing, trends in this component of biological diversity, which will require more studies to be fully understood. This study also found the possible presence of *B. dendrobatidis* on some individuals, a result that, if confirmed by further analysis, will constitute the first report of this pathogen in the Province of Trento, and might have important repercussions on amphibian population conservation and management.

6.3 IMPLICATIONS FOR WETLANDS CONSERVATION

Regarding wetland conservation, the results of this doctoral thesis support the fundamental role of existing protected areas for wildlife conservation, in terms of number of amphibian species detected inside these areas compared to artificial water bodies or other wetlands not subjected to protection by law. However, as the study presented here was limited to only 18 protected areas and is a snapshot picture of amphibian communities, a wider (in terms of areas included), long-term monitoring is recommended to comprehensively assess the effectiveness of protection measures adopted. Nevertheless, it should be noted that three of the rarest amphibian species living in PAT (*L. vulgaris, R. dalmatina* and *T. carnifex*) were found inside Natura2000 sites, a consideration that additionally reinforces the importance of the latter.

In addition, this study highlighted that farm ponds are also relevant breeding sites for amphibians of several species, including the rare *S. atra*. This type of freshwater body has been overlooked up to now as important amphibian habitat, given the consistent disturbance caused by cattle and other farm animals either directly through walking in and around the ponds, or indirectly by polluting the water with urine and feces. However, it should be noted that some of these areas, especially if located near mountain huts at moderately-high elevation, are often owned by farmers, which could make any attempt at regulating farming around those sites rather challenging (although more sustainable methods could be associated with marketing farm products). Nevertheless, the possibility of suggesting minor interventions or *ad-hoc* management practices, such as the construction of wooden fences that allow cattle to drink from the farm pond without entering in and disturbing the water, should be presented as guidelines to the local authorities. To understand stakeholder interests and willingness to implement these ‘best practices’, disciplines such as economics and social studies could provide the correct tools as well as invaluable insights (e.g., Multi-Criteria Decision Making Analysis).

6.4 CURRENT LIMITATIONS AND FUTURE DIRECTIONS

eDNA protocols and their application to the study of genetic diversity still require a more in-depth evaluation in comparison with traditional methods. The issue of false negative errors, for instance, concerns species presence data in particular. As mentioned in section 6.2, the absence of two species from my dataset could be due to actual absence of the species in the samples sites as well as be a false negative error related to the specific limitations of eDNA sampling (i.e., spatial replicates may have been insufficient) or to sampling conditions (i.e., some species might be more affected by climate phenomena such as the drought in 2021). In order to rule out the possibility of errors, therefore, intensive traditional monitoring data might provide useful validation for particular wetlands/species. In addition, considering the results of both single-species and multi-species eDNA monitoring (Chapters 2 and 3, respectively), sampling design may need to be adjusted according to each species' seasonal activity as well as to the climate variations during sampling season. In the case of this study area, for instance, for sites known to host species with non-overlapping reproductive periods (e.g., *R. temporaria*: early spring; *P.* kl. *esculentus*: late spring/early summer), two or three temporal replicates should be considered, in order to ensure appropriate representation of all species and to take into account unexpected climate events such as droughts or heavy rains.

This thesis also highlighted the difficulty of separating DNA sequences of target species (and therefore haplotypes) from false positive errors (i.e., degraded sequences present in the environment, PCR or sequencing errors, and so on). Again, a comparison with tissue-based genetic data could help discriminate between the two. Also, additional studies are needed to deepen our understanding of molecular mechanisms that lead to false positives and, subsequently, to develop statistical methods to isolate true positives.

In principle, routine wetland eDNA monitoring across the entire Province, and beyond, is now possible, thereby creating the possibility of establishing a network of updated data on species presence and their genetic diversity that could mimic the Natura2000 network. In fact, the markers I developed can be applied to other European species of the Families and Genera considered in my study: Bombinatoridae, Bufonidae, Hylidae, Pleurodelinae, Salamandrinae and the Genera Rana and Pelophylax. Furthermore, this approach could easily be adapted for the detection of invasive species (amphibian or otherwise), and/ or for monitoring the spread of pathogens such as Bd and Bsal. Considering the impact of these organisms on amphibian conservation, this application could become a part of the protection measures already in place inside protected areas.

The application of landscape planning approaches to biological data, in this case, has been limited to amphibian species richness and presence data in the Province of Trento, but could in the future be expanded to include further biological levels, taxonomic groups and geographical scope. Firstly, the same workflow could be applied to haplotypes instead of species (i.e., using the number of haplotypes and haplotype presence data, or even genetic diversity indices such as haplotype or nucleotide diversity), with the aim of highlighting patterns in genetic diversity distribution across alpine wetlands in relation to anthropic presence and activities. Secondly, this approach could be extended to other species with similar lifestyles (i.e., fish, crayfish, insects) or that make use of wetlands for short periods (i.e., birds, mammals), in order to increase robustness of conclusions by providing results across several taxa. Thirdly, this multidisciplinary methodology could easily be utilized outside PAT, again providing an exceptional example of a productive dialogue between disciplines.

The results of the final chapter of this multidisciplinary work have direct applications to sustainable development. For instance, the artificial sites showing the highest number of species could become positive examples for the creation of specific credits linked to amphibian conservation, in the context of GBC evaluations. More in general, the variables that will be identified as the most impacting on amphibian diversity will be useful to construct ‘ecological condition indices’, powerful transdisciplinary tools with applications in environmental and landscape impact assessment, urban ecology and policy development.

6.5 CLOSING REMARKS

In conclusion, this thesis has demonstrated how different disciplines can interact with each other, corroborating overall results and broadening the scope and applications of the entire research project. As biodiversity continues its downward spiral, it is becoming increasingly evident that humans, too, will be greatly affected by worldwide biodiversity loss. For this reason, it is now imperative that people from all areas and backgrounds join their expertise and different tools in order to address this crisis. In fact, it is impossible, and would be unjust, to achieve certain targets (i.e., 30% of European territory under protection law;

conversion of some agricultural lands to wetlands) without taking into account the socioeconomic context as well as the specific needs of local populations, which must be properly understood and addressed. Therefore, while local, single-species or case-specific studies are still fundamental to increase the global knowledge in a step-by-step fashion, only a global, all-round, human-, environmental- and climate-just, transdisciplinary effort could hope to halt, and possibly invert, the current trend of biodiversity decline. In a praise of frogs, as a song of a salamander, but also a beg for toads and all other ‘foul and loathsome beasts’, this thesis hopes to provide a first step in this direction.

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I would like to thank the coordinator of the ESW PhD course, prof. Paola Spinozzi, for the helpful advice, and in particular for putting me in contact with prof. Maragno in order to expand the Landscape Planning component of my project. Thanks also to prof. Jorge S. Burns for frequently challenging me to broaden the scope and applications of my thesis.

I want to thank all present and former members of the Conservation Genomics Research Unit (FEM) for the welcoming environment and the stimulating conversations. In particular, I will never be able to thank Matteo Girardi enough, without whom this project would probably never have seen the light of day. I hope you know by now how much I appreciate the knowledge you shared with me, your steady support and how you always push me to do better. I guess even bickering with you is fun, although sometimes frustrating! Warmest thanks also to Stefano Casari: with your seraphic demeanor and pragmatic attitude, your presence has helped me to calmly address the most annoying issues, including the above-mentioned colleague! Many thanks also to Giulio Galla for his much-appreciated support both during field sampling and data analysis. I want to thank both my master's students, Irene Lo Presti and Federico Polli, for giving me the opportunity (and challenge) to share my knowledge, and in particular Irene for field support during the second year of sampling. Thanks also to Andrea Gandolfi, Barbara Crestanello, Chiara Rossi, Alexis Marchesini, Emily Pascoe and all students at FEM for the scientific discussions and time shared together.

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Many warm thanks to all my colleagues from the ESW PhD course for sharing this journey with me: I have no doubts that no one understands how challenging these past years were more than you do. Special thanks to Caterina Rondoni, most of all for ‘infecting’ me with (a certain degree of) optimism and willingness to be politically more active, but also for your kindness and braveness. Your work is a constant inspiration for me. I also want to thank Francesca A. Lioce for helping me navigate the bureaucracy of this course and for the encouragement during the final stages of drafting my thesis, as well as Victor H. M. Mora, Cristina Sagnotti, Matteo Lombardi, Andrea Raisi, Giovanni Piva, Sara D’Addario and Mahim Mohiuddin. Cheers to us, for all the ups and downs we shared!

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Speaking of strange loves and strange creatures, there are a lot of friends who supported me during these years, maybe even more than they were aware of. In fact, I'm very grateful to all people who showed interest in my research, as thinking back on your appreciative comments gave me a reason to push forward during the most difficult and disheartening times. Warm thanks to Carla and Brigida for the encouraging talks; to Alberto, Fra, Angy, Leo, Gio, Matilde and Elisa for sticking around even after ten (and still counting!) years (yes, we're getting that old); to Arianna for always lending an ear to my ranting about work-related stuff; to Ilaria, Luca, Davide and Bea for helping me NOT think about work while being such a fun D&D party (I promise I'll soon get back to being a decent DM); to Giorgia, Marco, Giulio and Irene for being sympathetic to the struggles of a PhD journey, and to Davide for being another wonderful and most creative D&D player despite the many misunderstandings we had. Honorable mention for Gonza, whose patience, after listening to hours of my vocal messages, is now legendary. If I still have my sanity, I know it's mostly your… fault (just kidding). Thanks a lot also for the precious help of bibliography editing for a couple of chapters! Many thanks also to my Shifu Giuseppe Bon, and to Gian, Diego, and my Muay Thai fighter friends of the Italia Poon Ze Team, for teaching me so much while helping me relieve the stress of these last months. Noemi, you're the best sparring partner I could wish for!

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LEGAL SOURCES

International law

Convention on Wetlands of International Importance Especially as Waterfowl Habitat (Ramsar Convention), Ramsar, 2 February 1971

Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES or Washington Convention), Washington, 3 March 1973, the United Nations Environment Programme

Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention), Bern, 19 September 1979, Council of Europe, ETS No. 104

Convention on Biological Diversity, Rio de Janeiro, 5 June 1992, the United Nations Environment Programme

Vision for the Bern Convention for the period to 2030, Strasbourg, 3 December 2021, Council of Europe, T-PVS(2021)14

Kunming-Montreal Global Biodiversity Framework, Montreal, 19 December 2022, Conference of the Parties to the Convention on Biological Diversity, CBD/COP/DEC/15/4

Strategic Plan for the Bern Convention for the period to 2030, Strasbourg, 1 December 2023, Council of Europe, T-PVS(2023)18

European law

Council Directive 79/409/EEC of 2 April 1979 on the conservation of wild birds (‘Birds’ Directive), the Council of the European Communities; later substituted by the Directive 2009/147/EC of the European Parliament and of the Council of 30 November 2009

Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora (‘Habitats’ Directive), the Council of the European Communities; latest amended by Council Directive 2013/17/EU of 13 May 2013

Council Regulation (EC) No 338/97 of 9 December 1996 on the protection of species of wild fauna and flora by regulating trade therein, amended recently by the Commission Regulation (EU) 2023/966 of 15 May 2023

Regulation (EU) 2018/841 of the European Parliament and of the Council of 30 May 2018 on the inclusion of greenhouse gas emissions and removals from land use, land use change and forestry in the 2030 climate and energy framework, and amending Regulation (EU) No 525/2013 and Decision No 529/2013/EU (Text with EEA relevance); amended by Regulation (EU) 2023/839 as regards the scope, simplifying the reporting and compliance rules, and setting out the targets of the Member States for 2030

Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions of 20 May 2020 ('EU Biodiversity strategy for 2030), European Commission, COM/2020/380 final

Proposal for a REGULATION OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL on nature restoration of 22 June 2022 ('Nature Restoration Law'), European Commission, COM/2022/304 final; which is still to be approved by the European Council

National law

L. 5 agosto 1981, n. 503, *Ratifica ed esecuzione della convenzione relativa alla conservazione della vita selvatica e dell'ambiente naturale in Europa, con allegati, adottata a Berna il 19 settembre 1979*, ratifying the 'Bern' Convention

L. 8 August 1985, n. 431, *Conversione in legge, con modificazioni, del decreto-legge 27 giugno 1985, n. 312, recante disposizioni urgenti per la tutela delle zone di particolare interesse ambientale. Integrazioni dell'articolo 82 del Decreto del Presidente della Repubblica 24 luglio 1977, n. 616*, or *legge 'Galasso'*, subjecting all Ramsar areas to landscape restrictions

L. 6 December 1991, n. 394, *Legge Quadro sulle Aree Protette*, regulating protected areas

D.P.R. 13 March 1976, n. 448, *Esecuzione della Convenzione relativa alle zone umide d'importanza internazionale, soprattutto come habitat degli uccelli acquatici, firmata a Ramsar il 2 febbraio 1971,* ratifying the Ramsar Convention

D.P.R. 11 February 1987, n. 184, *Esecuzione del Protocollo di emendamento della Convenzione internazionale di Ramsar del 2 febbraio 1971 sulle zone umide d'importanza internazionale, adottato a Parigi il 3 dicembre 1982,* ratifying the Ramsar Convention

D.P.R. 8 September 1997, n. 357, *Regolamento recante attuazione della direttiva 92/43/CEE relativa alla conservazione degli habitat naturali e seminaturali, nonché della flora e della fauna selvatiche,* ratifying EU ‘Habitats’ Directive

Constitutional Law 11 February 2022, n. 1, *Modifiche agli articoli 9 e 41 della Costituzione in materia di tutela dell'ambiente. (22G00019)*, amending articles 9 and 41 of the Italian Constitution

Ministerial Decree 3 August 2023, n. 252, adopting the ‘2030 Biodiversity National Strategy’

Provincial law

L.p. 9 December 1991, n. 24, *Norme per la protezione della fauna selvatica e per l'esercizio della caccia* (‘Provincial law on hunting’)

L.p. 23 May 2007, n. 11, *Governo del territorio forestale e montano, dei corsi d'acqua e delle aree protette* (‘Provincial law on forests and nature protection’), addressing the subjects of both land use and protected habitats management, modified with the l.p. 21 July 2016, n. 11

D.P.P. 3 November 2008, n. 50-157/Leg., *Regolamento concernente le procedure per l'individuazione delle zone speciali di conservazione e delle zone di protezione speciale, per l'adozione e l'approvazione delle relative misure di conservazione e dei piani, nonché la composizione, le funzioni e il funzionamento della cabina di regia delle aree protette e dei ghiacciai*, on regulation of Natura2000 sites

D.P.P. 26 October 2009, n. 23-25/Leg., *Regolamento di attuazione del titolo IV, capo II (Tutela della flora, fauna, funghi e tartufi) della legge provinciale 23 maggio 2007 n. 11 (Legge provinciale sulle foreste e sulla protezione della natura)*, on the ‘Provincial law on forests and nature protection’

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