

Bufotes and *Pelophylax* as Models for Semi-Arid Terrestrial and Aquatic Life: A Hypothesis-Generating Comparative Review of Ecological, Physiological and Candidate-Gene Evidence

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How to cite this paper: Degani, G. and Bercovich, D. (2026) *Bufotes* and *Pelophylax* as Models for Semi-Arid Terrestrial and Aquatic Life: A Hypothesis-Generating Comparative Review of Ecological, Physiological and Candidate-Gene Evidence. *Open Journal of Animal Sciences*, 16, 415-440.

<https://doi.org/10.4236/ojas.2026.164027>

Received: September 10, 2026

Accepted: October 17, 2026

Published: October 20, 2026

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Abstract

Amphibians are highly sensitive to environmental change because their life cycles, physiology, reproduction and survival depend strongly on water availability. The genera *Bufotes* and *Pelophylax* represent contrasting ecological strategies among anurans. The green toad *Bufotes sitibundus* is widely distributed across Mediterranean, semi-arid and arid regions and can persist in dry terrestrial habitats, whereas the Levant water frog *Pelophylax bedriagae* is closely associated with permanent freshwater habitats. This article is explicitly a hypothesis-generating comparative review rather than a report of demonstrated genomic adaptation. It separates three classes of evidence throughout: 1) demonstrated observations obtained directly from the two focal Israeli species, chiefly ecological, distributional and physiological data, including published measurements of oxygen consumption and carbon dioxide production under thermal and dehydration stress; 2) inference from related Eurasian taxa such as *Bufotes viridis*, *B. variabilis*, *Pelophylax ridibundus*, *P. lessonae* and *P. kurtmuelleri*; and 3) general amphibian or vertebrate background knowledge. Candidate genes associated with water balance, osmoregulation, heat and oxidative stress, hypoxia, immunity, skin-barrier function, metabolism and endocrine regulation are therefore presented as explicit, testable predictions and not as established species-specific differences, because no direct expression, sequence or functional data are yet available for either focal species. Because *Bufotes* and *Pelophylax* are distantly related genera, ecological contrast between them cannot

by itself distinguish convergent or lineage-specific genomic adaptation from inherited phylogenetic difference. Current evidence supports marked ecological and physiological differentiation between the two genera, but direct whole-genome comparisons of *B. sitibundus* and *P. bedriagae* remain unavailable. Comparative genome sequencing, population-genomic analyses and functional studies are therefore required to test the proposed candidate pathways and to identify lineage-specific mechanisms of adaptation.

Keywords

Bufotes sitibundus, *Pelophylax bedriagae*, Amphibians, Arid Adaptation, Aquatic Adaptation, Comparative Genomics, Candidate Genes, Hypothesis-Generating Review, Levels of Evidence, Phylogeography, Aquaporins, Heat-Shock Proteins, Osmoregulation, Israel

1. Introduction

Amphibians occupy an exceptionally wide range of ecological habitats, including permanent lakes and rivers, temporary ponds, humid forests, grasslands, agricultural landscapes, semi-arid environments and deserts [1] [2]. Their highly permeable skin, biphasic life cycle, dependence on external water for reproduction and ectothermic physiology make them particularly sensitive to environmental variation and valuable model organisms for studying ecological adaptation and evolutionary processes [3]. However, these same biological characteristics also make amphibians among the vertebrate groups most vulnerable to habitat degradation, climate change, environmental pollution, emerging infectious diseases and habitat fragmentation [4] [5].

Israel represents an exceptional natural laboratory for investigating amphibian adaptation because one of the world's steepest climatic gradients occurs over a relatively small geographic area. Climatic conditions change from humid Mediterranean habitats in northern Israel through semi-arid regions to the extreme desert environments of the Negev and Arava. Along this environmental gradient, amphibian species exhibit marked differences in habitat preference, reproductive strategy, dispersal capacity, physiology and tolerance to water limitation [1] [2].

The Levant water frog, *Pelophylax bedriagae* (Camerano, 1882), remains closely associated with permanent freshwater habitats, including springs, streams, ponds, reservoirs and wetlands throughout its life [1] [2] [6] [7]. A recent ecological synthesis proposed that *B. sitibundus* represents a useful model for studying adaptation to semi-arid and arid habitats, whereas *P. bedriagae* provides an appropriate model for investigating genomic and physiological specialization to permanent aquatic environments [1] [2].

The genus *Bufotes* comprises several species of green toads distributed throughout Europe, western Asia, Central Asia and parts of North Africa. Molecular phylogenetic, cytogenetic, ecological and phylogeographic studies have substantially

improved understanding of species relationships within the genus, particularly in *Bufo viridis* and *Bufo variabilis* [8]-[10], and comparable work on other west-Palearctic toads illustrates how Pleistocene refugia and secondary contact have shaped present-day lineage structure in the group [11]. Furthermore, the publication of a chromosome-level reference genome for *B. viridis* [12] has provided an important genomic resource for comparative analyses across the genus and offers a valuable framework for future investigations of *B. sitibundus*.

The genus *Pelophylax* includes the Palearctic water frogs, which are widely distributed throughout Europe, western Asia and the Middle East. Taxonomy within the genus is particularly complex because several species form hybridogenetic systems and exhibit extensive mitochondrial introgression. *Pelophylax bedriagae* belongs to the eastern Mediterranean lineage and has historically been treated within, or closely associated with, the *Pelophylax ridibundus* species complex [8] [9]. Recent molecular studies have identified multiple mitochondrial lineages closely related to *P. ridibundus*, *P. kurtmuelleri* and *P. bedriagae*, highlighting the importance of combining mitochondrial and nuclear markers for resolving species boundaries, reconstructing evolutionary history and assessing population structure [8] [9] [13].

Comparing the genera *Bufo* and *Pelophylax* provides an opportunity to investigate the molecular basis of adaptation to contrasting environmental conditions. Species of *Bufo* generally exhibit greater tolerance to dehydration, prolonged terrestrial activity and temporary breeding habitats, whereas species of *Pelophylax* remain strongly dependent on permanent freshwater ecosystems [6] [14]. These ecological differences generate the hypothesis that natural selection has acted on genes associated with water conservation, osmoregulation, thermal tolerance, oxidative stress, hypoxia, epidermal barrier function, immune regulation and energy metabolism [1] [2] [15].

Comprehensive whole-genome comparisons between *Bufo sitibundus* and *Pelophylax bedriagae* are not available. This review therefore integrates molecular, genomic, phylogenetic, ecological and physiological evidence from the focal Israeli species with data from closely related Eurasian taxa, including *Bufo viridis*, *B. variabilis*, *Pelophylax ridibundus*, *P. lessonae* and *P. kurtmuelleri*. Direct evidence from the focal species is distinguished from comparative inference based on homologous genes and related taxa [1] [2]. This approach identifies conserved and potentially lineage-specific mechanisms associated with adaptation to semi-arid terrestrial environments and permanent aquatic habitats, while defining priorities for future comparative genomic and functional studies.

This review compares the ecological, phylogenetic and genomic characteristics of *Bufo* and *Pelophylax*. It integrates evidence from mitochondrial and nuclear markers [16] [17], evaluates candidate genes and molecular pathways potentially involved in adaptation to semi-arid terrestrial and permanent aquatic habitats, and proposes a comparative framework for future genomic research on Israeli amphibians.

Distribution patterns were compiled from published herpetological atlases, the IUCN Red List, AmphibiaWeb, the Israel Nature and Parks Authority, and relevant phylogeographic studies (Figure 1). *Pelophylax bedriagae* is mainly associated with permanent freshwater systems, whereas *B. sitibundus* occupies a broader range of Mediterranean, semi-arid and arid habitats and breeds primarily in temporary or semi-permanent water bodies.

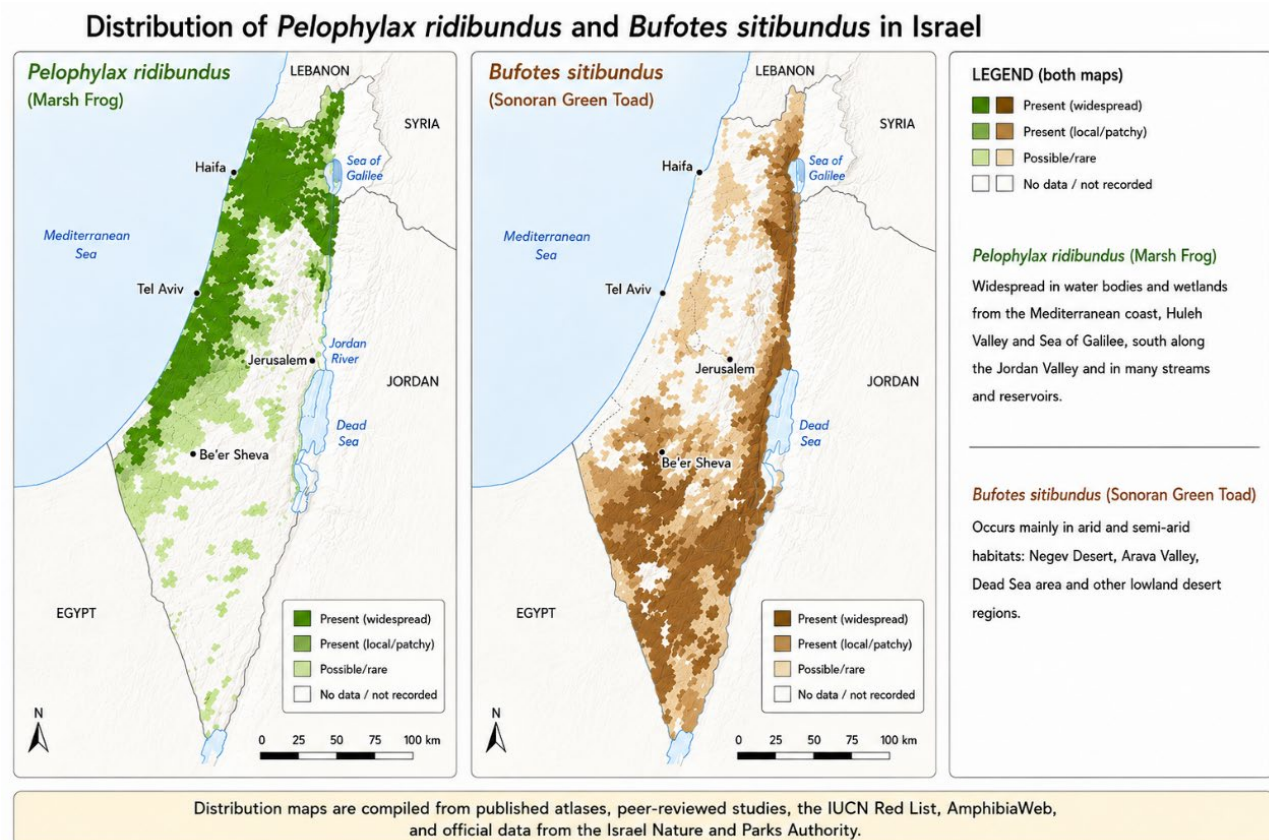


Figure 1. Distribution of *Pelophylax ridibundus* (Marsh Frog) and *Bufo sitibundus* (Green Toad) in Israel.

1.1. Scope of the Review and Status of Its Central Claim

The central claim of this review is deliberately restricted. We do not claim to demonstrate genomic adaptation in *B. sitibundus* or *P. bedriagae*. What is demonstrated here is a set of ecological, distributional and physiological contrasts between the two species, several of which were measured directly in Israeli animals. What is proposed here is a structured, prioritized set of candidate genes and molecular pathways that could plausibly underlie those contrasts and that can be tested by future work. The two are kept separate throughout the text, and the reader should treat every gene name in Section 7 as a prediction rather than as a finding unless direct expression, sequence or functional evidence is cited alongside it. No such direct molecular evidence currently exists for either focal species.

A second and equally important limitation concerns what an ecological contrast

between two genera can and cannot show. *Bufo* (Bufonidae) and *Pelophylax* (Ranidae) are distantly related lineages whose most recent common ancestor lies deep within the Neobatrachia [18] [19]. The two genera therefore differ in a very large number of characters, most of which have nothing to do with present-day habitat use and simply reflect inherited phylogenetic history. Skin thickness and glandular architecture, limb proportions, webbing, karyotype and genome size all differ between bufonids and ranids as a general rule, irrespective of whether a particular species is arid-adapted or aquatic. Consequently, an observed difference between *B. sitibundus* and *P. bedriagae*, whether physiological or, in future, genomic, cannot by itself be attributed to habitat association: the same difference is expected under the null hypothesis of phylogenetic inertia alone.

Distinguishing habitat-driven adaptation from phylogenetic background requires designs that this review can recommend but not supply. The most informative are: 1) within-genus comparisons along the Israeli climatic gradient, for example among *B. sitibundus* populations from Mediterranean, semi-arid and desert-margin sites, where phylogenetic background is approximately constant and habitat varies; 2) phylogenetically replicated cross-genus comparisons, in which several independent terrestrial-aquatic species pairs are examined so that convergence can be tested statistically; and 3) explicit phylogenetic comparative methods applied to trait and sequence data across a broader anuran sample. Until such designs are implemented, statements in this review about “lineage-specific” or “convergent” adaptation should be read as hypotheses to be tested, not as conclusions.

1.2. Levels of Evidence Used in This Review

To avoid conflating observation with inference, every substantive statement in this review belongs to one of three evidence levels. These levels are signalled in the text by explicit wording, and the reader can identify the level of any claim from that wording alone.

Level 1—Direct evidence from the focal taxa (*B. sitibundus* or *P. bedriagae*, including material published under earlier names). This is the strongest category and is comparatively small. It comprises distributional and habitat data for Israeli populations [1] [2] [6] [14]; larval ecology and development in Israeli winter ponds [6] [14]; urea accumulation during terrestrial acclimation in the green toad [20]; respirometric measurements of oxygen consumption and carbon dioxide production across ambient temperatures in hydrated and dehydrated animals of both species [21]; and mitochondrial 12S and 16S rRNA sequences generated from Israeli *P. bedriagae* and from Israeli green toads [16] [17]. Statements resting on Level 1 evidence are introduced in the text by phrases such as “has been measured in”, “was observed in” or “direct evidence from the focal species shows”.

Level 2—Evidence from related taxa within *Bufo* or *Pelophylax*. This category supplies most of the molecular and genomic material in the review, because

genomic resources for the focal species are essentially absent. It includes the chromosome-level reference genome of *B. viridis* [12], phylogenetic and phylogeographic analyses of the *B. viridis* subgroup [10] and of the *P. ridibundus* complex [9] [13], and population-genomic work on hybridogenetic *Pelophylax* systems [8]. Level 2 evidence constrains what is plausible for the focal species but does not establish it, because the congeners differ from the focal taxa in ploidy, range, climate and, for several *Pelophylax* lineages, reproductive mode. Statements at this level are introduced by “in related species”, “in congeners” or “by extrapolation from”.

Level 3—General amphibian or vertebrate evidence. This is the weakest category with respect to the focal species and supplies the mechanistic background: aquaporin-mediated water uptake across ventral skin in semiterrestrial and aquatic anurans generally [22] [23]; heat-shock and cellular stress responses across ectotherms [24]–[26]; antioxidant, hypoxia-signalling, innate-immune, metabolic and endocrine pathways characterized largely in model vertebrates [27]–[37]; and general amphibian environmental physiology [3] [15] [18] [38]. Because these pathways are conserved across vertebrates, their presence in *Bufo* and *Pelophylax* can be assumed, but nothing about habitat-specific regulation in either genus follows from that conservation. Statements at this level are introduced by “in amphibians generally”, “across vertebrates” or “conserved across taxa”.

All candidate-gene assignments in Section 7 rest on Level 3 evidence, occasionally supported at Level 2. None currently rests on Level 1. They are accordingly labelled as predictions throughout, and Section 7.9 sets out what would be required to raise them to Level 1.

1.3. Taxonomic Nomenclature and Assignment of Cited Datasets

Nomenclature in both genera has changed repeatedly, and several of the primary datasets used here were published under names that are no longer valid. To avoid ambiguity, the following conventions are applied consistently throughout this review.

Pelophylax bedriagae (Camerano, 1882) is used for the Levant water frog of Israel and the adjacent eastern Mediterranean. Material published as *Rana ridibunda*, *Rana bedriagae*, *Rana levantina* or *Pelophylax ridibundus* from Israeli localities is referred here to *P. bedriagae*, and the original name is given in parentheses at first mention of each dataset. *Pelophylax ridibundus* (Pallas, 1771) is retained in its restricted modern sense for the marsh frog of central and eastern Europe and western Asia and is treated as a distinct species, not as a senior synonym of the Israeli taxon. Where a cited study analysed a broader assemblage, the term “*P. ridibundus* species complex” is used and the Israeli taxon is not assumed to be represented unless the study sampled Levantine localities.

Bufo sitibundus (Pallas, 1771) is used for the green toad of Israel. Material published as *Bufo viridis*, *Pseudepidalea viridis* or *Bufo variabilis* from Israeli localities is referred here to *B. sitibundus*, again with the original name given in

parentheses at first mention. *Bufotes viridis* is retained for the European green toad in its restricted sense, including the individual from which the reference genome was assembled [12]; that genome is therefore Level 2 evidence for *B. sitibundus*, not Level 1.

Assignment of the principal datasets used here is as follows. The respirometry of Degani and Meltzer [21], published as *Bufo viridis* and *Rana ridibunda*, was performed on animals collected in northern Israel and is assigned to *B. sitibundus* and *P. bedriagae* respectively. The urea-accumulation study of Degani *et al.* [20], published as *Bufo viridis*, is assigned to *B. sitibundus*. The mitochondrial sequence data of Degani *et al.* [16], published as *Pseudepidalea viridis* (syn. *Bufo viridis*), are assigned to *B. sitibundus*; those of Degani *et al.* [17], published as *Rana bedriagae*, are assigned to *P. bedriagae* and are listed in **Table 1**. The larval-ecology studies [6] [14] were conducted in Israeli winter ponds and are assigned to the focal species accordingly. By contrast, the phylogeographic and population-genomic datasets of Dubey *et al.* [8], Ualiyeva *et al.* [13] and Dufresnes *et al.* [9] sample predominantly non-Levantine lineages; they are used here as Level 2 evidence and are not treated as data on the focal Israeli populations.

2. Ecological Strategies of *Bufotes* and *Pelophylax*

The genera *Bufotes* and *Pelophylax* represent contrasting ecological strategies among anurans and provide useful models for studying amphibian adaptation along the pronounced climatic gradient of Israel. Their distributions, life-history traits, reproductive strategies and physiological characteristics reflect different evolutionary responses to terrestrial and aquatic habitats. *Bufotes sitibundus* is primarily terrestrial and is well adapted to Mediterranean, semi-arid and arid environments. It occurs in woodlands, agricultural landscapes, open grasslands, rocky hillsides and desert margins from northern Israel to the Negev [1] [2] [9]. The broad distribution of *B. sitibundus* indicates tolerance of seasonal drought, elevated temperatures and limited water availability [1] [2] [9]. *Bufotes sitibundus* reproduces mainly in temporary ponds, rain-filled pools, reservoirs and other ephemeral water bodies during the rainy season, and rapid larval development increases the probability of completing metamorphosis before these habitats dry [14].

In contrast, *Pelophylax bedriagae* is strongly associated with permanent freshwater ecosystems, including streams, rivers, ponds, lakes, marshes, springs, reservoirs and irrigation canals. In Israel, it is concentrated mainly in northern and central regions, including the Hula Valley, the Sea of Galilee drainage basin, the Jordan River system and permanent wetlands along the Mediterranean coastal plain [1] [6]. Adults remain closely associated with water throughout the year and possess strong swimming ability, which may facilitate dispersal through connected freshwater systems. Breeding generally occurs in permanent or long-lasting water bodies, where larval development is less constrained by rapid desiccation [1]-[3].

These ecological differences are reflected in morphology and physiology. *Bufotes* has relatively thick, keratinized skin and numerous parotoid and granular glands that contribute to chemical defence and may reduce evaporative water loss [3]. Its relatively long limbs facilitate terrestrial locomotion. *Pelophylax* has smoother, more permeable skin that supports cutaneous respiration in aquatic environments, together with elongated hind limbs and well-developed interdigital webbing adapted for swimming [18]. It should be noted, however, that several of these characters, in particular skin thickness, glandular architecture and webbing, distinguish bufonids from ranids as families and are therefore confounded with phylogeny; they cannot be interpreted as habitat-driven adaptations on the basis of this two-species comparison alone (Section 1.1).

Contrasting habitat requirements are also expected to influence population structure and gene flow. *Bufotes* populations may become differentiated when breeding sites are isolated temporary ponds separated by unsuitable terrestrial habitat, thereby reducing connectivity and increasing population subdivision [9] [10]. *Pelophylax* populations may retain greater connectivity through river networks and permanent wetlands, although fragmentation, introductions and hybridization can modify this pattern [9]. Both expectations derive from Level 2 evidence in congeners and remain untested in Israeli populations of either focal species.

Overall, *B. sitibundus* represents a predominantly terrestrial strategy characterized by tolerance of drought, environmental unpredictability and reproduction in temporary water bodies, whereas *P. bedriagae* represents an aquatic strategy characterized by persistent association with permanent freshwater habitats. Together, these genera provide complementary models for investigating ecological adaptation, physiological evolution and population differentiation across the Mediterranean-to-desert climatic gradient of Israel.

3. Phylogenetic Evidence from Mitochondrial and Nuclear Markers

Phylogenetic studies using mitochondrial and nuclear DNA markers have substantially improved understanding of the evolutionary history, taxonomy, population structure and ecological differentiation of *Pelophylax* and *Bufotes*. The focal species, *P. bedriagae* and *B. sitibundus*, belong to lineages with contrasting habitat associations and therefore provide a useful comparative framework for examining amphibian evolution across Israel's environmental gradient [3] [10].

3.1. Mitochondrial DNA Markers

Mitochondrial markers, including cytochrome *b* (*cytb*), 12S rRNA, 16S rRNA, cytochrome *c* oxidase subunit I (COI), NADH dehydrogenase subunit 2 (ND2) and the control region (D-loop), are widely used to reconstruct phylogenetic relationships and historical biogeography because they are maternally inherited and gen-

erally lack recombination [1] [2] [39]. In the *Pelophylax ridibundus* species complex, analyses of ND2, COI, *cytb* and 16S sequences have revealed deeply divergent mitochondrial lineages associated with historical isolation, recolonization and secondary contact [9] [13]. Studies of the *Bufo viridis* species complex similarly support *B. sitibundus* as a distinct evolutionary lineage distributed across the Levant, Anatolia, Iran and Central Asia [8]-[10]; comparable patterns of refugial isolation and secondary contact have been described in other west-Palearctic toads [11] (Figure 2). Mitochondrial 12S and 16S rRNA sequences generated directly from Israeli *P. bedriagae* (published as *Rana bedriagae*) constitute Level 1 evidence for the focal taxon and are listed with their GenBank accession numbers in Table 1 [17].

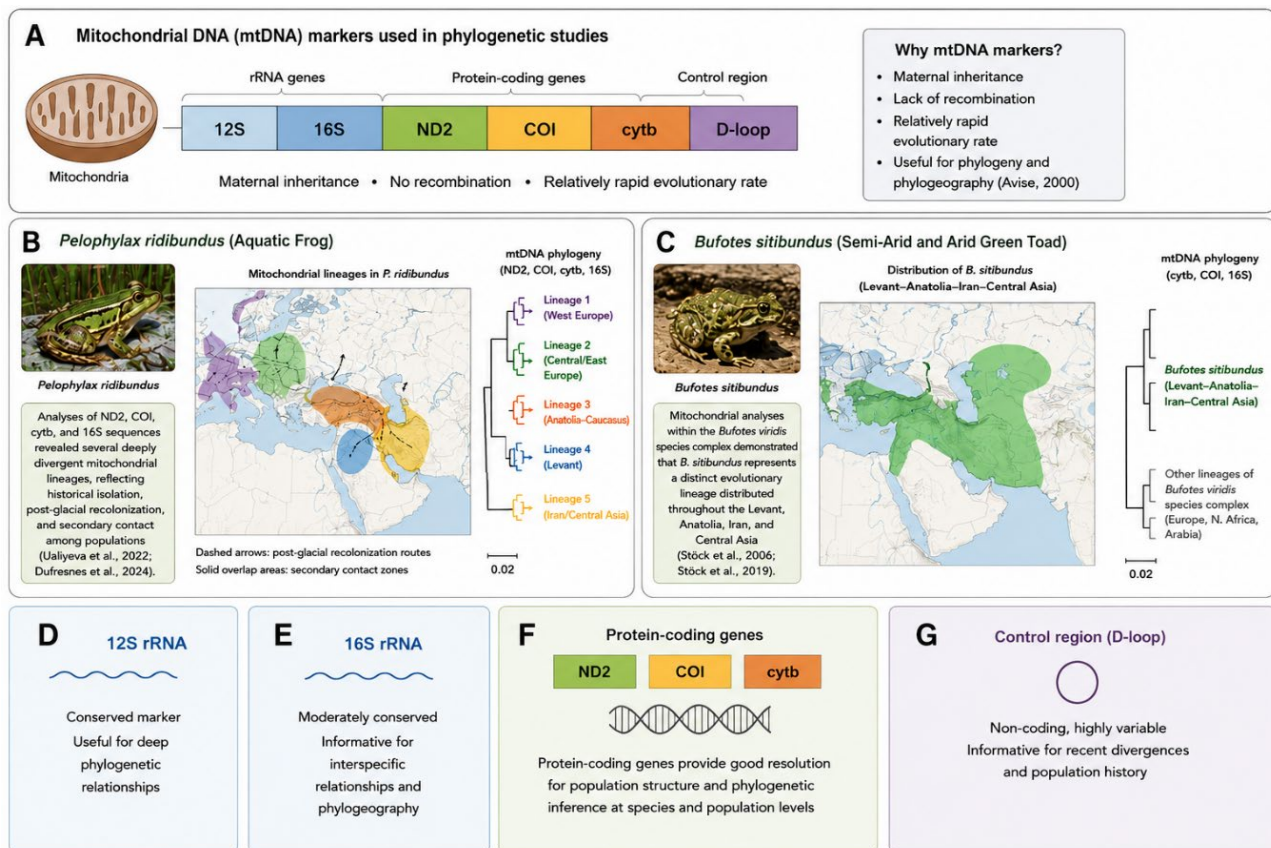


Figure 2. Comparative mitochondrial DNA (mtDNA) markers and phylogeographic patterns of *Pelophylax bedriagae* and *Bufo sitibundus*.

The figure summarizes the major mitochondrial DNA markers (12S, 16S, ND2, COI, *cytb* and D-loop) commonly used in amphibian phylogenetic studies. It compares the mitochondrial lineages and geographic distributions of *P. bedriagae* (25 samples from 5 sites) and *B. sitibundus* (50 samples from 24 sites), illustrating their evolutionary history and population divergence. The lower panel highlights the main applications of each mtDNA marker for phylogenetic, phylogeographic and population-genetic analyses [8]-[10].

Table 1. Accession numbers of mitochondrial 12S and 16S rRNA sequences of *Pelophylax bedriagae* (published as *Rana bedriagae*), from Degani *et al.* [17].

Breeding Sites	12S GeneBank acc. No.	16S GeneBank acc. No.
A-Lhavot Pond	FJ595206	FJ595211
B-Wasset Pond	FJ595208	FJ595213
C-Fara Pond	FJ595204	FJ595209
D-Navoraya Spring	FJ595207	FJ5952127
E-Jauda Pond	FJ595205	FJ595210

Note. Sequences were obtained from Israeli populations and are assigned here to *P. bedriagae* following the conventions in Section 1.3. This dataset is Level 1 evidence for the focal taxon.

3.2. Nuclear DNA Markers

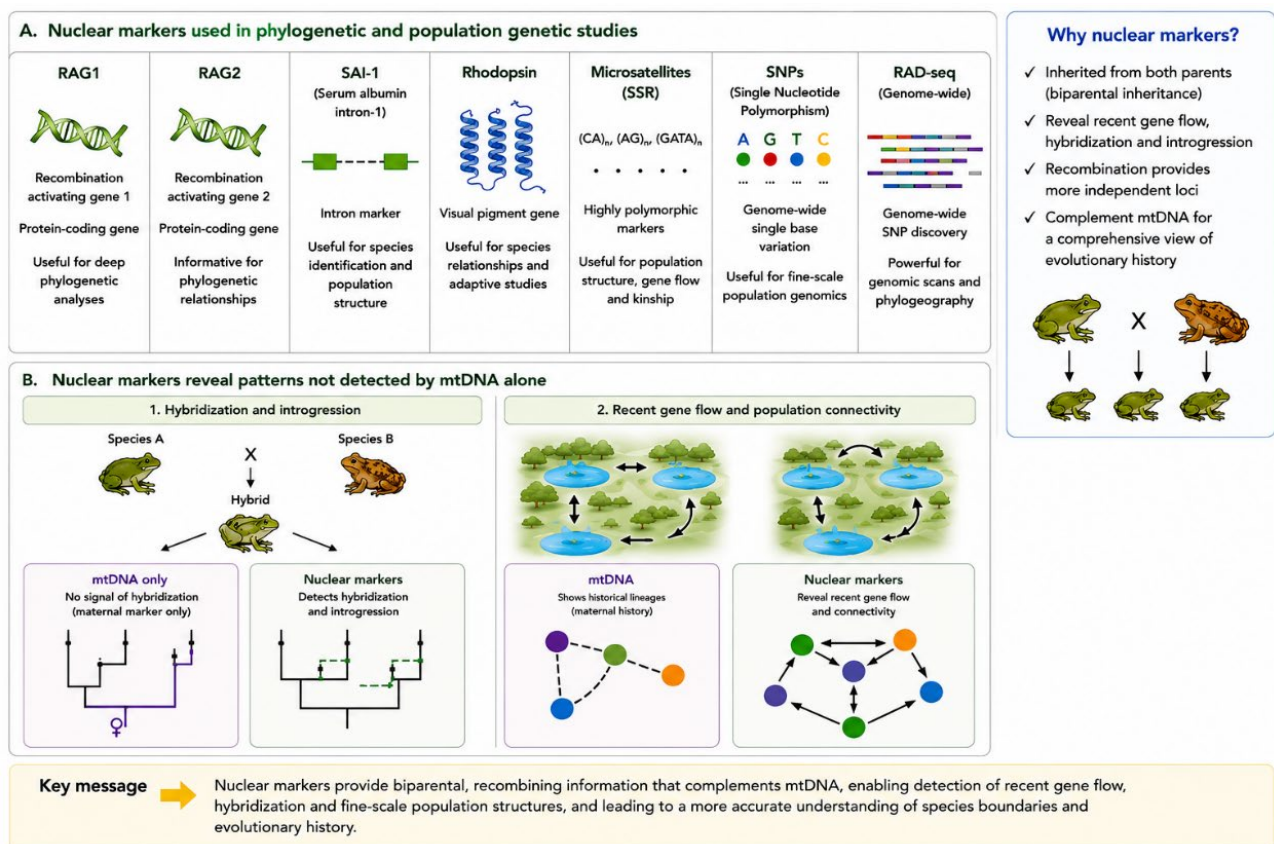


Figure 3. Nuclear DNA markers complement mitochondrial analyses for resolving phylogeny, hybridization, and population structure in *Pelophylax ridibundus* and *Bufo sitibundus*.

Nuclear markers complement mitochondrial data because they are inherited from both parents and provide information on recombination, recent gene flow, hybridization and introgression. Commonly used markers include RAG1, RAG2, serum albumin intron 1 (SAI-1), rhodopsin, microsatellites and single-nucleotide

polymorphisms (SNPs) [19], together with genome-wide reduced-representation approaches such as RAD-seq [40] [41]. Nuclear analyses have helped resolve taxonomic uncertainty within the *Pelophylax ridibundus* complex and have detected hybridization and introgression that may be obscured by mitochondrial data alone [9] [13]. Combined mitochondrial and nuclear analyses have likewise clarified species boundaries within *Bufo* and demonstrated the contribution of historical hybridization to present-day phylogeographic patterns [8] [10] (Figure 3).

The complementarity of the two marker systems is summarized in Figure 3. Hybridization and introgression can remain undetected when only maternally inherited mtDNA is analysed but are readily identified using biparentally inherited nuclear loci; recent gene flow and fine-scale population connectivity are likewise resolved more accurately with nuclear markers, because these reflect recombination and inheritance from both parents, whereas mtDNA records historical maternal lineages only. No RAD-seq or SNP dataset has yet been published for Israeli populations of either focal species, so all statements in this subsection concerning the focal taxa are Level 2 extrapolations.

In Figure 3, nuclear DNA markers complement mitochondrial analyses for resolving phylogeny, hybridization and population structure in *Pelophylax bedriagae* (25 samples from 5 sites) and *Bufo sitibundus* (25 samples from 5 sites). 1) Nuclear markers used in phylogenetic and population-genetic studies. RAG1 and RAG2 are conserved protein-coding genes commonly used for deep phylogenetic analyses; SAI-1 (serum albumin intron 1) is an intron marker useful for species identification and population differentiation; rhodopsin is a nuclear gene applied in phylogenetic and adaptive studies; microsatellites (SSR) are highly polymorphic markers suitable for assessing genetic diversity, kinship and population structure; SNPs provide thousands of genome-wide markers for fine-scale population-genomic analyses; and RAD-seq enables large-scale SNP discovery across the genome and is widely used for phylogeography, adaptation and evolutionary genomics. 2) Nuclear markers reveal patterns not detected by mitochondrial DNA, including hybridization, introgression, recent gene flow and fine-scale population structure. When combined with mitochondrial markers they provide a more complete reconstruction of species boundaries and evolutionary history [8] [19] [40] [41].

4. Distribution and Connectivity of *Pelophylax bedriagae* in Israel

In Israel, *Pelophylax bedriagae* is largely associated with permanent freshwater habitats, including the Hula Valley, the Jordan River system, the Sea of Galilee drainage basin, permanent streams, reservoirs, springs and wetlands of northern and central Israel [1] [6] [14] [42] (Figure 4). The continuity of aquatic habitats can promote dispersal and gene flow among populations, potentially reducing genetic differentiation relative to amphibians that breed in isolated temporary ponds. This is an expectation derived from general amphibian landscape genetics (Level

3) rather than a demonstrated property of Israeli populations, and it should be tested directly using population-genomic data from Israeli populations.

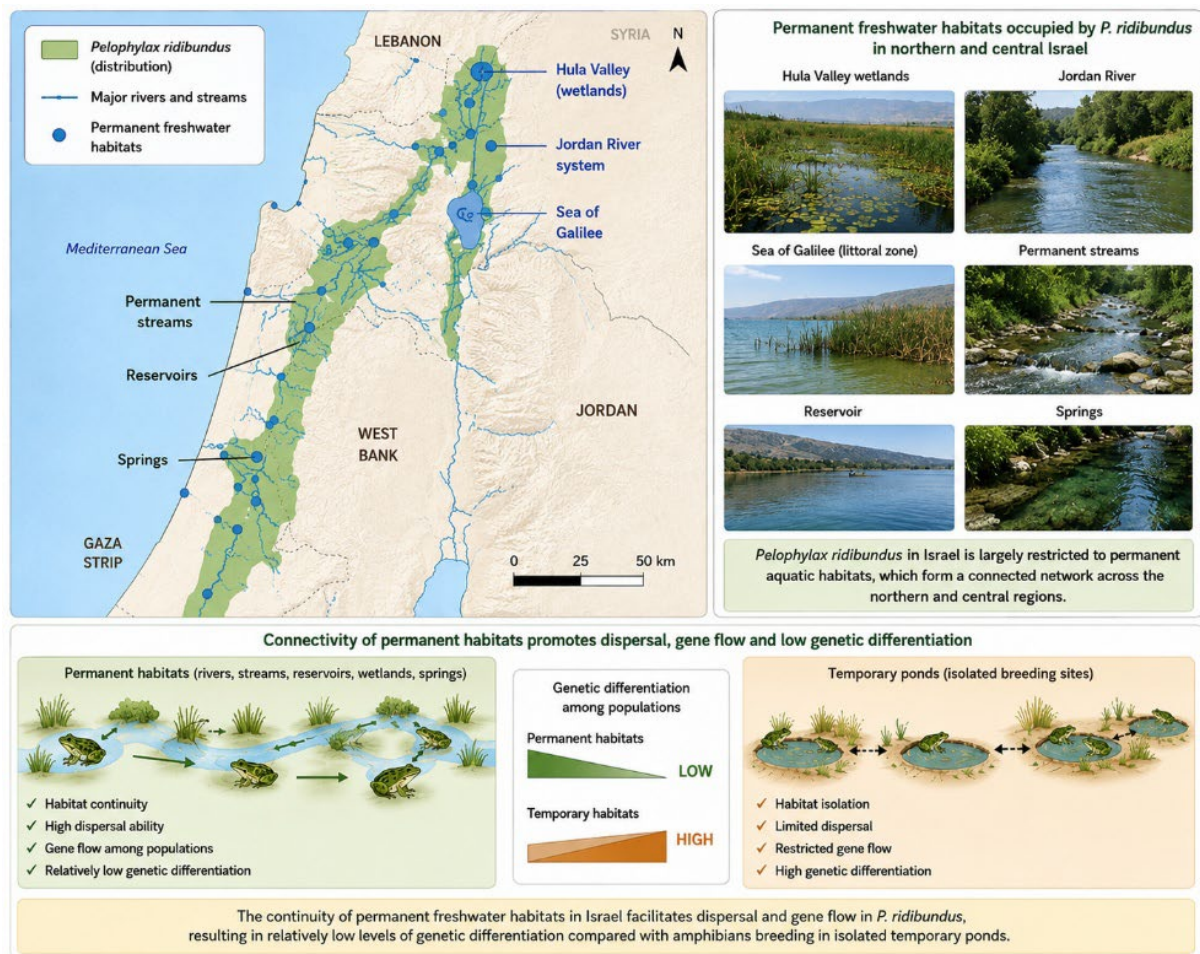


Figure 4. Distribution of *Pelophylax ridibundus* in Israel: Permanent Freshwater Habitats Promote Gene Flow and Reduce Genetic Differentiation.

Figure 4 shows distribution and aquatic habitat associations of *Pelophylax bedriagae* in Israel. The figure illustrates the distribution of *P. bedriagae* in Israel and its close association with permanent freshwater habitats, including the Hula Valley, the Jordan River system, the Sea of Galilee drainage basin, permanent streams, reservoirs, springs and wetlands [1]. The continuity of these aquatic habitats may facilitate dispersal and gene flow among populations by maintaining ecological connectivity [3]. In contrast, amphibian populations breeding in isolated temporary ponds generally experience lower connectivity and may consequently show greater population-genetic differentiation [1] [2]. The distribution pattern shown here therefore highlights the potential importance of permanent and interconnected aquatic habitats in shaping population connectivity and genetic structure in *P. bedriagae*. These relationships are hypothesized rather than demonstrated for the Israeli populations.

5. Distribution and Habitat Isolation of *Bufotes sitibundus* in Israel

Bufotes sitibundus occupies a broad ecological range extending from Mediterranean habitats in northern Israel through agricultural landscapes to semi-arid and arid regions of the Negev Desert [1] [2] (Figure 5). Because reproduction occurs mainly in temporary ponds and ephemeral rain pools, breeding populations may become geographically isolated after these sites dry. This fragmented breeding system is predicted to restrict gene flow, promote local adaptation and increase genetic differentiation, making *B. sitibundus* a useful model for studying the genomic consequences of habitat isolation [10].

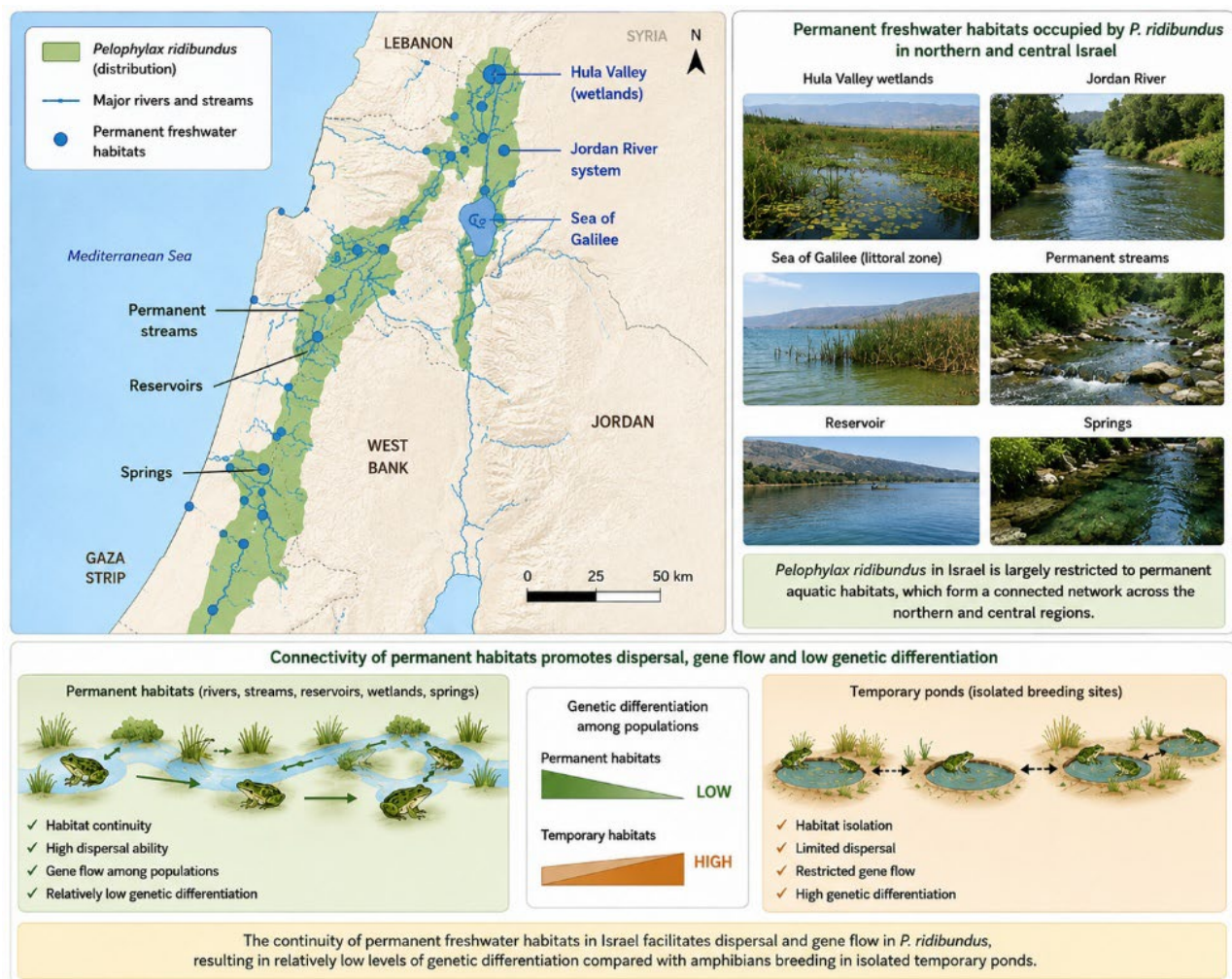


Figure 5. Ecological Distribution of *Bufotes sitibundus* in Israel: Habitat Isolation Promotes Local Adaptation and Genetic Differentiation.

Figure 5 shows Distribution and habitat isolation of *Bufotes sitibundus* in Israel. The figure illustrates the broad distribution of *B. sitibundus* from Mediterranean habitats in northern Israel through agricultural areas to the semi-arid and arid Negev Desert. It shows that the species breeds mainly in temporary ponds

and ephemeral rain pools, which become isolated after drying. This fragmented breeding system is expected to limit gene flow among populations, promote local adaptation and increase genetic differentiation, making *B. sitibundus* a useful model for studying habitat isolation and population divergence [1] [2]. The distributional data are Level 1 evidence; the inferred consequences for gene flow are predictions awaiting population-genomic testing.

6. Integrating Mitochondrial and Nuclear Evidence

Mitochondrial markers reconstruct maternal evolutionary history and historical biogeography, whereas nuclear markers provide insight into biparental ancestry, recent connectivity, hybridization and gene flow. Integrating both marker systems therefore provides the most robust framework for understanding the evolutionary history of *P. bedriagae* and *B. sitibundus* (Figure 6). Their contrasting ecological strategies, permanent aquatic association versus predominantly terrestrial life in semi-arid environments, make them valuable comparative models for studying adaptation, phylogeography and diversification along Israel's environmental gradients.

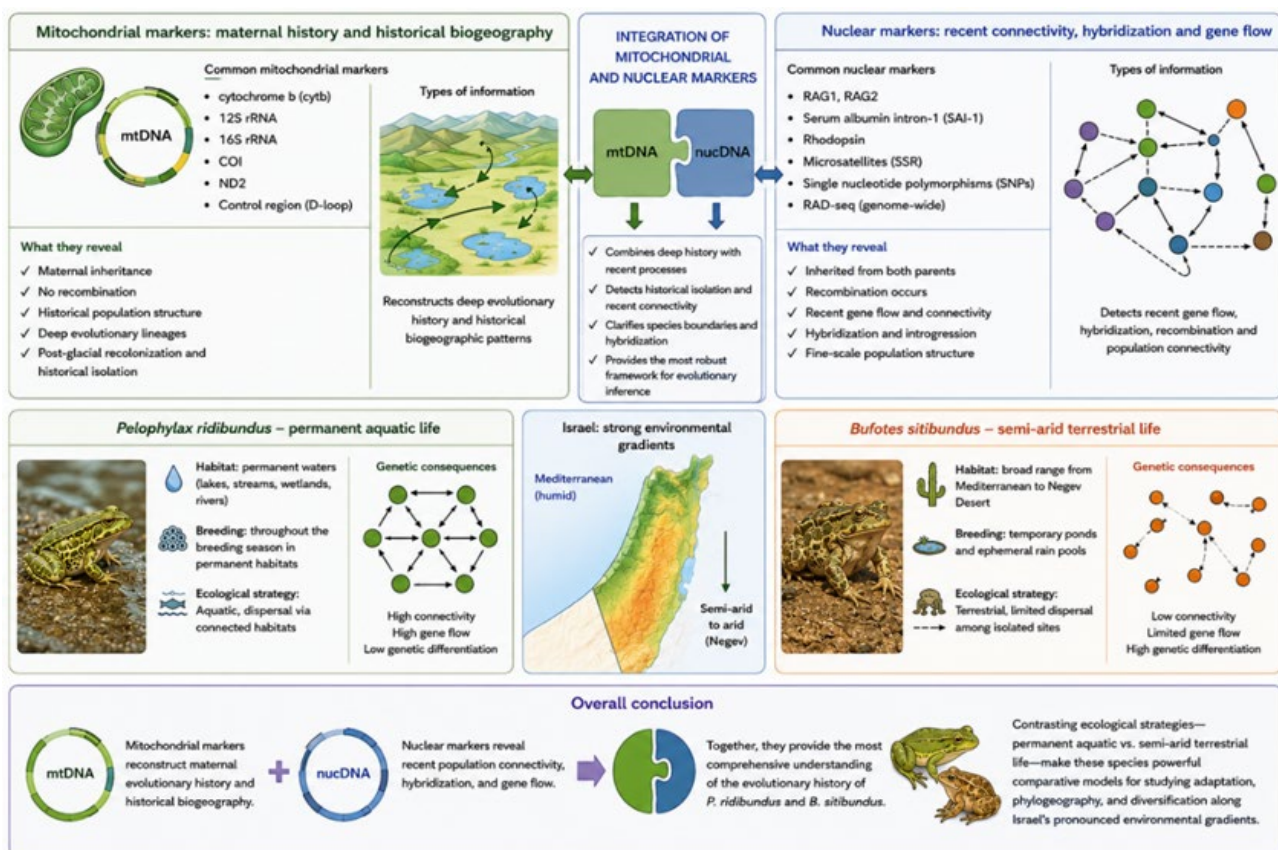


Figure 6. Integration of mitochondrial and nuclear DNA Markers for understanding the evolutionary history of *Pelophylax ridibundus* and *Bufotes sitibundus*.

Figure 6 shows integration of mitochondrial and nuclear DNA markers for understanding the evolutionary history of *Pelophylax bedriagae* (25 samples from 5

sites) and *Bufo sitibundus* (25 samples from 5 sites). The figure summarizes the complementary roles of mitochondrial and nuclear DNA markers in reconstructing evolutionary history. Mitochondrial markers reveal maternal ancestry and historical biogeography, whereas nuclear markers identify recent gene flow, hybridization and population connectivity. The figure also compares the contrasting ecological strategies of the two species and shows how combining both marker systems provides the most comprehensive framework for studying adaptation, phylogeography and diversification across Israel's environmental gradients.

Direct whole-genome comparisons between *B. sitibundus* and *P. bedriagae* are not yet available. The candidate pathways discussed in the following section should therefore be regarded as testable hypotheses derived from comparative evidence in related amphibians and in vertebrates generally, rather than as demonstrated species-specific genomic differences.

7. Candidate Genes and Molecular Pathways: Predictions for Habitat-Specific Adaptation

The contrasting ecological niches occupied by *B. sitibundus* and *P. bedriagae* are reflected in morphology and physiology and are predicted to involve molecular pathways regulating water balance, cellular stress, metabolism, immunity and endocrine function. Because genomic data for the focal Israeli species are unavailable, evidence from closely related Eurasian anurans (Level 2) and from amphibians and vertebrates generally (Level 3) is used to identify candidate genes and to generate explicit hypotheses for future testing [3] [38]. Every gene named below is a candidate, not a finding. For each pathway, we state the prediction, the evidence level on which it rests and the observation that would be required to confirm or refute it. Section 7.3 is the exception and is presented separately because it reports measured physiological data from the focal species themselves.

7.1. Water Conservation and Osmoregulation

Maintaining water balance is one of the greatest physiological challenges for amphibians. In terrestrial species, water uptake and conservation are regulated primarily through aquaporin (AQP) water channels expressed in the pelvic skin, urinary bladder and kidneys, which facilitate rapid water transport while limiting dehydration during prolonged exposure to dry environments. Vasotocin-regulated AQP2-like proteins contribute to water reabsorption, whereas AQP5-like proteins contribute to skin permeability [22] [23]. These mechanisms are characterized in anurans generally (Level 3) and have not been examined in either focal species. Direct evidence from *B. sitibundus* is limited to the whole-organism level: green toads from Israel accumulate urea during terrestrial acclimation, which reduces the osmotic gradient driving evaporative water loss [20]. This is a Level 1 observation of a physiological outcome; the transporters and channels responsible have not been identified in this species.

Pelophylax bedriagae inhabits permanent freshwater habitats, where dehydra-

tion risk is generally lower than in terrestrial environments [3]. The principal prediction is therefore not the absence of osmoregulatory genes, which are broadly conserved across amphibians, but habitat-associated differences in their regulation, tissue-specific expression or sequence variation [23]. In particular, aquaporins and other genes involved in transepithelial water and ion transport are predicted to exhibit differential expression or regulation in response to contrasting hydric environments. Direct comparative genomic and transcriptomic analyses of *P. bedriagae* and terrestrial *Bufo* species are required to test this hypothesis. The most informative test would be quantitative comparison of AQP transcript abundance in pelvic skin and urinary bladder between hydrated and dehydrated animals of both species, ideally replicated across the Israeli climatic gradient so that habitat and phylogeny are not confounded.

Candidate genes (predicted): *AQP1*, *AQP2-like (AQP-h2)*, *AQP3*, *AQP5-like (AQP-h3)*, *AVPR2-like*, *SLC9A3*, *ATP1A1*. Evidence level: 3, with no supporting Level 1 or Level 2 data in either genus.

7.2. Heat Stress and Cellular Protection

Semi-arid habitats expose *Bufo* to elevated temperatures and prolonged solar radiation. Heat-shock proteins protect cellular proteins from denaturation and maintain protein folding under thermal stress, and HSP70 and HSP90 are induced by thermal stress in many ectotherms, including amphibians [24]–[26]. This is Level 3 evidence. Terrestrial *Bufo* populations are predicted to show stronger or more rapidly inducible heat-shock responses than aquatic *Pelophylax* populations, but this prediction has not been tested in the focal species, and no heat-shock expression data exist for either. A direct test would compare induction kinetics and threshold temperatures for HSP70 and HSP90 transcripts and proteins in tissue from both species under controlled thermal ramps.

Candidate genes (predicted): *HSP70 (HSPA1A)*, *HSP90AA1*, *HSF1*. Evidence level: 3.

7.3. Metabolic Responses to Temperature and Dehydration: Direct Physiological Evidence from the Focal Species

This subsection differs from the remainder of Section 7 in that it reports measured data from the two focal species rather than predictions. It is the principal body of Level 1 physiological evidence available for the comparison.

Degani and Meltzer [21] examined the effects of environmental temperature and dehydration on energy metabolism in two amphibian species occupying contrasting ecological habitats: the terrestrial green toad, *Bufo sitibundus* (published as *Bufo viridis*), and the semi-aquatic marsh frog, *Pelophylax bedriagae* (published as *Rana ridibunda*). Oxygen consumption was measured across a range of ambient temperatures before and after the animals were exposed to dehydration.

At moderate ambient temperatures ranging from 14°C to 27°C, the two species

exhibited generally similar metabolic rates, indicating that their metabolic responses were comparable under relatively mild thermal conditions. Clear interspecific differences emerged as environmental temperatures increased.

At higher temperatures, between 27°C and 36°C, *P. bedriagae* (the semi-aquatic frog) showed a greater metabolic rate than *B. sitibundus* (the terrestrial toad). This difference was evident in both hydrated and dehydrated animals, suggesting that the contrasting metabolic responses were associated primarily with species-specific physiological characteristics rather than with hydration status alone [21].

Measurements of carbon dioxide production were consistent with the oxygen-consumption results. At elevated temperatures, CO₂ production was also higher in *P. bedriagae* than in *B. sitibundus*. Together, these findings indicate that the terrestrial toad maintains a comparatively lower metabolic rate when exposed to high environmental temperatures, whereas the semi-aquatic frog exhibits a stronger increase in metabolic activity [21].

These physiological differences are consistent with adaptation to the contrasting ecological conditions occupied by the two species. The relatively reduced metabolic response of *B. sitibundus* at high temperatures may contribute to its ability to tolerate terrestrial environments in which elevated temperatures and limited water availability frequently occur. In contrast, the higher metabolic activity observed in *P. bedriagae* is consistent with its greater dependence on aquatic or semi-aquatic habitats, where access to water reduces some of the physiological constraints associated with dehydration and exposure to high temperatures.

Two qualifications should be emphasized. First, the observed difference in thermal metabolic response is a demonstrated phenotypic contrast; the genes or regulatory mechanisms responsible for it were not investigated and remain entirely unknown. No inference about metabolic gene sequence or expression in either species follows from these measurements. Second, because the comparison involves a single bufonid and a single ranid, the difference cannot be attributed to habitat association rather than to family-level physiological differences (Section 1.1). Replication across additional terrestrial-aquatic species pairs, and across *B. sitibundus* populations spanning the Israeli climatic gradient, would be required to establish that the contrast is habitat-driven.

7.4. Oxidative Stress Defence

Thermal stress, dehydration and fluctuating oxygen availability increase the production of reactive oxygen species (ROS). Amphibians counteract oxidative damage through antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GSR) and peroxiredoxins detoxify ROS and protect lipids, proteins and DNA [26] [27]. These functions are conserved across vertebrates (Level 3). The prediction that terrestrial *B. sitibundus* sustains higher constitutive or inducible antioxidant capacity than aquatic *P. bedriagae* is untested; enzyme activity assays and transcript quantification in liver, muscle and skin under controlled heat and dehydration treatments would provide

the first direct test.

Candidate genes (predicted): *SOD1*, *SOD2*, *CAT*, *GPX1*, *GSR*, *PRDX1*. Evidence level: 3.

7.5. Hypoxia Responses

Aquatic anurans encounter hypoxic conditions in eutrophic ponds, beneath ice and during prolonged submergence, and oxygen-sensing pathways centred on hypoxia-inducible factor 1 α mediate the transcriptional response to low oxygen availability in vertebrates generally, regulating anaerobic glycolysis, angiogenesis and erythropoiesis [28] [29]. The prediction arising from the ecological contrast is that *P. bedriagae* relies more heavily on these pathways than does the predominantly terrestrial *B. sitibundus*, which instead encounters desiccation and hyperthermia as its principal stressors. This prediction rests entirely on Level 3 evidence; no hypoxia-response data exist for either focal species. A direct test would compare HIF1A target-gene induction and blood lactate accumulation during controlled submergence or progressive hypoxia in the two species.

Candidate genes (predicted): *HIF1A*, *VEGFA*, *EPO*, *LDHA*, *EGLN1*. Evidence level: 3.

7.6. Skin-Barrier Function and Innate Immunity

Amphibian skin serves simultaneously as a respiratory surface, an osmoregulatory organ and the primary barrier against pathogens. Antimicrobial peptides, pattern-recognition receptors and complement components constitute the principal innate defences of anuran skin [30] [31], and gross differences in skin architecture between bufonids and ranids are well documented [18]. The thick, keratinized skin of *Bufo*tes is expected to reduce evaporative water loss and to provide mechanical protection, whereas the thin, highly permeable skin of *Pelophylax* facilitates cutaneous respiration but presents a more exposed surface to waterborne pathogens. These structural differences are, however, family-level characters (Section 1.1) and cannot be read as habitat adaptations within this two-species comparison. Whether the associated immune gene repertoires differ between the focal species, and whether any such difference tracks habitat rather than phylogeny, is unknown; skin transcriptome comparison under standardized pathogen challenge would be the appropriate first test.

Candidate genes (predicted): *TLR2*, *TLR4*, β -defensins, cathelicidins, lysozyme (*LYZ*), *IL1B*, *TNF*, *C3*. Evidence level: 3.

7.7. Energy Metabolism

Habitat differences are expected to influence energy metabolism in amphibians. Terrestrial *B. sitibundus* experiences intermittent food availability, dehydration and prolonged periods of inactivity, which would favour metabolic flexibility, efficient glycogen storage, lipid utilization and energy conservation. In contrast, aquatic *P. bedriagae* generally inhabits more stable environments with continuous

access to water and relatively predictable food resources, reducing the need for extreme metabolic adjustment [38]. The measured thermal metabolic contrast reported in Section 7.3 [21] is the one element of this picture that has been demonstrated in the focal species; the remainder is inference.

In vertebrates generally, such physiological differences are regulated by genes involved in energy sensing, glycolysis, mitochondrial respiration and fatty-acid oxidation [33] [43]. AMPK (*PRKAA1*) acts as the major cellular energy sensor, promoting ATP production while suppressing energy-consuming processes. *PPARGC1A* (*PGC-1 α*) regulates mitochondrial biogenesis and oxidative metabolism [32]. *CPT1A* controls the transport of long-chain fatty acids into mitochondria for β -oxidation. *HK1* catalyses the first step of glycolysis, *LDHA* supports anaerobic ATP production during oxygen limitation and *COX1* encodes a key subunit of cytochrome *c* oxidase in the mitochondrial electron transport chain.

Comparative analyses of these genes may reveal differences in expression, regulation, enzyme activity or sequence variation between *B. sitibundus* and *P. bedriagae*. No such comparison has been performed, and the gene list below is therefore a prediction generated by the phenotypic contrast in Section 7.3 rather than a molecular explanation of it. Direct comparative genomic, transcriptomic, proteomic and physiological studies are required to determine whether these pathways have undergone habitat-specific modification.

Candidate genes (predicted): *PRKAA1* (*AMPK*), *PPARGC1A* (*PGC-1 α*), *CPT1A*, *HK1*, *LDHA*, *COX1*. Evidence level: 3, with a supporting Level 1 whole-organism phenotype (Section 7.3) that does not by itself implicate any of these loci.

7.8. Endocrine Regulation

Endocrine pathways integrate environmental signals with physiological responses that regulate water balance, growth, metabolism, development and stress adaptation in amphibians [3] [34]. Arginine vasotocin (AVT) regulates osmoregulation by stimulating aquaporin-mediated water transport in the skin and urinary bladder [23]. Corticosterone, acting through the glucocorticoid receptor (*NR3C1*), coordinates physiological responses to dehydration and other environmental stresses [34]. Thyroid hormones, acting through *THRA* and *THRB*, control metamorphosis and tissue differentiation [35]. Growth hormone (*GHI*), insulin-like growth factor 1 (*IGF1*) and prolactin (*PRL*) regulate somatic growth, energy metabolism, osmoregulation and tissue maintenance [36] [37]. All of this is Level 3 evidence from amphibians and vertebrates generally.

Comparative analyses of these genes between *B. sitibundus* and *P. bedriagae* may reveal habitat-associated differences in endocrine regulation, gene expression or sequence variation. No endocrine measurements or expression data are available for either focal species, so the assignment below is predictive only. Plasma corticosterone and AVT profiling during standardized dehydration, coupled with receptor-transcript quantification, would supply the first direct evidence.

Candidate genes (predicted): *AVPR2-like*, *GH1*, *IGF1*, *PRL*, *THRA*, *THRB*, *NR3C1*. Evidence level: 3.

7.9. Current Genomic Evidence and Research Priorities

High-quality genomic resources remain unavailable for *B. sitibundus* and *P. bedriagae*. Most molecular inference therefore relies on related species, including *B. viridis*, *B. variabilis*, *P. ridibundus*, *P. lessonae* and *P. kurtmuelleri* [8] [10] [12] [13]. Genes involved in water transport, stress responses, immunity, metabolism and endocrine regulation are broadly conserved across anurans, but conservation alone does not demonstrate adaptive differentiation between the focal species (Figure 7).



Figure 7. Comparative molecular pathways proposed to underlie habitat-specific adaptation in the green toad (*Bufo sitibundus*) and the Levant water frog (*Pelophylax bedriagae*).

Four priorities follow directly from the evidence structure set out in Section 1.2. First, chromosome-level genome assemblies for *B. sitibundus* and *P. bedriagae*, anchored to the existing *B. viridis* assembly [12], would convert much of the Level 2 material in this review into Level 1 evidence. Second, population-genomic sampling of *B. sitibundus* across the Mediterranean-to-desert gradient would allow selection scans in which phylogenetic background is held approximately constant while habitat varies, which is the single most direct way to separate adaptation

from phylogenetic inertia. Third, tissue-specific transcriptomics under controlled dehydration, heat and hypoxia challenge would test the expression predictions in Sections 7.1, 7.2 and 7.4 - 7.8 without requiring a complete assembly. Fourth, phylogenetically replicated comparisons across additional terrestrial-aquatic anuran pairs would establish whether any difference recovered between the focal genera represents convergent habitat adaptation or a lineage-specific peculiarity. Until at least the second and third of these are undertaken, the candidate pathways proposed here remain untested hypotheses.

All pathways shown are candidate predictions, not demonstrated species-specific differences. **1) Water conservation and osmoregulation:** the terrestrial green toad is predicted to rely on efficient water conservation through aquaporins and ion transporters regulating water uptake across the pelvic skin, urinary bladder and kidneys, whereas the aquatic frog experiences lower dehydration stress [3] [26]-[28]. **2) Heat stress and cellular protection:** terrestrial habitats expose *Bufo* to elevated temperatures and solar radiation, predicting stronger induction of heat-shock proteins; candidate genes include *HSP70* (*HSPA1A*), *HSP90AA1* and *HSF1* [3] [24] [26]. **3) Oxidative stress defence:** heat and dehydration increase reactive oxygen species, predicting enhanced antioxidant defences in the terrestrial species; candidate genes include *SOD1*, *SOD2*, *CAT*, *GPX1*, *GSR* and *PRDX1* [3] [26] [27]. **4) Hypoxia responses:** aquatic frogs encounter hypoxic conditions and are predicted to depend more strongly on oxygen-sensing pathways; candidate genes include *HIF1A*, *VEGFA*, *EPO* and *LDHA* [3] [28] [29]. **5) Skin-barrier function and innate immunity:** the thick, keratinized skin of *Bufo* reduces evaporative water loss and provides protection against pathogens, whereas the thin, permeable skin of *Pelophylax* facilitates cutaneous respiration; candidate immune genes include *TLR2*, *TLR4*, β -defensins, cathelicidins, lysozyme, *IL1B*, *TNF* and *C3* [3] [30] [31]. **6) Energy metabolism:** the green toad is predicted to show greater metabolic flexibility in fluctuating terrestrial environments; candidate genes include *PRKAA1*, *PPARGC1A*, *CPT1A*, *HK1*, *LDHA* and *COX1* [3] [26] [33]. **7) Endocrine regulation:** hormonal pathways coordinate water balance, stress responses, growth, reproduction and metamorphosis; candidate genes include *AVPR2-like*, *GHI*, *IGF1*, *PRL*, *THRA*, *THRB* and *NR3C1* [3] [34]. **8) Current genomic evidence and research priorities:** genomic resources for the focal species remain unavailable, and the proposed pathways are inferred from related Eurasian species and from vertebrates generally; future whole-genome sequencing, population-genomic analyses and functional studies are required to validate them [8]-[10] [12] [13] [18].

8. Conclusions

Bufo sitibundus and *Pelophylax bedriagae* represent contrasting ecological strategies and provide complementary models for studying adaptation to semi-arid terrestrial and permanent aquatic environments. The demonstrated contrasts between them are ecological, distributional and physiological: differing habitat

and breeding-site associations across the Israeli climatic gradient, urea accumulation during terrestrial acclimation in the green toad, and a measured divergence in metabolic response to elevated ambient temperature that persists in both hydrated and dehydrated animals. The molecular content of this review is of a different kind. Comparative evidence from related taxa and from vertebrates generally identifies plausible pathways involving water and ion transport, heat-shock and antioxidant defences, hypoxia signalling, skin-barrier and immune function, energy metabolism and endocrine regulation, but no direct expression, sequence or functional data link any of these to habitat use in either focal species. These candidate pathways are therefore offered as an explicit, prioritized research agenda rather than as conclusions.

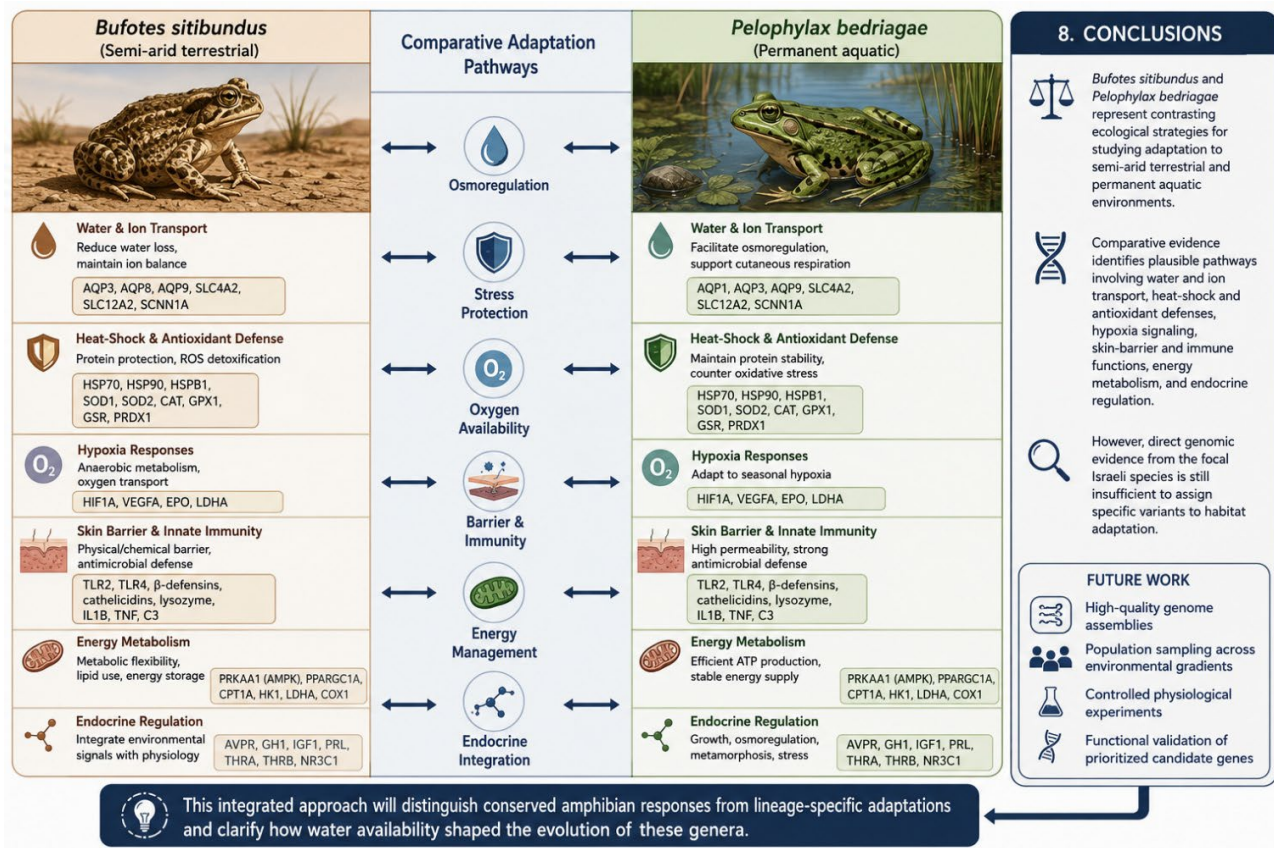


Figure 8. Conceptual model of habitat-specific physiological and molecular adaptations in *Bufootes sitibundus* and *Pelophylax bedriagae*: current evidence and future research directions.

Two constraints should govern how that agenda is pursued. First, because *Bufootes* and *Pelophylax* are distantly related, any difference recovered between them is confounded with phylogeny and cannot by itself demonstrate habitat-driven adaptation; within-genus contrasts along the Israeli gradient and phylogenetically replicated cross-genus comparisons are needed to break that confound. Second, conservation of a pathway across anurans is not evidence of its adaptive differentiation between the focal species. Future work should combine high-quality

ity genome assemblies, population sampling across environmental gradients, controlled physiological experiments and functional validation of prioritized candidate genes. This approach will distinguish conserved amphibian responses from lineage-specific adaptations and clarify how water availability has shaped the evolution of these genera (**Figure 8**).

Note. The two species represent contrasting semi-arid terrestrial and permanent aquatic strategies. Their proposed adaptations involve water and ion transport, stress and antioxidant protection, hypoxia responses, skin barrier and immunity, energy metabolism and endocrine regulation, with the principal candidate genes shown. Current evidence identifies plausible pathways but is insufficient to establish habitat-specific genomic variants, and the candidate genes shown are predictions. Future studies should combine genome assemblies, population sampling, physiological experiments and functional gene validation to distinguish conserved from lineage-specific adaptations.

Data Availability Statement

No new experimental data were generated in this study. This review is based on data previously published in the scientific literature concerning *Bufo* and *Pelophylax*. The published evidence was critically reviewed, comparatively analysed, synthesized and presented in a new integrative framework, including revised tables and graphical representations. All original data sources are cited in the manuscript and are available through the referenced publications. Accession numbers for the mitochondrial 12S and 16S rRNA sequences of *P. bedriagae* (published as *Rana bedriagae*) reported by Degani *et al.* [17] are listed in **Table 1**, in Section 3.1.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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