Interdisciplinary approach to solve unusual mortalities in the European common frog (*Rana temporaria*) in two high-mountain ponds affected by climate change

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A B S T R A C T

The global decline in amphibian populations is a major environmental issue. Chytridiomycosis, Ranaviruses and the red-leg syndrome have been identified in unusual mortality events. However, these infections do not account for all causes of declining amphibian populations. Moreover, several cases of amphibian mortality are difficult to solve without resorting to an interdisciplinary approach. Two cases of unusual mortality in *Rana temporaria* occurred at two high-mountain ponds (northwest Italy) in April and May 2021. Water and frog samples were analysed to understand the possible causes responsible for the unusual mortalities. Results of the main physicochemical (pH, conductivity, dissolved oxygen, chemical and biochemical oxygen demand) and nutrient (ammonia/ammonium, nitrite, nitrate, total phosphorus) parameters revealed a good condition of the water quality, with the absence of the main cyanotoxins (microcystins/nodularins). However, unseasonably high spring water temperatures were recorded in both ponds (12.73 °C and 14.21 °C for Frog Pond and Selleries Pond, respectively). Frogs (n = 50; snout–vent length: 7.0–9.8 cm; body mass: 85–123 g) collected from Frog Pond mainly presented bumps on the ventral cavity and dermal ulceration associated with the isolation of *Carnobacterium maltaromaticum*. On the other hand, frogs (n = 5; snout–vent length: 8.0–9.1 cm; body mass: 87–92 g) from Selleries Pond presented petechiae and dermal ulcerations on the rear limbs associated with the isolation of *Aeromonas salmonicida* and *A. sobria*. In both mortality events, the interdisciplinary approach revealed an association between frog mortalities and the isolation of bacteria. Isolated bacteria are considered opportunistic pathogens, and the high values of the water temperature has certainly led a stress on the frogs, favouring the spread of bacteria and the death of the frogs. Further studies are needed to assess the pathophysiological effects of the opportunistic bacteria here isolated, clarifying the interactions between emerging pathogens and climate change.

1. Introduction

Amphibian species are under greater threat than mammals or birds, with many species on the verge of extinction: 34.1% (2490 of 7296) of amphibian species are threatened with extinction, 5.7% (418) are known to be "Near Threatened", 16.3% (1193) are "Data Deficient", and

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54.8% (~4000 species) are in trouble (IUCN SSC, 2022). Among the factors contributing to the decline of amphibian populations are habitat destruction (leading cause of decline and extinction of amphibian population), pollution, introduction of alien species, climate change, infectious diseases, and overexploitation (collection for pet and food industries) (Green et al., 2020; Aliko et al., 2021). All these causes may be further complexed by interconnecting co-factors (Campbell Grant et al., 2020). Furthermore, depending on the habitat type, the causes of population decline differ from region to region and within populations of the same species (Valdez et al., 2021). Moreover, studies suggest interspecific and life-stage differences in how amphibians react to stressors, which have an impact from molecular to community level (Zheng et al., 2021).

Climate change and emerging infectious diseases are two far-reaching ecological challenges (Brooks and Boeger, 2019). Climate has a direct impact on disease outbreaks. Predictive models have shown that climate change together with infectious diseases contribute to current and future impoverishment in biodiversity (Ogden and Gachon, 2019). Cohen et al. (2017) reported that the patterns of amphibian decline can only be explained by a combination of increased temperatures and infectious disease, particularly in species adapted to relatively cold environments. Furthermore, in a later study (Cohen et al., 2019) suggested that hosts adapted to cold environments will be the most vulnerable to the combination of rising mean temperature and emerging infectious diseases.

For example, the chytrid fungus *Batrachochytrium dendrobatidis* infects the epidermal layer of amphibians and has been linked to global population declines (Collins, 2013). Cohen et al. (2017) studied 235 amphibian species in 598 globally distributed populations and discovered that *B. dendrobatidis* satisfies the thermal mismatch hypothesis (hosts are vulnerable to disease at temperatures where the performance gap between themselves and parasites is greatest).

Ranaviruses and the red-leg syndrome have been linked to large-scale amphibian extinctions and population declines (Cohen et al., 2019). Ranaviruses are double-stranded DNA viruses that can infect fish, amphibians, and reptiles (Family Iridoviridae; Subfamily Alphairidovirinae) (Snyder et al., 2022). Ranaviruses cause systemic infections with varying disease manifestations; clinical signs include buoyancy difficulty, anorexia, leg and body swelling, redness, haemorrhages, cutaneous erosions, and ulcerations (Davis et al., 2019). A common infection of frogs is the red-leg syndrome, which is caused by *Aeromonas hydrophila*, an opportunistic bacterial pathogen, and distinguished by redness on the underside of the amphibian's legs and abdomen (Pasteris et al., 2006; Densmore and Green, 2007).

The recent worldwide increase in unusual mortality incidents in frogs and toads has been reported to involve hundreds to thousands of specimens dying over several weeks (McCallum et al., 2018; Isidoro-Ayza et al., 2019; Khalifa et al., 2021; Park et al., 2021; Hartmann et al., 2022). Frogs that are dying or dead may or may not show visible external symptoms (i.e., discolored skin, ulcers, bleeding), but they may appear lethargic and disoriented, especially near pond edges. Though possible, there is no evidence that a single disease agent or environmental factor is to blame for the recent increase in mortality. In addition, some amphibian mortality incidents cannot be fully explained since any actions could be implemented within restricted timeframe for sampling and laboratory analyses. For such reasons, to assess complex environmental issues like global and climate change, an interdisciplinary approach is required to provide a fundamental comprehension of environmental systems and processes. Issues related to climate change are good examples of situations that necessitate complex synthesis of ideas from a wide range of disciplines and scientists.

The European common frog (*Rana temporaria*) is one of the most common amphibian species found throughout Europe, from Spain to Siberia. In Italy it is distributed in the Alps with some populations inhabiting lowlands and hills in Piedmont and the northern Apennines (province of Forlì, Emilia-Romagna Region) (Temple and Cox, 2009). Its habitats are located at elevations from sea level to 2700 m.

The IUCN Red List (IUCN, 2022) categorizes it as being of "Least Concern", indicating population stability without serious threats to this species. A closer look, however, will reveal dramatic changes in local populations. Those inhabiting the Italian Alps, for example, are threatened by the introduction of alien fish (mainly salmonids) to lakes for recreational purposes and the abandonment of pastoralism, resulting in the disappearance of pools for reproduction (Pastorino et al., 2020a).

Local agencies (i.e., park management authority) have reported recent unusual mortality incidents in adult *R. temporaria* at high-mountain ponds (Cottian Alps; northwest Italy). Between April and May 2021 two unusual mortality incidents in *R. temporaria* were reported at two high-mountain ponds (Frog Pond and Selleries Pond) in Piedmont (northwest Italy). The main hypothesis tested in this study was to verify if the use of an interdisciplinary approach based on the expertise of ecologists, veterinarians, and biotechnologists could solve the causes of these unusual incidents. We also hypothesized a link between the unusual mortality incidents and the combined effect of pathogens and increased temperature at the two ponds.

2. Material and methods

2.1. Study sites

Frog Pond (45°03′50.3″N 7°01′51.4″E) and Selleries Pond (45°02′52.5″N 7°07′15.5″E) are two high-mountain ponds located at 1916 m and 1985 m a.s.l., respectively, in the Cottian Alps (northwest Italy). Frog Pond is located in the municipality of Usseaux (Province of Turin, Piedmont) and Selleries Pond (45°02′52.8″N 7°07′16.5″E) in the municipality of Roure (Province of Turin, Piedmont) (Fig. 1 a, b). Selleries Pond is designated as a Special Area of Conservation (SAC-IT1110006 Orsiera Rocciavrè). Frog Pond is 125 m in perimeter, 1.5 m in maximum depth, and 766 m^2 in surface area. Selleries pond is 120 m in perimeter, 2 m in maximum depth, and 815 m^2 in surface area. Both ponds are covered by ice from December to March/April and serve as a breeding site for *Rana temporaria*. The benefit of being in remote areas is that the ponds are far away from direct anthropogenic impacts as wastewater treatment plants, agriculture and industrial activities that can cause a rapid and significant change of the water quality and aquatic communities. The two main anthropogenic activities in summer are trekking and pasturing.

2.2. Frog mortality and sampling

On April 2, 2021 an unusual mortality incident (>50%) involving adult frogs (*Rana temporaria*) occurred at Frog Pond. The dead individuals were recorded by local rangers and sampled by veterinarians of the University of Torino. About 50 dead individuals were collected with a hand net, placed in plastic bags, and frozen at −20 °C until laboratory analysis.

On May 15, 2021 an unusual mortality incident (~40%) involving adult frogs (*R. temporaria*) occurred at Selleries Pond. The event was reported by local ranges, but sampling was not performed immediately. A few days later (May 17, 2021), environmental scientists from Istituto Zooprofilattico Sperimentale del Piemonte, Liguria and Valle d'Aosta together with veterinarians from the local veterinary office (ASL-TO3) performed a walk-through and recorded about 20 dead individuals. Since many were badly decomposed, the researchers collected five moribund individuals with a hand net. All five presented with lethargy and two with extreme weight loss. They transported alive (cold box: +8 °C) to the laboratory (within 2 h), euthanized by tricaine methanesulfonate (MS-222; concentration: 6 g/L; Sigma Aldrich, Italy) and analysed.

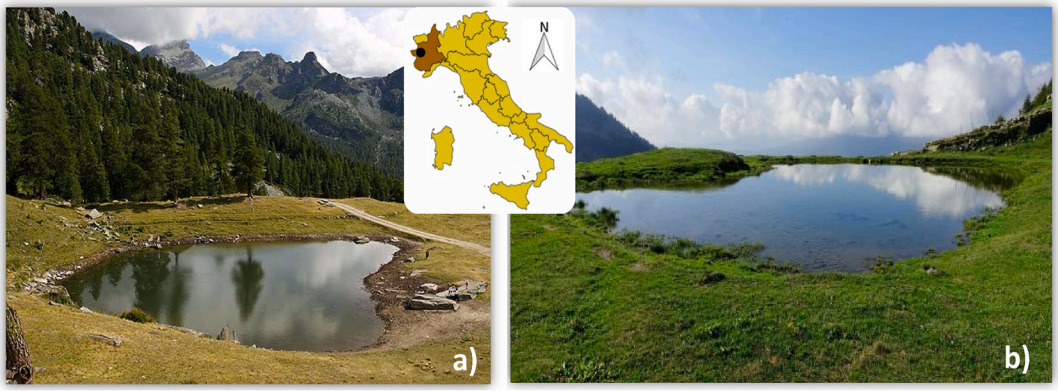

Fig. 1. Study sites in the Cottian Alps (northwest Italy): a) Frog Pond (45°03′50.3″N 7°01′51.4″E); b) Selleries Pond (45°02′52.8″N 7°07′16.5″E).

2.3. Water sampling and analysis

The main physiochemical water parameters were recorded at the two sites at the same time of the frog sampling. Water temperature (°C), pH (unit), conductivity (μs/cm) and dissolved oxygen (mg/L) were recorded in the field using a multiparameter portable meter (HI98194; Hanna Instruments Inc., USA). Three replicates were collected from each parameter to approximately 40 cm under the water surface. Samples of water were also collected using glass bottles (2 L; three replicates) from the two ponds and transported refrigerated (+4 °C) in laboratory to determine ammonia/ammonium (NH_3/NH_4^+; mg/L), nitrite (NO_2^-; mg/L), nitrate (NO_3^-; mg/L), total phosphorus (P; mg/L), chemical oxygen demand (COD; mg/L) and biochemical oxygen demand (BOD_5; mg/L). Analyses were performed using a multi-parameter bench photometer (HI83399, Hanna Instruments Inc., USA) and following standard methods (APHA, 1998; ASTM, 2015). The presence of the two main cyanotoxins (microcystins and nodularins) was also determined using a commercial kit (Microcystins-ADDA ELISA; Product No. 520011O; Abraxis, Warminster, PA, USA) based on an indirect competitive ELISA test, strictly following the methodology reported by the manufacturer (Abraxis, Warminster, PA, USA). Briefly, when present in a sample, the toxin competes with an immobilized analogue of the microcystins protein for the binding sites of the anti-microcystins/nodularins antibodies (Magara et al., 2022). A second antibody-horseradish peroxidase (HRP) label is then added once the plate has been cleaned. A color signal is produced following the addition of the substrate solution and a second washing step. After a predetermined amount of time, the color reaction is stopped, and the color was evaluated using an ELISA reader (Synergy HT, multimode microplate reader, BioTek Instruments, USA). Interpolation was used to determine the concentrations of the samples using the standard curve constructed with each run. The limit of detection (LOD) of this test was 0.10 μg/L.

2.4. Necropsy, parasitological examination, and tissue collection

Body mass (g) and snout–vent length (cm) were retrieved for each specimen. Necropsy was performed to inspect lesions or other pathological alterations, as detailed in Pessier and Mendelson (2010). Briefly, necropsy began with external examination of the specimen's general condition. The skin was inspected for potential discoloration, increased mucus, skin shedding or ulceration. Areas of skin from the ventral body and the lesion margins were scraped with a scalpel in preparation for wet mount examination to detect external protozoan parasites, including *Trichodina* spp. This was not carried out on the frozen frogs. Skin portions were sampled for histological examination (see Section 2.5). The individuals were then processed for internal examination. The animal was placed in dorsal recumbency and the ventral midline from the intermandibular region to the cloaca was incised with a sterile scalpel. The main internal organs (see other sections for details) were examined, dissected, and sampled for bacteriological, histological, and molecular analyses.

2.5. Histological examination

Brain, eye, heart, lung, spleen, liver, kidney, skin, and muscle were collected from each specimen and immediately fixed in 10% neutral buffered formalin. Standard paraffin wax techniques were used to process the samples, which were then cut into thick sections (4 ± 2 μm) with a microtome and stained with haematoxylin and eosin (HE). Microscopically, slides were examined at increasing magnification (10–40x) using a Nikon microscope (H550L, Nikon, Japan).

2.6. Bacteriological examination

Bacteriological examination of skin, kidney, lung, brain, and eyes was performed according to standardized protocols (Dökenel and Selmin, 2019; Pastorino et al., 2021). All primary cultures were incubated in triplicate on Columbia blood agar (CBA) and trypticase soy agar (TSA) for 72 h at 22 °C. Colonies were the sub-cultured and incubated at 22 °C for 24 h for biochemical and phenotypic characterization following standardized procedures (Santi et al., 2019; Pastorino et al., 2021). Bacterial identification was performed on a VITEK MS system (bioMérieux, France) using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Isolates underwent biomolecular characterization and kept at −80 °C in trypticase soy broth containing 20% glycerol for further analysis.

2.7. Biomolecular analyses

2.7.1. Ranavirus

DNA was extracted using the ReliaPrep™ gDNA Tissue Miniprep System Kit (3.C. Protocol for Buccal Swabs) from swabs performed on skin. The DNA was then quantified by nanophotometer and stored at −80 °C. The protocol for Ranavirus detection was based on the amplification of a portion of about 500 base pairs of the Ranavirus major capsid protein (*MCP*) gene (Mao et al., 1997; Allender et al., 2011; Stöhr et al., 2013) The PCR reaction was performed in a total volume of 25 μL containing 2 μL of template, 2.5 μL of buffer, 5 μL CG/Q solution, 1 μL dNTPs, 1.5 μL MgCl, 1.25 μL of primer F (5′-GACTTGGCCACTTATGAC-3′), 1.25 μL of primer R (5′-GTCTGGAGAAGAAGAA-3′) and 0.25 μL of Taq polymerase (HotstarTaq DNA Polymerase, Qiagen). The PCR program included: 95 °C for 10 min, 30 denaturation cycles at 95 °C for 30 s; annealing at 50 °C for 30 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 10 min. The PCR products were then revealed on 2% Gelgreen® stained agarose gel (Biotium, USA).

2.7.2. *Batrachochytrium dendrobatidis*

An end-point PCR protocol was used for *B. dendrobatidis* using the primers described by Boyle et al. (2004) that permit to amplify a portion of 160 base pairs straddling the ITS1-3 and 5.8 S regions. The PCR reaction was performed in a total volume of 25 μL containing 5 μL of template, 2.5 μL of buffer, 5 μL CG/Q solution, 1 μL dNTPs, 1.5 μL Mg^{2+}, 0.625 μL of ITS1-3 primer Chtry (5′-CCTTGATATAATACAGTGTGCCATATGTC-3′), 0.625 μL of 5.8 S Chytr primer (5′- AGCCAAGAGATCCGTTGTCAAA-3′) and 0.25 μL IU of Taq polymerase (HotstarTaq DNA Polymerase, Qiagen). The PCR protocol included: 95 °C for 10 min, 50 denaturation cycles at 95 °C for 30 s; annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 10 min. The PCR products were then revealed on 2% Gelgreen® stained agarose gel (Biotium, USA).

2.7.3. *Bacteria*

Biomolecular identification of strains of *Aeromonas* and *Carnobacterium* isolated from samples collected at the two study sites was carried out based on DNA extracted according to the protocol proposed by Pastorino et al. (2021).

2.7.3.1. Aeromonas. The *Aeromonas rpoB* gene, which encodes the b subunit of the multisubunit enzyme DNA-dependent RNA polymerase, was amplified using the primers and protocol described in Korczak et al. (2004). Except for strains 48853.4.1 and 4.2, for which a unique consensus sequence was deposited (the *rpoB* sequences of the two strains were identical to each other; see Results), all consensus sequences obtained from *Aeromonas* strains were deposited in GenBank (Accession number OM326891 - OM326894) (Accession number OM326891).

2.7.3.2. Carnobacterium. PCR amplification of the genus *Carnobacterium* was carried out using a multiplex PCR with primers for the intergenic spacer region (ISR) of the 16 S and 23 S rRNA genes, as well as the *pisA* precursor gene for the piscicolin 126 protein, as described in Pellé et al. (2005).

ISR and *pisA* sequences were compared to the nucleotide sequences in the GenBank database (BLAST). In previous studies (Pastorino et al., 2021 and unpublished data), different consensus sequences for the ISR region called Cm1, Cm2, Cm3, Cm4, and Cm5VM were identified (GenBank Accession numbers MW447308; MW447302; MW447306; MW438292; MZ147002). The sequences matching 100% the sequences listed above were not deposited in GenBank, while two new consensus sequences (Cm6Fr and Cm7Fr) were deposited.

2.7.3.3. Phylogenetic analysis. The Molecular Evolutionary Genetics Analysis software (MEGAX) was used to perform the neighbour-joining analysis (Kumar et al., 2018). Evolutionary distances were calculated using the maximum composite likelihood method (Tamura et al., 2004). A bootstrap test with 1000 replicates was run, with a cut-off of 70% to compute the bootstrap condensed tree. *Aeromonas: RpoB* gene sequences of 5 *consensus* obtained from the isolated strains and 17 *rpoB* sequences of different *Aeromonas* species were entered into the phylogenetic analysis. *Carnobacterium:* ISR gene sequences of the four different *consensus*, nine sequences of *C. maltaromaticum* present in GenBank, and twelve sequences of different species belonging to the genus *Carnobacterium* and other taxa were entered into the phylogenetic analysis.

2.8. *Ethical statement*

As required by local laws, permission for frog sampling was obtained from the Ente di Gestione delle Aree Protette delle Alpi Cozie (Authorization no. 106/2022). Directive 2010/63/EU and the ARRIVE guidelines were followed during the experimental procedures.

3. Results

3.1. *Water samples*

Values of the physicochemical and nutrient water parameters are reported in Table S1. Generally, values (mean values) were almost quite similar among the two ponds: temperature was slightly higher in Selleries Pond (14.21 °C) compared to Frog Pond (12.73 °C); pH values were closer to neutral in both ponds (7.14 and 7.03 for Frog Pond and Selleries Pond, respectively); conductivity values (22 μs/cm and 19 μs/cm for Frog Pond and Selleries Pond, respectively) were rather low, and a good oxygenation level was recorded in both ponds (7.52 mg/L and 6.23 mg/L for Frog Pond and Selleries Pond, respectively). A condition of oligotrophy emerged, with rather low values of ammonia/ammonium (0.01/0.01 and 0.02/0.02 mg/L for Frog Pond and Selleries Pond, respectively), nitrite (0.02 mg/L and 0.03 mg/L for Frog Pond and Selleries Pond, respectively), nitrate (1.34 mg/L and 2.12 mg/L for Frog Pond and Selleries Pond, respectively), and total phosphorus (0.01 mg/L for both ponds). BOD_5 (2.45 mg/L and 2.18 mg/L for Frog Pond and Selleries Pond, respectively) and COD (4.89 mg/L and 4.30 mg/L for Frog Pond and Selleries Pond, respectively) values were low, indicating a good water quality condition. Finally, all water samples tested negative for the presence of microcystins/nodularins.

3.2. *Frog samples*

3.2.1. *Frog Pond*

In the 50 frogs examined (snout–vent length 7.0–9.8 cm; range in body mass 85–123 g), the main finding (45/50 individuals) was reddish bumps (papules) on the ventral aspect (Fig. 2). Some (n = 30) also showed dermal ulceration. Females had a swollen abdomen and eggs were noted on opening the ventral cavity. No significant alterations or lesions of the internal organs were observed, nor was haemorrhaging or erythema noted. Ranavirus and *B. dendrobatidis* analyses tested negative in all specimens. In contrast, the bacteriological exam tested positive in the specimens' presenting papules and/or dermal ulcerations (n = 45). Brain, eye, and kidney tissues tested positive for *C. maltaromaticum*. All isolates were Gram-positive, non-motile bacilli that hydrolyzed esculin, produced arginine dihydrolase and 2,3-butanediol from glucose, and did not reduce nitrate. Indole, lysine decarboxylase, ornithine decarboxylase, urease, or citrate were not produced by any of the isolated strains. Acid was generally produced from glucose, mannitol, sucrose, and trehalose. Arabinose, galactose, inositol, maltose, raffinose, rhamnose, and sorbitol produced no acid. MALDI-TOF MS identified the isolates as *C. maltaromaticum* (ID 99.9%). All the isolated strains (Table 1) showed a fragment of 600 bp for the ISR characteristic of *C. maltaromaticum*, as described in Pellé et al. (2005). Only strain 40,363.14.6 was positive on *pisA* precursor gene amplification and showed a 300 bp band. The sequence had a BLASTn identity of 100% with the reference sequence AF275938.

Moreover, strains 40,363.14.6 and 40,363.14.7 (negative on *pisA* precursor gene amplification) came from the kidney of the same specimen. Table 1 presents the identity of each strain using BLASTn. The sequences obtained for strains 40,363.12.3, 12.5, 13.4, 14.3, and 14.5 were grouped together to form a new *consensus* called Cm6Fr and deposited in GenBank (Accession number OM304843). The same was done for strains 40,363.14.1 and 2 grouped in the consensus called Cm7Fr and deposited in GenBank (Accession number OM304842).

Phylogenetic analysis showed that the *C. maltaromaticum* strains were well separated from the other species and taxa and formed a defined clade with a high bootstrap (97%), supporting the biomolecular identification. This large clade was composed of five clusters in which distribution of the strains isolated from *R. temporaria* were in line with the results obtained by BLASTn analysis (Fig. 3). Cm7Fr was in the same cluster of MW447308 corresponding to Cm1 (same identity with MW447308 99.43%); Cm6Fr was in the same cluster as the MZ147002

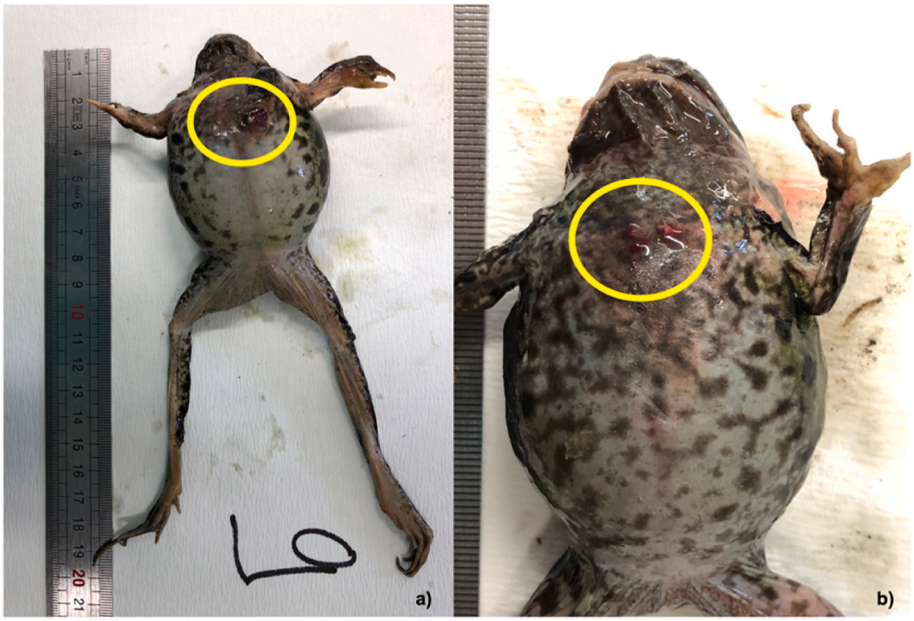

Fig. 2. Ventral view of *Rana temporaria* (female) from Frog Pond with a) dermal ulceration and (b) with reddish papules.

reference sequence, for which the identity was 99.81%.

3.2.2. Selleries Pond

All five frogs (snout–vent length 8.0–9.1 cm; range of body mass 87–92 g) presented both petechiae (Fig. 4a) and dermal ulcerations on the rear limbs (Fig. 4a), with one or two ulcers 20–30 mm in diameter (Fig. 4b) and a 2–3 mm area of grey discoloration around the dermal ulcer (Fig. 4b). Histological analysis of the skin revealed focal lesions characterized by hyperplasia of the epidermis with mild hyperkeratosis (Fig. 5a). Haemorrhages of in femoral musculature was observed in one individual. Histological analysis revealed multiple haemorrhagic foci associated with mild infiltration of inflammatory cells (Fig. 5b). No major alterations or lesions of the internal organs were observed and was confirmed by histological analysis. Parasitological (external exam), Ranavirus and *B. dendrobatidis* analyses tested negative in all specimens, whereas bacteriological exam tested positive in brain and eye of all five individuals.

Five of the six isolated strains (Table 2) were Gram negative bacilli that were motile and positive for catalase, cytochrome oxidase, nitrate reductase, glucose, and trehalose fermentation. One strain presents Gram-negative coccoid rods that were non-motile and positive for catalase, cytochrome oxidase, nitrate reductase, glucose, and trehalose fermentation. Generally, all the isolates did not produce acid from d-arabitol, dulcitol, erythritol, and xylose. MALDI-TOF MS identified five strains as *Aeromonas bestiarum* (ID 97.8%) and one strain as *Aeromonas salmonicida* (ID 99.9%). All six strains showed a fragment of 560 bp from the *rpoB* gene amplification. Five of the six were identified at the species level as *Aeromonas sobria* and one as *Aeromonas salmonicida* (BLASTn nucleotide sequence identity >99%) (Table 2).

Phylogenetic analysis revealed that *A. sobria* was clearly separated from the other species and formed a clade comprising the sequences found in this study and the two reference sequences, with a bootstrap value of 98% (Fig. 6).

Strain 48,847.21.2 identified as *A. salmonicida* with BLASTn identity of 99.81% (query coverage 96%) and matching the reference sequence AY851101, was outside the clade formed by the two clusters of *A. salmonicida* and *A. bestiarum* (Fig. 6).

4. Discussion

Amphibians are under constant threat by direct and indirect stressors (Zheng et al., 2021). Natural stressors (i.e., diseases) can be exacerbated by human-caused stressors (Narayan et al., 2019). For the present study we analysed two unusual mortality incidents of *Rana temporaria* that occurred at two high-mountain ponds. In many amphibian species there are multiple causal factors for the decline or mortality, which sometimes act in synergy (Walls and Gabor, 2019). For such reason, both environmental (water) and frog samples were analysed to try to understand the possible causes responsible for the unusual mortalities. Generally, physicochemical, and nutrient water parameters were in line with those reported for high-altitude aquatic ecosystems located in the western Alps (i.e., Pastorino et al., 2020b; Perilli et al., 2020). Values of dissolved oxygen concentrations, pH and conductivity recorded in both ponds were also in line with those commonly reported for mountain lakes located in granitic bedrocks (i.e., Füreder et al., 2006; Pastorino et al., 2020b; Perilli et al., 2020). Both ponds showed a low concentration of nutrients (i.e., nitrogen compounds and phosphorus) justifying the absence of cyanotoxins. Indeed, high levels of cyanobacterial biomass are mostly caused by nutrients from anthropogenic sources such agricultural runoff and wastewater (WHO, 2020). However, if we consider the season (spring) in which the mortalities occurred, water temperatures of both ponds were quite higher and comparable to those recorded in the summer season in other shallow high-altitude lakes located in the same area. For example, during August 2017, Pastorino et al. (2020b) in the Upper Balma Lake (2100 m a.s.l., Cottian Alps) recorded a mean water temperature of 15.62 °C (range 14.60–16.10 °C). The first case of unusual mortality occurred at Frog Lake occurred on April 2, 2021. On this path, the highest maximum air temperature recorded for spring in Piedmont occurred during the first week of April 2021, with an average maximum of 24.6 °C due to anticyclonic influence (ARPA, 2022). The second mortality incident (Selleries Pond) happened concurrently with an increase in air temperature in the first half of May 2021 (ARPA, 2022). Because high-mountain ponds are usually quite shallow (i.e., depth < -2.5 m), the water temperature can rise rapidly with increasing air temperature. In both cases, the increase in temperatures has certainly led a greater stress on the frogs, ensuing in a reduction of immune system defences. Indeed, in literature it is well documented that changes in temperatures can create variations from ideal immunity levels,

Table 1
Biomolecular identification of *Carnobacterium* spp. isolates from the mortality event at Frog Pond). *Cm6Fr and Cm7Fr consensus sequences have been deposited in GenBank (Accession Numbers OM304843 and OM304842, respectively).

ID	Genus and species	Source	BLASTn Identity (%)	ISR	Ref Seq for Identity
40363.12.1	*Carnobacterium maltaromaticum*	Brain	100%	Cm1	MW447308
40363.12.2	*Carnobacterium maltaromaticum*	Brain	100%	Cm1	MW447308
40363.12.3	*Carnobacterium maltaromaticum*	Brain	99.81%	Cm6*	MZ147002
40363.12.4	*Carnobacterium maltaromaticum*	Brain	100%	Cm1	MW447308
40363.12.5	*Carnobacterium maltaromaticum*	Brain	99.81%	Cm6Fr*	MZ147002
40363.12.6	*Carnobacterium maltaromaticum*	Brain	100%	Cm1	MW447308
40363.12.7	*Carnobacterium maltaromaticum*	Brain	100%	Cm1	MW447308
40363.12.8	*Carnobacterium maltaromaticum*	Brain	100%	Cm1	MW447308
40363.13.1	*Carnobacterium maltaromaticum*	Eye	100%	Cm1	MW447308
40363.13.2	*Carnobacterium maltaromaticum*	Eye	100%	Cm1	MW447308
40363.13.3	*Carnobacterium maltaromaticum*	Eye	100%	Cm1	MW447308
40363.13.4	*Carnobacterium maltaromaticum*	Eye	99.81%	Cm6Fr*	MZ147002
40363.13.5	*Carnobacterium maltaromaticum*	Eye	100%	Cm1	MW447308
40363.13.6	*Carnobacterium maltaromaticum*	Eye	100%	Cm1	MW447308
40363.13.7	*Carnobacterium maltaromaticum*	Eye	100%	Cm1	MW447308
40363.13.8	*Carnobacterium maltaromaticum*	Eye	100%	Cm1	MW447308
40363.13.9	*Carnobacterium maltaromaticum*	Eye	100%	Cm1	MW447308
40363.14.1	*Carnobacterium maltaromaticum*	Kidney	99.43%	Cm7Fr*	MW447308
40363.14.2	*Carnobacterium maltaromaticum*	Kidney	99.43%	Cm7Fr*	MW447308
40363.14.3	*Carnobacterium maltaromaticum*	Kidney	99.81%	Cm6Fr*	MZ147002
40363.14.4	*Carnobacterium maltaromaticum*	Kidney	100%	Cm1	MW447308
40363.14.5	*Carnobacterium maltaromaticum*	Kidney	99.81%	Cm6Fr*	MZ147002
40363.14.6	*Carnobacterium maltaromaticum*	Kidney	100%	Cm1	MW447308
40363.14.7	*Carnobacterium maltaromaticum*	Kidney	100%	Cm2	MW447302

increasing susceptibility of amphibians to infections (Raffel et al., 2006). Amphibians, due to their ectothermic nature, are highly dependent on several abiotic factors (i.e., temperature) and are impacted by global warming (Petrović et al., 2021). Although amphibians are known for their high tolerance and adaptation towards environmental stress due to their intrinsic biochemical and physiological mechanisms (i.e., Bagnyukova et al., 2003; Gavrilović et al., 2021), cold-adapted populations inhabiting mountain areas are often unable to withstand the physiological stress of rising temperatures, making these species vulnerable to pathogens and climate change (i.e., El-Sayed and Kamel, 2020; Brusch et al., 2022).

Such assumptions were confirmed by the results of microbiological and biomolecular analyses. *Carnobacterium maltaromaticum*, which represents the first report in amphibians, was reported in specimens of *R. temporaria* collected from Frog Pond. *Carnobacterium maltaromaticum* has been widely reported in teleost and cartilaginous fish from the United States, Africa, and Europe (Loch et al., 2008, 2011; Leisner et al., 2012; Mohamed et al., 2017). Loch et al. (2011) reported *C. maltaromaticum* infections in wild and reared rainbow trout, as well as in chinook and coho salmon broodstock. *Carnobacterium maltaromaticum* was isolated in 2018 from diseased rainbow trout on a Korean fish farm (Roh et al., 2020). Our observation of similar lesions on frog skin is shared by the description of severe ulcerative lesions on the trout's body surface (Roh et al., 2020).

Two new consensus sequences for *ISR* identified as Cm6Fr and Cm7Fr were found in *R. temporaria*. Carnobacteria, particularly *C. maltaromaticum* and *C. divergens* have been discovered in polar, temperate and sea environments, as well as in *Sphagnum* spp. ponds and in cold and alkaline tufa columns (Leisner et al., 2007). The mechanisms by which Carnobacteria occur and persist in the natural environment, as well as their genetics, remain unknown. Furthermore, there is a lack of knowledge about their microbial ecology and genomics. However, Leisner et al. (2008) suggested that chitin-containing insects (the major food source for amphibian) could act as a vector for Carnobacteria that catabolize the exoskeleton chitin. On this path, it is important to point out that Diptera Chironomidae larvae represents the main assemblage pattern of macroinvertebrates in high-mountain lakes (Perilli et al., 2020), and could be one of the main portals of entry for frog-related Carnobacteria. The presence of Carnobacteria in frogs' eyes and brain tissue could be interpreted as immune-evasion mechanisms, such as a macrophage-mediated Trojan-horse effect, which has been described for other Gram-positive bacteria (Thulin et al., 2006). For example, *Streptococcus iniae* in fish has been shown to enter and multiply within macrophages, where the pathogen gains an efficient mechanism for translocation into the central nervous system (Zlotkin et al., 2003). The eye provides a safe haven for bacteria, from which it can then infect wild animal populations under unfavourable environmental conditions (i.e., spawn events, rise in temperature) as occurred at Frog Pond during this unusual mortality incident. In a recent study, Pastorino et al. (2021) reported the same bacterium in the eyes of healthy salmonids (*Salmo trutta* and *Salvelinus fontinalis*) captured in three high mountain lakes also located in the Cottian Alps. Since the salmonids were captured and analysed during the ice-free season and in favourable environmental conditions, the absence of lesions and/or pathological alterations is well justified. But isolation of the bacterium in the fish eyes strengthen our hypothesis of a Trojan-horse effect, which merits further study.

Carnobacteria have been described with various bacteriocins (Leisner et al., 2012). Bacteriocins are antimicrobial peptides or proteins (ribosomally synthesized) produced by bacteria that kill other microorganisms (Leisner et al., 2012). They are considered as a successful strategy for population maintenance to reduce the number of competitors for more nutrients and living space in environments (Yang et al., 2014). About 99% of bacteria can produce at least one bacteriocin, but the selective forces driving their evolution remain unknown (Leisner et al., 2012). Here, we screened *C. maltaromaticum* strains for the presence of *pisA* in order to compare our findings to those of Loch et al. (2008) who isolated a *C. maltaromaticum*-like bacterium from systemically infected lake whitefish (*Coregonus clupeaformis*). Two strains isolated from the same organ (kidney) of the same specimen differed completely: 40,363.14.6 was characterized by ISR Cm1 and produced a *pisA* precursor, whereas 40,363.14.7 was characterized by ISR Cm2 and lacked *pisA*. They were displayed in two different clusters in the phylogenetic analysis. As previously stated, we isolated only one strain (40,363.14.6) that was positive for *pisA* precursor gene amplification. Bacterial interactions frequently influence the evolution of bacteriocin production, which is related to the metabolic cost of their production (Leisner et al., 2012). For example, Piscicolin 126 (*pisA*), a class II bacteriocin, has been shown to be bactericidal against other Gram-positive bacteria such as *Listeria monocytogenes* (Linke et al., 2014). Thus, under some circumstances, the strain 40,363.14.6 discovered in this study may have an inhibitory effect to other bacteria. However, further investigations are needed since we have no information about the microbial community composition and ecology of Frog

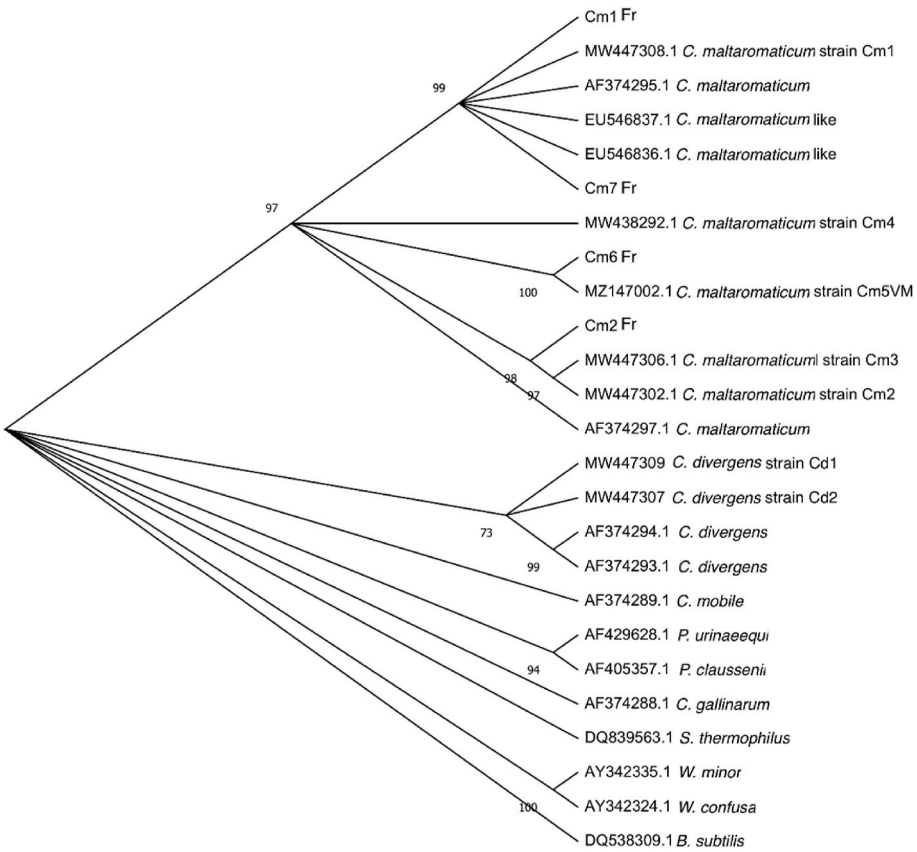

Fig. 3. Phylogenetic relationship (neighbour-joining method; MEGAX software) of *Carnobacterium* spp. and other taxa. A bootstrap test of 1000 replicates was performed. The bootstrap condensed tree (cut-off of 70%) is shown. Fr = strains isolated in the present study.

Fig. 4. *Rana temporaria* from Selleries Pond: (a) petechiae and (b) dermal ulceration on the rear limbs.

Pond.

The second unusual mortality incident occurred at Selleries Pond. The dead frogs were not immediately sampled. A few days later five moribund specimens of *R. temporaria* were collected for analysis. On this path, it is important to point out that the time of death of frog was concentrated within few hours since no dead individuals were observed two days before the mortality event (park ranger; personal communication). They presented with symptoms and clinical signs very similar to the red leg disease caused by *Aeromonas hydrophila* (Densmore and Green, 2007). Lethargy, skin ulcerations, and characteristic cutaneous pinpoint haemorrhages of the legs and abdomen are pathognomonic clinical signs of this disease, which has been reported in several amphibian species (Densmore and Green, 2007).

Since the early 2000s, there have been periodic mass die-offs of *Rana temporaria* tadpole populations in Alpine areas in Brescia Province (northern Italy) associated with *A. hydrophila* and *A. sobria* (Tiberti, 2011). *Aeromonas* species can be found in a variety of aquatic environments, including freshwater and brackish water, also in environments characterized by nutrient-limited conditions (Sadique et al., 2021), as those recorded in the high-mountain ponds here considered. Among the known *Aeromonas* spp., motile aeromonads such as *A. hydrophila* and *A. sobria* are Gram-negative bacilli that cause motile *Aeromonas* infection (MAI) in aquatic organisms (Noga, 2010). The bacteria are considered opportunistic since MAI outbreaks are usually associated with stress, handling, low dissolved oxygen levels or poor nutritional status (Santi et al., 2019). Unfavourable environmental conditions are

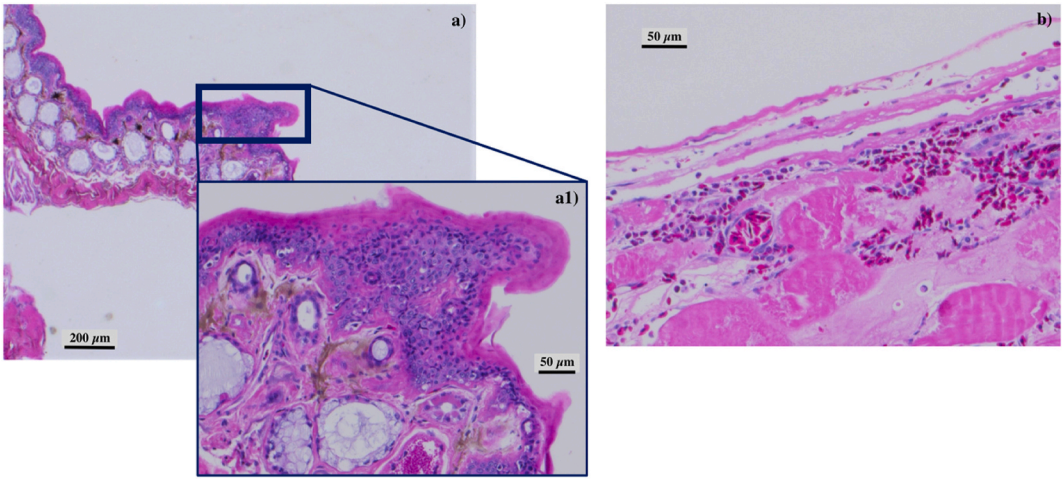

Fig. 5. a) Skin section (HE) of *Rana temporaria* showing a focal lesion characterized by hyperplasia of the epidermis with mild hyperkeratosis; b) skeletal muscle section (HE) of *Rana temporaria* showing multiple haemorrhagic foci associated with mild of inflammatory infiltrate.

Table 2
Biomolecular identification of *Aeromonas* isolates in *Rana temporaria* from Selleries Pond.

ID	Genus and species	Source	Identity (%)	BLASTn Ref Seq for identity	GenBank Accession Number
48831.4.1	*Aeromonas sobria*	Brain	99.58	MG098881 – AY851119	OM326891
48831.4.2	*Aeromonas sobria*	Brain	99.81	MG098881 – AY851119	OM326892
48847.21.1	*Aeromonas sobria*	Brain	99.42	MG098881 – AY851119	OM326893
48847.21.2	*Aeromonas salmonicida*	Eye	99.81	AY851101	OM326894
48853.4.1	*Aeromonas sobria*	Brain	99.61	MG098881 – AY851119	OM326890
48853.4.2	*Aeromonas sobria*	Brain	99.61	MG098881 – AY851119	OM326890

predisposing factors for the onset of MAI (Santi et al., 2019). Thus, the increase in temperature registered in the first days of May 2021 has probably favoured the spread of *Aeromonas* strains. Indeed, when water temperatures rise, opportunistic bacteria as motile aeromonads pose a serious threat to aquatic organisms (Reda et al., 2022).

One isolated strain belonged to *A. salmonicida*, an obligate pathogen which is the causative agent of the furunculosis, one of the most important diseases of salmonids. Lesions are typical of bacteria septicaemia, with the presence of skin ulcers (Noga, 2010). No reports of *A. salmonicida* infection on amphibian are available to date for comparison, but it is well known that *A. salmonicida* could persist for a long time in sediments of low-nutrient freshwater such as those found in high-mountain ponds which are nutrient-poor environments (Morgan et al., 1991; Pastorino et al., 2020b).

The use of a standardized protocol to identify bacteria is essential for accurate diagnosis. One major issue in the identification of *Aeromonas* spp. is that some species are phenotypically very similar, which is why the 16 S rRNA gene is commonly used for the study of phylogenetic relationships among bacterial taxa (Küpfer et al., 2006). 16 S rRNA gene sequencing was only useful for defining *Aeromonas* isolates at the genus level. For this reason, more discriminating genetic markers of housekeeping genes have been used: the *gyrB* gene, encoding for the B-subunit of DNA gyrase (Yamamoto and Harayama, 1995). Use of the *rpoB* gene, previously reported for phylogenetic purposes on archaea and bacteria, was used for the first time in 2006 to study the relationship between *Aeromonas* strains (Küpfer et al., 2006). The *rpoB* gene was chosen here for its capability to discriminate among controversial taxa of the genus *Aeromonas,* and between *A. bestiarum* and *A. salmonicida* in particular. Phylogenetic analysis showed that the *rpoB* gene was highly efficacious in discriminating between the two isolated species of *Aeromonas* (*A. sobria and A. salmonicida*). *A. sobria* was well discriminated by phylogenetic analysis, while *A. salmonicida*, identified through BLASTn with a high identity (99.81% with a query coverage of 96%) was outside the clade of *A. salmonicida* and *A. bestiarum* reference sequences, probably due to the high variability in the region not covered by the BLAST analysis.

5. Conclusion

Finding from this study reveals that the use of an interdisciplinary approach helped to clarify the causes of two unusual mortality incidents at two high-mountain ponds, which are key components of the alpine ecosystem. Indeed, necropsy, histology, microbiology, and molecular biology revealed an association between frog mortalities and isolation of bacteria in coincidence with unseasonably high spring temperatures. Predicting the synergistic effects of climate change and disease on cold-adapted species is a conservation priority that will help us to better understand disease dynamics in understudied species such as the European common frog in high-mountain ponds. On this path, it is urgent to assess the oxidative stress ecology of frog living in mountain environment, considering that it plays a significant function in controlling the developmental processes and frog reactions to environmental alterations (Prokić et al., 2019). Further studies are needed to clarify the interactions between emerging pathogens and climate change, also considering the routes of entry and the mode of transmission of opportunistic bacteria (i.e., analysing aquatic and terrestrial insects). Moreover, further experimental studies (under controlled conditions) are needed to clarify the pathophysiological effects, time of death and lethal dose of *C. maltaromaticum, Aeromonas sobria* and *A. salmonicida* in frogs and tadpoles exposed to different temperatures.

Finally, there has been little research on healthy frogs to date and wildlife disease in amphibians is understudied due to the lack of synergy among researchers and public agencies. Standardized protocols for sampling and processing dead frogs need to be jointly developed by public agencies and scientists (i.e., veterinarians, biologists, ecologists, biotechnologists) with a view to improve the study of amphibians in alpine environments affected by climate change.

Credit author statement

Paolo Pastorino: writing-original draft; Investigation;

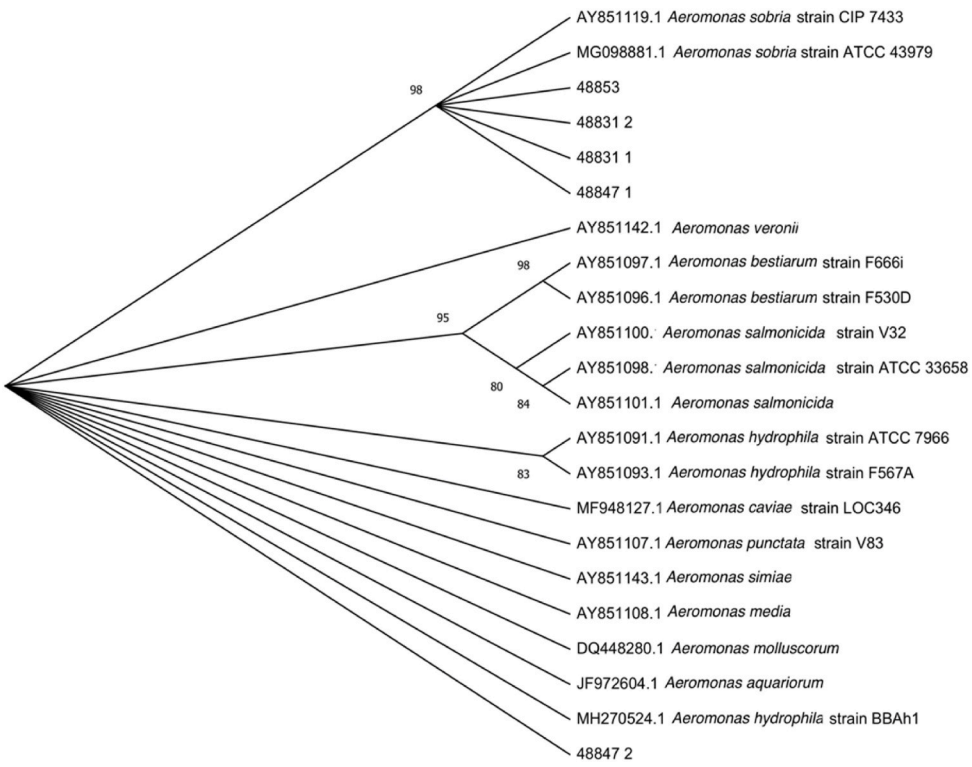

Fig. 6. Phylogenetic relationship (neighbour-joining method; MEGAX software) of *Aeromonas* spp. A bootstrap test of 1000 replicates was performed; the bootstrap condensed tree (cut-off 70%) is shown.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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