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Elizabethkingia miricola infection in multiple anuran species

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Abstract

This report describes an outbreak of *Elizabethkingia miricola* in northern leopard frogs (*Lithobates pipiens*) and three other species of frogs and toads held in captivity in Germany. The authors examine several treatment options and underline the difficulties in treating larger numbers of individuals with antimicrobials applied through bathing. Whole genome sequencing of three bacterial isolates emphasizes their relatedness to other frog isolates and leads us to conclude that *E. miricola* is an emerging and difficult to treat pathogen with a broad host range across anuran species. Moreover, ambiguities in identification of flavobacteria associated with disease in frogs reported in the literature make it seem possible that *E. miricola* has been overlooked as an anuran pathogen in the past.

KEYWORDS

amphibian, anuran, bacterial infection, *Bombina microdeladigitora*, *Elizabethkingia miricola*, frog, leopard frog, *Lithobates pipiens*, *Pipa parva*, *Theloderma bicolor*, toad

1 | INTRODUCTION

Infectious diseases have been linked to an unprecedented decline in amphibians worldwide. The largest infectious threats to amphibians are constituted by fungi of the genus *Batrachochytrium* (Fisher & Garner, 2020) and members of the family *Iridoviridae* known as ranaviruses (Price et al., 2017). Despite this dominance of fungi and viruses as infectious threats to amphibians, bacterial infection is an important and frequently diagnosed problem in amphibian medicine (Hemingway, Brunner, Speare, & Berger, 2009; Whitaker & Wright, 2019).

Elizabethkingia miricola is a gram-negative bacterium occasionally isolated from infections in humans (Lin, Lai, Yang, & Huang, 2019). In the last years, severe infections in two species of true frogs (*Ranidae*) were reported from China (Hu, Yuan, Meng, Wang, & Gu, 2017; Huang et al., 2019; Lei et al., 2018). To date, no *E. miricola* infection has been described in non-ranid frogs or outside of China. Here, we report an outbreak and treatment of a severe *E. miricola* infection in four only distantly related anuran species held in captivity in Germany. Furthermore, we discuss the potential role of *E. miricola* in older reports of bacterial disease in frogs and provide evidence of failure to identify *E. miricola* as a frog pathogen in the literature.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult northern leopard frogs (*Lithobates pipiens*) were wild-caught in the United States of America (no exact location provided) and imported via the German pet trade. Two newly acquired females imported in 2019 were introduced into an established breeding group after 2 weeks of quarantine.

Chapa bug-eyed frogs (*Theloderma bicolor*) were F1 offspring from parents wild-caught in Vietnam (no exact location provided). Parental animals were obtained from the same source and housed in the same collection as the northern leopard frogs mentioned above.

Vietnamese warty toads (*Bombina microdeladigitora*) were wild-caught in Vietnam (no exact location provided) and imported via the German pet trade. Four animals were imported in 2018 and did not present any signs of bacterial infection prior to the introduction of four additional animals imported in 2019. The toads were obtained from the same source as the frogs mentioned above, but housed in a different collection.

Sabana Surinam toads (*Pipa parva*) were captive bred in Germany and housed in the same collection as *B. microdeladigitora*. According to the owner, equipment such as forceps and nets was shared between the different species.

2.2 | Treatment

For antimicrobial bath treatment, frogs were moved to 11 L polypropylene boxes with a level of 2 cm aqueous antibiotic solution (details on preparation see Appendix S1). Frogs were continuously kept in antibiotic solution and could not leave the water while under treatment.

Parenteral treatment was applied as subcutaneous injection into the dorsal lymph sac of the animals. For more details on animal husbandry and treatment see the respective sections of the Appendix S1.

2.3 | Histopathology

Deceased animals were fixed as full carcasses in 4% formalin or 70% ethanol. Full body cross sections were obtained after fixation for at least 48 hr, embedded in paraffin and stained with standard haematoxylin and eosin stain.

Animals showed degenerative and inflammatory lesions in several organ systems (Table 2; Figure 3).

2.4 | PCR and qPCR

qPCR analysis was performed to exclude *Batrachochytrium dendrobatidis* infection with a previously published Primer/Probe set (Boyle, Boyle, Olsen, Morgan, & Hyatt, 2004). DNA extracted from 50 mg of frog skin was used for this method. The reaction was performed on an ABI OneStep Plus cycler (Thermo Fisher) using the fast mode for TaqMan analysis with the SensiFAST Probe Hi-ROX Kit (Bioline). A standard curve was generated by diluting a synthetic gBlock containing primer- and probe-binding sites as published previously (Standish et al., 2018). A pure culture *B. dendrobatidis* (isolate JEL 423) DNA extract served as positive control.

By PCR, using three previously published primer sets (Mao, Hedrick, & Chinchar, 1997; World Organisation for Animal Health, 2019), ranavirus infection was excluded. DNA extracted from 50 mg of frog liver and kidney was used for this method. DNA extracted from A6 cell cultures infected with frog virus 3 served as positive control.

To detect bacteria in skin swabs of frogs and environmental water samples, two primers sets specific for *E. miricola* were designed. Emir_RagA_Fwd 3′-GCATACCATGGCAAGTACTTTATG-5′ and Emir_RagA_Rev 3′-CGCAGGATACAGCTCAAGCT-5′, product size ~700 bp as well as Emir_Bla_Fwd 3′-GGAGTTGTAGTTATAGATTGCCC-5′ and Emir_Bla_Rev 3′-CATGCCCCGCGACTATATA-5′, product size ~500 bp.

The Emir RagA primer set was used primarily to detect *E. miricola* in the sample types mentioned above. The Emir Bla primer set was used for confirmation in questionable cases. Serial dilution of an *E. miricola* pure culture in aquarium water confirmed the sensitivity of the PCR reaction with the Emir RagA primer set to be around 10 colony-forming units per ml of water using the DNA extraction procedure described below. For further confirmation, three randomly selected PCR products obtained from skin samples and all PCR products obtained from water samples were send for Sanger Sequencing and returned with 100% nucleotide identity compared to the whole genome sequenced isolates.

PCR was performed with DreamTaq DNA Polymerase (Thermo Fisher) according to the manufacturers recommendations, and exact conditions can be found in Appendix S1, Table A6.

2.5 | DNA extraction from frog tissues

DNA was extracted from liver, kidney and skin of diseased frogs using the innuSPEED Tissue DNA Kit (Analytik Jena) according to the manufacturer's instructions with the modification that the proteinase K digest was prolonged from 30 min to 1 hr. 50 mg of tissue was used per extraction. DNA from the aforementioned organs of 16 individual *L. pipiens* (8 individuals prior to 1st enrofloxacin bath treatment and 8 other individuals that died after the end of this treatment) and one individual of *P. parva*, *T. bicolor* and *B. microdeladigitora* was extracted and subjected to PCR and qPCR analysis as described.

2.6 | DNA extraction from skin swabs and water samples

Skin swabs were collected for DNA extraction as described for detection of *B. dendrobatidis* (Kriger, Hines, Hyatt, Boyle, & Hero, 2006), and DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen).

For DNA isolation, water samples of 50 ml were centrifuged at 4,500 *g* for 30 min, the supernatant was aspirated and the bottom 1.5 ml transferred into microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) and frozen at −20°C for further analysis. Once thawed, samples were centrifuged at 15,000 *g* for 10 min and the bottom 200 µl used for DNA extraction with the DNeasy Blood & Tissue Kit (Qiagen).

2.7 | Bacterial isolation, identification and antibiotic susceptibility testing

Immediately after death, frogs were dissected for macroscopic inspection, and samples for microbiological examination were

obtained aseptically using sterile cotton swabs with Amies agar for transport (Sarstedt). Alternatively, whole frogs were frozen at −20°C immediately after found dead, transported to the lab on dry ice and dissected in a frozen state to avoid contamination from meltwater.

The samples were analysed by aerobic cultivation after direct inoculation on suitable agar plates (all agar purchased from Oxoid). For the detection of aerobic bacteria, Columbia blood agar (5% sheep blood), Gassner agar and Brilliance UTI Clarity agar were inoculated within 12 hr after sampling in the case of swab samples or immediately after dissection in the case of previously frozen frogs for 24–48 hr (aerobic, 36°C and ambient temperature). For the evaluation of the presence of obligate anaerobic bacteria, the inoculation was carried out on Columbia blood agar (5% sheep blood) with added L-cysteine (Merck), haemin (Sigma-Aldrich), vitamin K1 (Roche) and lysed sheep blood 0.5% (Oxoid) and on an additionally plate with the same agar and added gentamicin (Hexal), both for 48–72 hr (anaerobic, 36°C). Species identification was performed by colony morphology evaluation and via matrix-assisted laser desorption/ionization-time of flight mass spectrometry-based SIG-identification with Bruker Microflex LT in combination with Flex Control (flexControl Version 3.4) and BIOTYPER (MBT Compass 4.1) software (Bruker Daltonics). The original database content comprises five spectra of *Elizabethkingia* in total and two spectra of *E. miricola*.

The determination of minimal inhibitory concentrations (MIC values) of antimicrobial agents was performed by broth microdilution according to the technical workflow given in the CLSI document M100 Ed.29 (CLSI, 2019) using sensititre™ microtitre plates (BMD1; Thermo Fisher). These microtitre plates corresponded to those that were used in the German National Monitoring program GERM-Vet and contained oxacillin, penicillin, ampicillin, amoxicillin/clavulanic acid, imipenem, ceftiofur, cefquinome, cefalothin, cefotaxime, cefoperazone, erythromycin, tylosin tartrate, tulathromycin, tilmicosin, clindamycin, pirlimycin, tiamulin, ciprofloxacin, enrofloxacin, marbofloxacin, nalidixic acid, gentamicin, kanamycin, streptomycin, neomycin, tetracycline, doxycycline, sulphamethoxazole/trimethoprim, florfenicol, linezolid, vancomycin and quinupristin/dalfopristin in a twofold dilution series. (Scholtzek et al., 2019).

2.8 | Semi-quantitative assessment

For a semi-quantitative assessment of bacterial growth, the following categories are defined: +++ (>100 colony forming units [cfu] grown per agar plate), ++ (up to 100 cfu/plate), + (up to 30 cfu/plate) and ± (up to 5 cfu/plate).

2.9 | Bacterial DNA isolation, sequencing and phylogeny

Bacterial DNA was isolated using the MasterPure Complete DNA and RNA Purification Kit (Epicentre, Illumina Company). Sequencing libraries were constructed using the Nextera XT library preparation Kit according to the manufacturer's instructions (Illumina). Afterwards, the 300 bp paired-end reads were generated using an Illumina MiSeq sequencer. The raw reads were de novo assembled using MIRA v4.0. The whole genome sequences (WGSs) of the three analysed isolates were deposited in GenBank with the following Accession numbers: JAAOKZ000000000 (IMT47318), JAAOKY000000000 (IMT47357), JAAOKX000000000 (IMT47538).

The phylogenetic tree was constructed using the CSIPhylogeny software tool (Zankari et al., 2012) at default settings online at www.genomicepidemiology.org with *E. miricola* strain F13 (GenBank accession number CP040450) as reference. The resulting single-nucleotide polymorphism alignment served as basis to construct a tree in Geneious 10.1.3 using RAxML 8.2.11 at default setting without additional bootstrap replicates.

The three *E. miricola* WGSs were screened for virulence factors using the 'virulence factor database (VFDB)' (Liu, Zheng, Jin, Chen, & Yang, 2019) as downloaded on 22 April 2020 as nucleotide sequences. The VFDB was searched against the three WGS (which were set as BLAST databases in Geneious 10.1.3) using blastn with default settings. Results with a nucleotide sequence identity of >60% were treated as hits and are summarized in Appendix S1, Table A5.

Acquired resistance genes were identified with ResFinder (Identification of acquired antimicrobial resistance genes; Zankari et al., 2012) and confirmed in Geneious 10.1.3 using reference gene sequences from the Reference Gene Catalog of NCBI (Database version: 2020-01-22.1).

3 | RESULTS

In April 2019, two wild-caught female northern leopard frogs (*L. pipiens*), imported from the United States, were introduced into a group of nine adult frogs of the same species. Following Amphiplex treatment (Trudeau et al., 2010), egg masses were deposited about 1 week following introduction into the breeding group. Five days later, one newly acquired female presented signs consistent with bacterial infection including oedema, ascites and protrusion of the urinary bladder. The frog died 1 week after the onset of illness. Meanwhile, three frogs of the established breeding group showed depression, unilateral opacity of the eye, later blindness, abdominal swelling, petechial haemorrhage of the skin, torticollis and incoordination (Figure 1a–c). Bacteria isolated from liver and kidney of the dead frog were identified as *E. miricola*. Based on MIC values (Table 1), fluoroquinolone treatment was considered the most promising treatment option. Following dosing suggestions from literature (Whitaker & McDermott, 2019), all ten remaining frogs were started on enrofloxacin treatment (5 mg/kg, subcutaneous administration [s.c.] q24 hr), within 3 days after start of enrofloxacin treatment, two frogs died and two previously healthy individuals developed signs of disease. On day three, the enrofloxacin dose was raised to 30 mg/

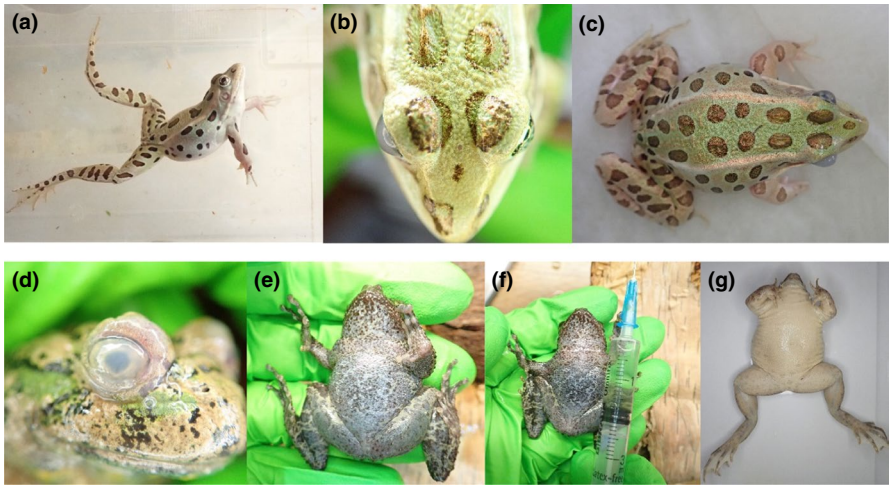

FIGURE 1 Clinical signs of disease in different anurans. (a) Torticollis in *Lithobates pipiens*. (b) Unilateral blindness/opacity of the eye in *L. pipiens*. (c) Abdominal swelling and unilateral blindness in *L. pipiens*. (d) Opacity of the eye in *Bombina microdeladigitora*. (e) Abdominal swelling due to ascites in *Theloderma bicolor*, (f) the same animal after aspiration of approximately 2ml fluid from the coelomic cavity. This animal lived for three more days after the picture was taken, but died despite daily drainage of the abdominal cavity and antibiotic treatment. (g) Swelling of the body in *Pipa parva*

kg s.c. q24 hr for all frogs. One more frog died 1 day after start of the elevated dose; all other animals improved and were treated for 7 days total. One frog had to be euthanized 1 week after the end of the treatment due to the loss of one eyeball and continued circling movements.

Eggs were separated from the adults immediately after deposition and developed normally; 254 tadpoles were raised to metamorphosis with losses of around 5%. Upon metamorphosis, many tadpoles developed massive swelling of the abdomen, incoordination, malformation of the eye and unilateral blindness and died within hours to days after leaving the water. Gross mortality reached 56% at this point. Some of the remaining 112 froglets developed similar signs and were started on enrofloxacin treatment (50 mg/L continuous bath, changed every 24 hr). Six frogs died within 1 day after the start of the treatment, in all other animals, all symptoms, except some cases of unilateral blindness, resolved completely within 5 days of treatment. Treatment was continued for 10 days total. On day three post-treatment, one frog died without previous signs of illness. Five days post-treatment, the acute death of six frogs was recorded, three of them presented cutaneous petechial bleeding on the hind limb ('red leg syndrome'). On day six, eleven frogs died, most of them with similar signs. Microbiological examination confirmed the presence of *E. miricola* in liver and kidney of deceased frogs. Thus, enrofloxacin bathing was considered inefficient for eradication of *E. miricola* and 88 remaining animals were started on minocycline treatment (50 mg/L continuous bath, changed every 12 hr). Minocycline is successfully used to treat *Elizabethkingia* infection in humans (Lin et al., 2019). Within 3 days after the start of the treatment, all signs of disease resolved, and no animal died under treatment. Treatment was continued for 10 days total. Similar to the situation after the first treatment, the acute death of two frogs was recorded 5 days post treatment. Until day eight post-treatment, a total of 21 frogs had succumbed to disease. Microbiological examination of internal organs of deceased frogs confirmed the presence of *E. miricola*, antimicrobial susceptibility testing revealed increased MIC values for tetracyclines, macrolides and amphenicols (Table 1). Despite this, the MIC for florfenicol justified rational antimicrobial treatment with this drug, all remaining 67 frogs were started on 150 mg/L (continuous bath, changed every 24 hr). Four frogs died within the first 3 days of treatment and the florfenicol concentration was raised to 300 mg/L for the rest of the animals. The treatment was continued for 10 days total. Similar to the situation following the earlier treatments, animals started to die without previous signs of disease on day four after the end of treatment. Again, *E. miricola* was isolated from liver, kidney and brain of deceased animals. During this study, several organs of a total of 21 *L. pipiens* were microbiologically examined, *E. miricola* was isolated from at least one organ of 14 animals (67%), and frequently high bacterial loads and pure or near-pure culture of *E. miricola* was obtained. Detailed results on all bacteria isolated during this study as well as a semi-quantitative assessment of their respective abundance can be found in Appendix S1, Table A1–A4.

32 surviving frogs, about half of which showed clinical signs, were started on parenteral enrofloxacin therapy (50 mg/kg, s.c. q24 hr plus 50 mg/L continuous bath, changed every 24 hr). Enrofloxacin was chosen for parental therapy based on the still favourable MIC values for this substance. All frogs survived treatment, developed normally and remained clinically healthy for 3 months after the end of the therapy (Figure 2). To ascertain clearance of *E. miricola* from frogs and environmental samples, we developed a PCR to detect the presence of *E. miricola* in skin swabs and environmental samples before and after parental treatment with enrofloxacin. Using this PCR, we confirmed the presence of *E. miricola* in 2 of 2 water samples collected from apparently healthy tadpoles that developed disease during metamorphosis as well as from one sample collected from sick adult frogs prior to treatment and 2 of 2 water samples collected from juvenile frogs during onset of disease following minocycline

TABLE 1 Minimal inhibitory concentrations (MIC, mg/L) of tested antimicrobials in three *Elizabethkingia miricola* isolates from *Lithobates pipiens*

Antibiotic	MIC – Isolate 1 (mg/L)	MIC – Isolate 2 (mg/L)	MIC – Isolate 3 (mg/L)
Erythromycin	2	16	32
Tilmicosin	4	8	16
Tulathromycin	≥64	≥64	≥64
Tylosin	8	16	16
Tiamulin	≥128	≥128	≥128
Linezolid	4	8	16
Vancomycin	32	16	16
Clindamycin	2	2	4
Pirlimycine	8	8	16
Quinupristin/dalfopristin	≥64	≥64	≥64
Penicillin	≥64	≥64	≥64
Ampicillin	≥128	≥128	≥128
Oxacilline + 2% NaCl	≥16	≥16	≥16
Ampicillin/Clavulanate	16/8	16/8	16/8
Cephalothin	≥258	≥258	≥258
Cefoperazone	≥64	32	32
Cefotaxime	32	32	32
Cefquinome	16	8	16
Ceftiofur	8	4	8
Imipinem	≥64	≥64	32
Colistin	≥128	≥128	≥128
Trimethoprim/sulfamethoxazole	4/76	8/152	32/608
Chloramphenicol	8	≥32	≥32
Florfenicol	1	8	16
Streptomycin	32	64	64
Neomycin	≥128	≥128	≥128
Gentamicin	256	32	128
Tetracycline	32	32	64
Doxycycline	1	4	8
Nalidixic acid	8	8	16
Ciprofloxacin	0.25	1	2
Marbofloxacin	0.12	0.25	0.5
Enrofloxacin	0.06	0.12	0.12

Note: Isolate 1 (IMT47521) was obtained from the initial case, a wild-caught adult female frog that died 7 days after first clinical signs of bacterial infection. Isolate 2 (IMT47538) originates from a juvenile frog that succumbed to disease 5 days after the end of minocycline treatment. Isolate 3 (IMT47430) was obtained from a juvenile frog that died on day three of treatment with florfenicol. Antimicrobials used in this study are indicated by dark grey shading. Light grey shading of doxycycline indicates the use of the pharmacologically similar minocycline.

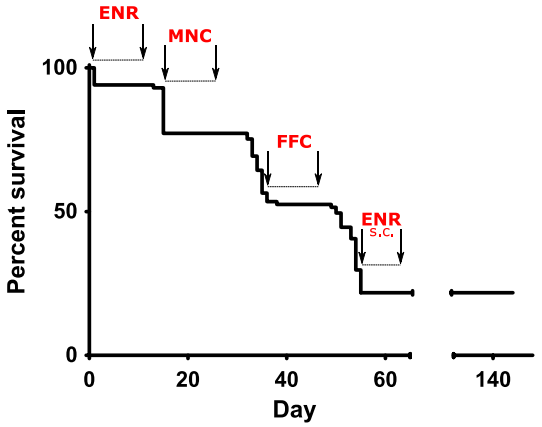

FIGURE 2 Survival of 112 *Lithobathes pipiens* post metamorphosis. Time of treatment is indicated with a dotted line between black arrows, and the method of treatment is presented in red abbreviations of the antibiotic used. All antibiotics were applied as continuous bath unless otherwise noted. ENR, enrofloxacin; FFC, florfenicol; MNC, minocycline; s.c. subcutaneous administration

and florfenicol bathing treatments. Similarly, DNA extracted from skin swabs of diseased frogs were positive for *E. miricola* DNA in 10 of 16 cases (63%) while DNA extracted from skin swabs of healthy frogs following parenteral enrofloxacin treatment tested negative for *E. miricola* in 16 of 16 cases.

During the outbreak described above, *E. miricola* infection was also associated with disease featuring similar clinical signs in different anuran species. Opacity of the eye (Figure 1d) and abdominal swelling were observed in a group of eight Vietnamese warty toads (*B. microdeladigitora*) with three fatal cases. Two out of twelve Chapa bug-eyed frogs (*T. bicolor*) died with severe ascites (Figure 1e, f) and opacity of the eye. In a group of 28 Sabana Surinam toads (*P. parva*), six animals showed abdominal swelling (Figure 1g) and meteorism causing the animals to float on the water and die. *E. miricola* was isolated from individuals in each of these groups. The outbreak could be controlled with enrofloxacin injection (50 mg/kg s.c., q24 hr, 7d) for *B. microdeladigitora* and *T. bicolor*. In *P. parva*, no further cases developed after sanitation of the tanks and daily water exchange for 10 days.

Histopathologic changes diagnosed in deceased frogs included unilateral otitis interna, endophthalmitis, keratitis, hepatitis, pneumonia, myositis, enteritis, nephritis and meningitis (Table 2; Figure 3). Deceased frogs tested negative for *Batrachochytrium* as well as ranavirus infection by qPCR or PCR (Boyle et al., 2004; Mao et al., 1997; World Organisation for Animal Health, 2019). Virus isolation was attempted in a variety of amphibian cells but results remained negative for visible cytopathic effects in all cases (see Appendix S1 for detailed information).

Phylogenetic analysis of WGSs revealed a close relationship not only between bacteria isolated in this study, but also to other *Elizabethkingia* isolates (Figure 4). The three isolates of this study, IMT47318, IMT47357 and IMT47538 covered the reference genome (GenBank accession number CP040450) by 99.89%, 99.67% and

TABLE 2 Full list of anurans examined in histopathology with their respective diagnoses

Species	Figure	Histopathology
Theloderma bicolor ($n = 1$)	Figure 3i	Hepatitis
		Myocarditis
		Nephritis
		Pneumonia
	Figure 3a	Otitis interna and osteomyelitis
		Optic nerve neuritis and uveitis
Lithobates pipiens ($n = 1$)		Myositis
		Ganglioneuritis
	Figure 3b	Meningitis
	Figure 3d	Keratitis
L. pipiens ($n = 1$)		Myocarditits and epicarditis
	Figure 3e	Nephritis
	Figure 3g	Pneumonia
		Endophthalmitis
		Otitis interna and osteomyelitis
	Figure 3f	Myositis
	Figure 3c	Uveitis
L. pipiens ($n = 1$)		Gastritis (and gastric wall oedema)
	Figure 3h	Enteritis
		Nephritis
		Otitis interna and osteomyelitis
		Phtysis bulbi with granulation tissue
		Ductus deferens adenoma
L. pipiens ($n = 6$)		Autolysis typical for aquatic habitat, otitis interna and osteomyelitis (2/6), endophthalmitis (2/6)
L. pipiens ($n = 2$)		Autolysis typical for aquatic habitat (1/2), glossitis (1/2)
L. pipiens ($n = 3$)		Glossitis (3/3)
L. pipiens ($n = 1$)		Autolysis typical for aquatic habitat
L. pipiens ($n = 4$)		Autolysis typical for aquatic habitat (3/4), endophthalmitis (1/4)
Pipa parva ($n = 1$)		Autolysis typical for aquatic habitat
Bombina microdeladigitora ($n = 1$)		Autolysis typical for aquatic habitat

99.38%, respectively. Within the phylogenetic tree, the analysed isolates are present in a cluster consisting of anuran isolates and clearly distinguished from a second cluster formed by eleven human *E. miricola* isolates. Three resistance genes were detected in the three analysed Elisabethkingia miricola WGSs: the subclass B3 metallo-beta-lactamase encoding gene blaGOB-19 (GenBank accession number MK955938.1), the subclass B1 metallo-beta-lactamase encoding blaBlaB-16 (GenBank accession number MK955937.1) both conferring resistance to carbapenems and the class A extended-spectrum beta-lactamase encoding gene blaCME-1 conferring resistance to cephalosporins (GenBank accession number AJ006275.1). The later had a nucleotide sequence identity of only 90.1% compared to the respective gene in the whole genome sequenced isolates, which was identical to the gene present in *E. miricola* strains F13 and FL160902. Thus, this presumably new resistance gene variant of $bla_{\text{CME-1}}$ is already published in GenBank, but not yet described and examined for its ability to confer resistance to beta-lactams.

The 16S rRNA sequences of the three analysed isolates were derived from the respective annotated whole genome nucleotide sequences (GenBank accession numbers NZ_JAAOKZ000000000; NZ_JAAOKY000000000; NZ_JAAOKX000000000) in GenBank and compared with the NCBI nucleotide database using blastn in default settings. All three blastn comparisons resulted in a 100% nucleotide sequence identity with *E. miricola* FL160902 (GenBank accession number CP040516.1).

4 | DISCUSSION

The authors investigated a disease outbreak associated with the presence of *E. miricola* in several internal organs of different anuran species. The isolates sequenced in this study form a sub-cluster that separates them from the sequences of Chinese isolates and places them in close relation with *E. miricola* isolate CIP108653 (GenBank accession number FTRC00000000.1). The annotation of this isolate (BioSample SAMEA4026803) states its belonging to strain LDVH-337.01 isolated from *Xenopus laevis* (misspelled as *laevi*; Pipidae) in France in the year 2002. Although no further information on this isolate is provided in the database entry, the same strain identifier is used in GenBank entry AY468482.1 where the organism is identified as *Chryseobacterium meningosepticum* isolated from *X. laevis* with haemorrhagic septicaemia; no isolate identifier is provided. The reference given in this entry (Bernardet et al., 2005) adopts the previous change in nomenclature (Kim, Kim, Lim, Park, & Lee, 2005) and names the bacterium *E. meningoseptica* 'strain' CIP108653 and points out that the same pathogen has been isolated from bullfrogs (*L. catesbeianus*) in Taiwan. The confusion surrounding isolate CIP108653 may have obscured the existence of pathogenic *E. miricola* infection in frogs prior to the first description from true frogs in China in 2017 (Hu et al., 2017). A similar example of uncertain identification of *E. meningoseptica* can be found in a report describing bacterial infection of tiger frogs (*Hoplobatrachus tigerinus*; Xie et al., 2009). Here, bacteria are identified as *E. meningoseptica* based on 16S rRNA sequences. Importantly, the isolates show only 98.6%–98.7% sequence identity with the *E. meningoseptica* type strain (ATCC 13,253) but up to

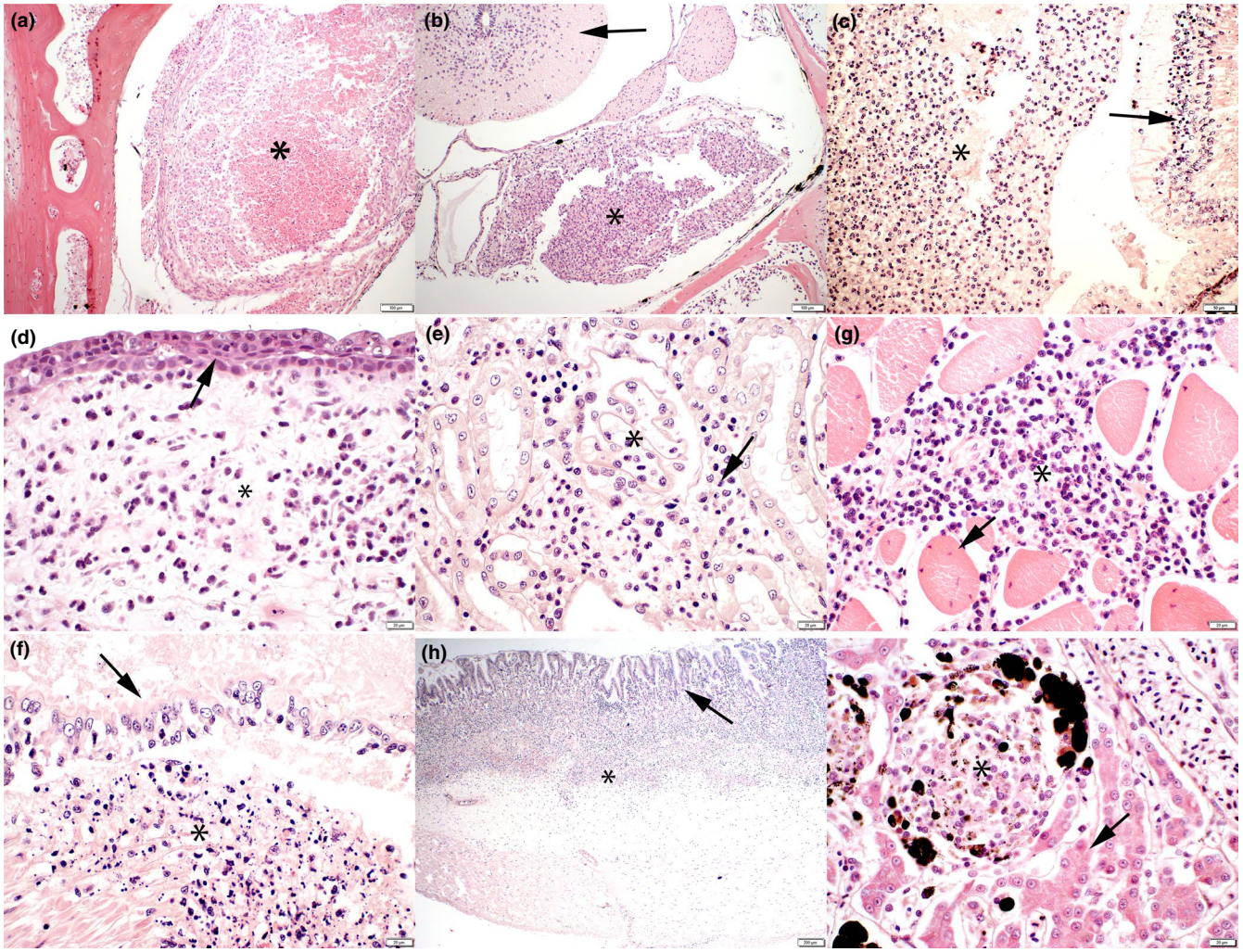

FIGURE 3 Histopathologic lesions associated with *Elizabethkingia miricola* infection in frogs. (a) *Theloderma bicolor*, inner ear: chronic, necrotizing to granulomatous otitis interna (*). HE stain. (b) *Lithobathes pipiens* (FFC-150), meninges: subacute meningitis (*) affecting the brain stem (black arrow). HE stain. (c) *L. pipiens* (ENR-5), eye: subacute, necrotizing endophthalmitis (*). Retina (black arrow), HE stain. (d) *L. pipiens* (FFC-150), eye: chronic keratitis (*). Hyperplastic corneal epithelium (black arrow), HE stain. (e) *L. pipiens* (ENR-5), kidney: mild interstitial nephritis (black arrow), glomerulum (*), HE stain. (f) *L. pipiens* (ENR-5), lung: acute, necrotizing pneumonia (*). Bronchial epithelium (black arrow), HE stain. (g) *L. pipiens* (ENR-5), skeletal muscle: subacute to chronic myositis (*). Myocyte (black arrow), HE stain. ENR-5, sample obtained from animals that died under 5 mg/kg parenteral enrofloxacin treatment; FFC-150, sample obtained from animals that died under 150 mg/L florfenicol bathing treatment; HE, haematoxylin and eosin

100% sequence identity with isolate NTU 870424-IL, which was isolated in 1986 from diseased *L. catesbeiana* in Taiwan and previously described as 'Flavibacterium ranacida' (Chung, 1990; Faung, Chiu, & Wang, 1996). This isolate was reclassified as *E. meningoseptica* in the same study that misidentified *E. miricola* isolate CIP108653 as *E. meningoseptica* based on 16S rRNA sequences (Bernardet et al., 2005). Given the changes in nomenclature and the sometimes challenging identification of *Elizabethkingia* isolates (de Carvalho Filho, Marson, & Levy, 2017; Han et al., 2016; Matyi, Hoyt, Ayoubi-Canaan, Hasan, & Gustafson, 2015; Rahim, Gupta, & Aggarwal, 2018), it cannot be excluded that some historic descriptions of bacterial infections similar to the condition described here (Chung, 1990; Green et al., 1999; Mauel, Miller, Frazier, & Hines, 2002; Olson, Gard, Brown, Hampton, & Morck, 1992; Taylor, Simmonds, & Loeffler, 1993) are in fact related to *E. miricola* infection. This would make *E. miricola* a more important amphibian pathogen than previously thought. This option, however, remains speculative and may be impossible to investigate retrospectively. In the view of the similarity of 16S rRNA sequences and the problems with the annotation in GenBank described above, 16S rRNA sequencing does not necessarily provide definite species identification for *Elizabethkingia* isolates obtained from frogs.

Considering these uncertainties, it seems even more important to report the highly pathogenic *E. miricola* infection in multiple amphibians described here. Our findings suggest pathogenicity of *E. miricola* for a large variety of anuran species. While *E. miricola* infection developed most rapidly in *L. pipiens* (Ranidae), it was also associated with clinical disease and mortality in the not closely related *B. microdeladigitora* (Bombinatoridae), *P. parva* (Pipidae) and *T. bicolor* (Rhacophoridae). The bacterium seems thus capable of causing

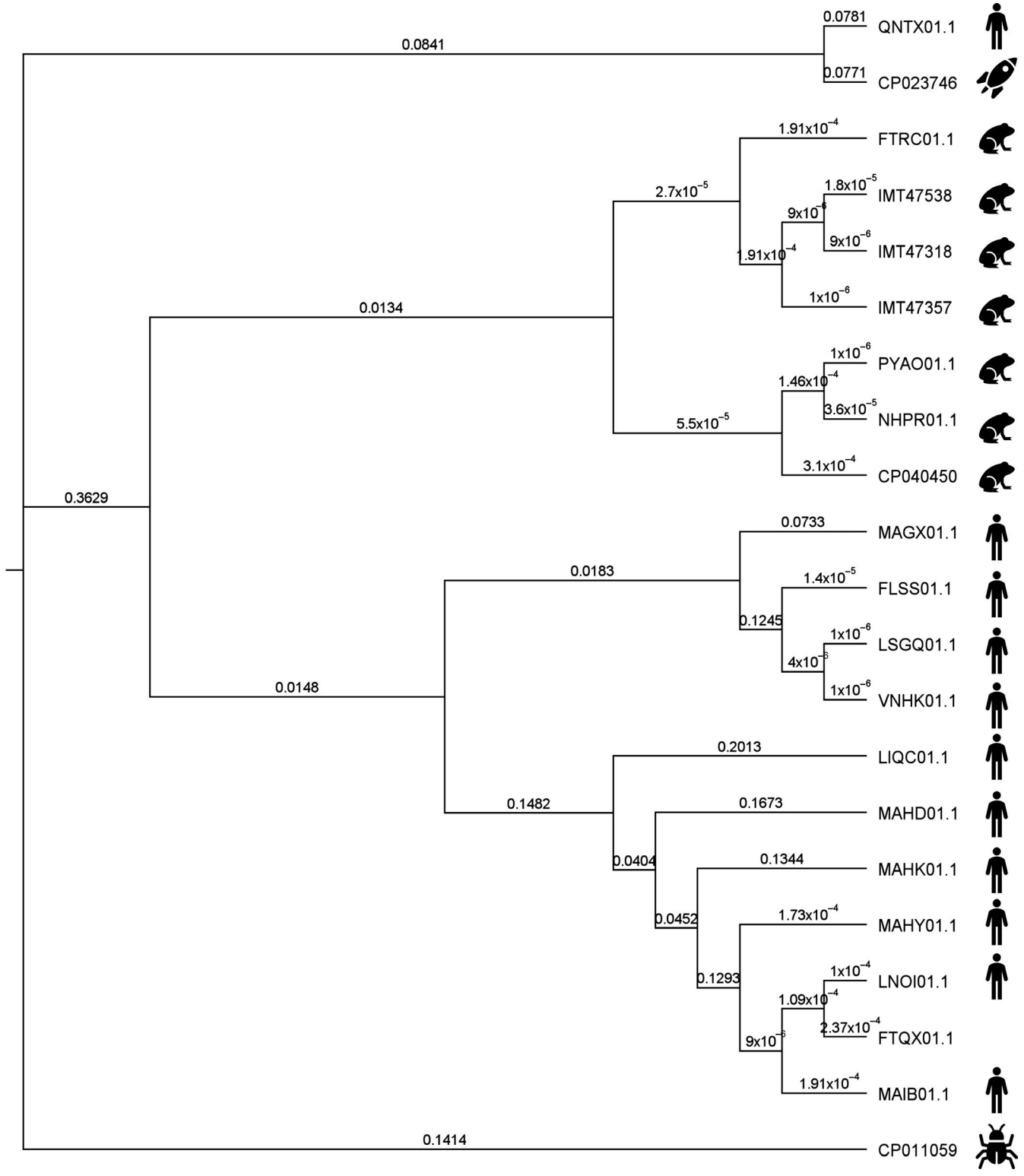

FIGURE 4 Phylogenetic tree of *Elizabethkingia miricola* isolates from this study. Phylogenetic tree of *E. miricola* isolates from this study (IMT47538 (*Lithobates pipiens*), IMT47318 (*Pipa parva*) and IMT47357 (*L. pipiens*)). The three isolates of this study covered the reference genome CP040450 by 99.89%, 99.67% and 99.38%, respectively. Within the phylogenetic tree, the analysed isolates are present in a cluster consisting of frog originating isolates and show the closest relationship to each other, followed by other isolates of frog origin. The second cluster consists of eleven human *E. miricola* isolates and is clearly distinguished from the frog-isolated ones. The two more unrelated isolates QNTX01.1 and CP023746 were isolated from human and condensation water of the Russian space station MIR, respectively. The remaining outlying isolate CP011059 was isolated from the wood-feeding termite *Reticulitermes speratus*. The following pictograms are used to allow visual identification of *E. miricola* origin Human Frog Space Station 'Mir' Insect. No origin could be identified for isolate CIP111047 (FTQX01.1)

severe infections in members of all three suborders within the order Anura.

Twelve virulence-associated genes were identified in the analysed genomes using the VFDB (Appendix S1, Table A5). Those virulence factors are identified by their sequence similarity to the known reference sequence alone. So far, no functional analysis of virulence factors of *E. miricola* has been published; hence, the characterization of the identified genes as true virulence factors cannot be based on the sequence analysis done here.

Our findings suggest that *E. miricola* is an emerging pathogen of anurans with a wide range of hosts, highly resistant to antimicrobials and thus difficult to treat. We recognize that bacterial infections such as described here may be aggravated by environmental factors such as suboptimal housing, high density of frogs and unbalanced diet of infected animals. Recurrence of disease after clinical cure under antimicrobial treatment was observed despite strict hygiene and argues for persistence in compartments that are difficult to reach with antimicrobial bathing. We did observe increased MIC values for several antimicrobials in bacteria that were isolated from frogs that had previously undergone antimicrobial treatment. In absence of pharmacokinetic data for transcutaneous application of antimicrobials, bathing treatments cannot be considered a rational approach and must be discouraged, especially in the context of highly drug resistant pathogens such as *E. miricola*. In this case, parenteral antimicrobial treatment of single frogs guided by susceptibility testing and pharmacokinetic considerations but with elevated doses proved to be the only viable treatment option in most species. Notably, the dispersal of the disease in *P. parva* could be controlled without pharmacological intervention. Since relatively high doses of bacteria seem to be required to induce disease (Lei et al., 2018), hygiene and management measures may be a possible way to manage disease outbreaks in certain species. This is also suggested by a literature source describing a *Flavobacterium meningosepticum* outbreak in *X. leavis* (Green et al., 1999). In the light of our findings, *E. miricola* infection should be considered an important differential diagnosis in mortality events in anuran species.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

All work was performed in accordance with relevant institutional and legislative regulations.

DATA AVAILABILITY STATEMENT

Whole genome sequences from all isolates sequenced in this study are deposited at GenBank and can be found under the following accession numbers: JAAOKZ000000000 (IMT47318), JAAOKY000000000 (IMT47357) and JAAOKX000000000 (IMT47538).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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