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FULL LENGTH RESEARCH PAPER

Molecular evidence for the predation of Critically Endangered endemic *Aphanius transgrediens* from the stomach contents of world wide invasive *Gambusia affinis*

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Abstract

Predation and competition among native and invasive species are difficult to study in aquatic environments. Identification of preys from semi-digested body parts sampled from stomach contents of the predator is very challenging. Recent studies were mainly based on use of DNA extracted from stomach content to identify the prey species. This study presents the molecular evidence that reveals the predation of critically endangered *Aphanius transgrediens* by world-wide invasive *Gambusia affinis* for a better understanding of the link between the invasion and the extinction of native species in freshwater ecosystems. DNA samples were extracted from semi-digested stomach contents of the invader and short fragments of mitochondrial *NADH1* gene were amplified using species-specific primers designed in this study to make identification at species level. Existence of both the prey and the predator species were also confirmed using environmental DNA extracted from water samples.

Keywords

Diet analysis, eDNA, freshwater, molecular identification, *NADH1*

History

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Introduction

Freshwater ecosystems are one of the most complex and threatened biodiversity sources on our planet (Malmqvist & Rundle, 2002). Freshwater fish species comprise approximately 25% of vertebrate species and are considered as one of the most endangered taxa of the world (Darwall et al., 2008). Redundance of endemic species found in the Mediterranean basin makes this fauna a hot spot for especially threatened freshwater fish species (Myers et al., 2000). More than 70% of these endemic freshwater fish species were flagged as Endangered, Critically Endangered or even already extinct (Hermoso & Clavero, 2011).

Introduction of non-native species and the process followed by the extinction of native species is recognized as a major threat to biodiversity and the balance of the ecosystem (Clavero & García-Berthou, 2005). Successful non-native species becomes invasive for the ecosystem and their poorly understood impacts could be crucial in most of the cases (Moyle & Light, 1996). Especially, predatory fish species can cause a significant decrease in the abundance or even extinction of native fish species (Krueger & May, 1991). Although adults of many native fish species are large enough to be considered as out of target, predation of eggs, larvae and juveniles of native fish species may affect recruitment of populations. Studies on conservation ecology showed that the main causes of species extinctions are related with invasions by non-native species (Fritts & Roda, 1998; Wilcove et al., 1998).

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At this point, predation becomes more of an issue in the balance of natural communities, species abundances, their distributions and compositions (Power, 1992). Moreover, predation has been implicated as the main mechanism behind species extinction process in invaded communities (Mooney & Cleland, 2001; Moyle & Light, 1996). When native fish populations are exposed to predatory non-native fishes, predation of eggs, larvae and juveniles will cause a decrease in recruitment (Krueger et al., 1995) and this will lead to a collapse in native population (Dudley & Matter, 2000). Predatory impacts are not always as a result of large piscivorous fish species, but also small omnivorous fish species should be considered too. Extinction records of native fishes, amphibians and invertebrates exists as a result of predation by introduced mosquitofish, *Gambusia affinis* (Courtenay & Meff, 1989; Goodsell & Kats, 1999; Meffe, 1985; Schoenherr, 1981). Mosquitofish are viciously feeding on eggs, larvae and juveniles of species from a wide range of taxon (Goodell et al., 2000) which makes them one of the 100 worst invasive species worldwide (Lowe et al., 2000).

Interspecific relationships like predation and competition are difficult to study, especially in aquatic ecosystems. Though, determination of predation–prey relationship based on feeding habits could be used as an important tool in management of ecosystems (Carreon-Martinez et al., 2011). Identification of semi-digested remnants of fish from stomach contents of the predator is very challenging (Heath, 1992). Several studies suggested using more steady parts such as otoliths and eye lenses (Nakaya et al., 2004; Wennhage & Pihl, 2001), but it is not always possible to make identification from these parts for every species. Also identification of these remnants is time consuming and need great expertise on morphology of prey species (Albaina et al., 2010).

Recently, studies based on the use of stomach content DNA to identify prey species becomes more prevalent as this approach

Table 1. Sampling locations and occurrence (visual/molecular) of target species.

	A. transgrediens		*G. affinis*		
Location	Visual	eDNA	Visual	eDNA	Coordinates (Springs near Lake Acı, Akpınar)
Spring 1	+	+	+	+	37°49′02.59″ N 29°55′35.11″E
Spring 2	+	+	+	+	37°49′06.15″ N 29°55′40.49″E
Spring 3	−	+	+	+	37°49′08.77″ N 29°55′45.43″E
Spring 4	−	+	−	−	37°49′14.16″ N 29°55′50.55″E
Spring 5	+	+	+	+	37°49′23.20″ N 29°56′04.26″E
Spring 6	−	+	+	+	37°49′29.74″ N 29°56′23.96″E

successfully detects the prey species from small quantities of degraded DNA molecules, extracted from the stomach (Symondson, 2002). DNA-based prey identification is especially useful in situations like small species, cryptic species and preys lacking parts for morphologic examination (Dunshea, 2009; Harper et al., 2005; Sheppard & Harwood, 2005). Detection of cryptic and endemic species becomes unnecessary as environmental DNA (eDNA) approach becomes more common. eDNA is the DNA (could be mitochondrial or nuclear) of an organism which is released into environment. In freshwater ecosystems, eDNA is diluted and transported by hydrological movements. Recent studies on molecular ecology gives promising results with more precise detection rates of freshwater fish species using eDNA (Keskin, 2014; Taberlet et al., 2012; Thomsen et al., 2012). Both DNA-based detection of predation–prey relationship and detection of freshwater fish species are the most recent approaches in molecular ecology and could be used together for a better understanding of interrelationship of freshwater fish species.

In 1992, one of the most invasive freshwater fish species, *Gambusia affinis* (Baird & Girard, 1854) was detected in one of the nearby springs at the southwest region of Lake Acı (Wildekamp et al., 1999). This introduced Poeciliid fish is known to be a significant threat for native species in terms of competive exclusion and direct predation on juveniles and fry. *G. affinis* species were mostly introduced to non-native habitats for mosquito control. During rainy seasons, hyper saline habitat of Lake Acı becomes fresher and permits *G. affinis* to migrate between other springs of the basin. This migration allows *G. affinis* populations to spread and occupy the entire basin which should be regarded as a serious threat to native freshwater species (Wildekamp & Valkenburg, 1994), especially on native *Aphanius transgrediens* (Ermin, 1946).

The aim of this study is twofold: First, to prove predation of fry and larvae of *A. transgrediens* by *G. affinis* in order to reveal the inextricable relation between introduction of invasive species and the extinction of native species. This linkage, between the invasions and the extinction of natives has not been evaluated with an evidence based approach yet. This paper describes the molecular evidence that reveals the predation of native *A. transgrediens* by invasive *G. affinis* based on semi-digested DNA samples extracted from the stomach content of the invader. To achieve this, species-specific primers that will amplify only a short and a unique fragment of the DNA was designed to allow species level molecular identification. Second, an uninvasive, species level molecular identification method (eDNA) has been applied to reveal existence of the endemic native *A. transgrediens* species in the springs of Lake Acı, without sampling or even visual detection of this Critically Endangered freshwater fish. The importance of designing species-specific primers, selection of proper genes and fragment size to amplify from degraded DNA and applicability of eDNA approach for species level identification of freshwater fish species were also discussed within this context.

Methods

A total of 50 *G. affinis* specimens were collected from the Lake Acı springs, near Akpınar. Water samples that will be used in eDNA extraction were also collected from the same six springs. Ten water samples and ten *G. affinis* specimens were collected from each spring except spring 4 as *G. affinis* was not found there. Locations of the springs and visual/molecular detection information are given in Table 1. Water samples were taken to sterile containers with 2 L volumes. Three different controls (negative field controls, negative transport controls and negative equipment controls) containing only deionized water were used for environmental samples (Goldberg et al., 2013). Both the negative controls and positive controls (containing water sampled from a different pond without the existence of any target species) were treated same with the actual samples. Environmental samples and *G. affinis* specimens were transferred immediately to laboratory on ice.

Dissections were performed in a sterilized laminar flow hood to avoid a possible contact with contaminant DNA. Gut samples were homogenized according to Baerwald et al. (2012). Samples were disrupted using Tissue Lyser II with ATL Lysis Buffer (Qiagen, Hilden, Germany) and proteinase K. After the homogenization process, samples were digested overnight at 56 °C. DNA was extracted from these digested samples using DNeasy Blood and Tissue Kit (Qiagen), according to manufacturers' animal tissue protocol. A blank extraction was conducted along with every DNA extraction as a negative control.

Environmental samples were filtered using Sterivex GP (Millipore, Billerica, MA) with 0.22 µm pore size. DNA extractions of environmental samples were conducted directly from membranes of these filtering units with PowerWater Sterivex DNA Isolation Kit (MoBio, Carlsbad, CA), according to manufacturers' protocol. Template eDNA samples were stored at −20 °C until the PCR.

Different-sized fragments of *NADH1* gene of mitochondrial DNA were amplified by PCR. Species-specific primer pairs (Figure 1) were designed to amplify only the DNA of target species from complex eDNA and stomach content. Specifity of primer pairs were tested *in silico* using the ecoPCR (Dejean et al., 2011) and primerBLAST (Jerde et al., 2011) software. Both the environmental DNA and the DNA from the stomach content of *G. affinis* specimens was possibly degraded, primers were designed to amplify short fragments of 133, 328, 383 and 601 base pairs long. *NADH1* gene was selected as the target mitochondrial gene, as it is the only region that contains species-specific conserved regions to amplify for both *G. affinis* and *A. transgrediens*, and previously submitted records of *NADH1* sequences were found in GenBank database to compare with our results in order to make identification at species level. Primers, fragment lengths and PCR conditions are given in Table 2.

Multiple tubes approach with triplicates was used in PCR process (Taberlet et al., 1996). This approach allows us to take results of nine duplications of PCR products from eDNA and

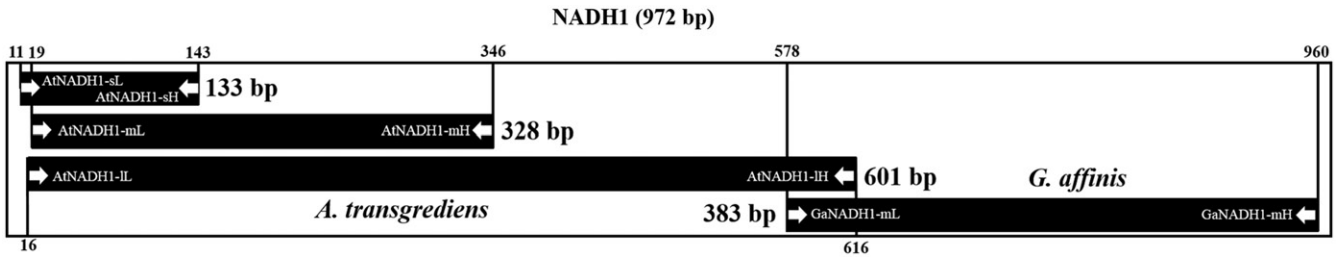

Figure 1. Species-specific primer pairs designed to amplify different size fragments of mitochondrial *NADH1* gene.

Table 2. Species-specific designed primer sequences, fragment length, reaction contents and PCR condition.

Primer sequence	Amplified fragment (bp)	Tm (°C)	PCR condition	Reaction content
AtNADH1-sL: 5′-CACTTCATGCAATTATTAACCCCC-3′ AtNADH1-sH: 5′-TATGGTCCTACTACATTGGGGC-3′	133	59	95 °C (2 min)	Total Volume: 25 µl
AtNADH1-mL: 5′-GCAATTATTAACCCCCTTGCGCT-3′ AtNADH1-mH: 5′-GGCTAGAGATGGCTAGGACAAA-3′	328	61	40 Cycles of: 95 °C (30 sec)	10 mM Tris–HCl (pH 8.3)
AtNADH1-lL: 5′-CATGCAATTATTAACCCCCTTGCG-3′ AtNADH1-lH: 5′-TTAGGTCAAAGGGGGCTC-3′	601	61	59–61 °C (45 sec) 72 °C (1 min)	50 mM KCl 3 mM $MgCl_2$ 0.3 mM dNTP 1 mM F-R primer 0.005 mg BSA
GaNADH1-mL: 5′-TCTCTACCCTGGCCGAGAC-3′ GaNADH1-mH: 5′-AAAGCTTGAGAAAGCTAGAGGTAGA-3′	383	60	72 °C (10 min)	0.5 U DNA Polymerase 5 µl DNA

stomach content DNA samples. If any reaction out of three resulted in negative PCR yield, sample was reanalyzed in triplicate. Amplified fragments were ran on 2% agarose gel and visualized under UV to check the proper amplicon length. Purification of the PCR products was performed with Wizard SV Genomic DNA Purification Kit (Promega, CA). Purified PCR yields were sequenced using ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) platform.

Nucleotide sequences were compared with publicly available sequences downloaded from GenBank database using MEGA 6 software (Tamura et al., 2013). All sequences were submitted to GenBank after they were aligned and trimmed to same length. Shortest fragments (133 bp) were completed to 200 bp *in silico* (using nucleotide sequences of longer fragments from this study) before submitting to GenBank, as GenBank does not accept submission of fragments shorter than 200 bp.

Results

A total of 50 stomach contents and 60 water samples were used in DNA extraction. All of the stomach content samples yielded amplifiable DNA for PCR. No DNA extraction could be performed for stomach content samples from spring 4 as no *G. affinis* specimens were collected from there. A total of 90 PCR's were performed for each spring, as we work in triplicates, resulted in 450 reactions (10 samples, 3 different-sized fragments, 3 repeats, 5 stations). None of the PCR amplifications produced double bands with any of the primer sets, clearing away the necessity for a cloning process in order to determine the nucleotide sequence of different length PCR products. Among these, 357 PCR (79.33%) produced successful amplifications. Mean PCR success according to fragment lengths were 87.33% for the small fragment (133 bp), 80.67% for the medium fragment (328 bp) and 70.67% for the longer fragment (601 bp). Positive PCR percentage for each sample is given in Table 3. PCR products of *A. transgrediens* were in expected lengths, as estimated by agarose gel electrophoresis. PCR amplification success according to target fragment length is also calculated. Shorter fragments were found to be more effective as expected. Amplification success percentage was calculated as 87.33%, 80.67% and 70.67% for fragment lengths of 133, 328 and 601 base pairs, respectively.

Water samples collected from six stations were found positive for eDNA, which could be used in PCR. Each DNA extraction process was performed in triplicates. All the DNA extraction attempts were successful for samples collected from stations 1, 2 and 5. Only one attempt of DNA extraction was successful for samples collected from station 4 and two successful attempts for the sample from stations 4 and 6. As a result, amplifiable eDNA was extracted from all the samples belonging to stations where the target species were visually observed. eDNA was also extracted from the samples from stations without visual occurrence of one or both of the target species (Table 1). No trace of contaminant DNA was found as none of the control samples contains amplifiable DNA. A total of 60 PCR amplification based on multi tube approach was performed for samples from each station to a sum of 360 reactions in total (10 samples, 6 stations, 3 repeats, 2 species). Each PCR reaction was performed as triplicates in order to confirm the specifity of primer sets designed to amplify 328–383 base pairs long fragment of mitochondrial *NADH1* gene and every positive amplification was used in DNA sequence analysis. None of the PCR products given double bands as every PCR product checked on agarose gel electrophoresis. All of the PCR products were in expected lengths (328 bp for *A. transgrediens* and 383 bp for *G. affinis*) for both of the target species. Number of successful PCR reactions was 306 out of 360 (85.00%). Average PCR success according to target species were 144 out of 180 (80.00%) attempts for *A. transgrediens* and 162 out of 180 (90.00%) for *G. affinis*. Only samples of spring 4 did not produce any positive amplification for *G. affinis*. Positive eDNA controls were unable to give any amplification using species-specific primers designed in this study. It is important to confirm

Table 3. PCR success of environmental DNA and stomach content DNA.

Location	Species	Sample	Fragment (bp)	Success rate (%)
Spring 1	*A. transgrediens*	Stomach content	133	96.67
Spring 1	*A. transgrediens*	Stomach content	328	90.00
Spring 1	*A. transgrediens*	Stomach content	601	70.00
Spring 1	*A. transgrediens*	eDNA	328	93.33
Spring 1	*G. affinis*	eDNA	383	96.67
Spring 2	*A. transgrediens*	Stomach content	133	93.33
Spring 2	*A. transgrediens*	Stomach content	328	90.00
Spring 2	*A. transgrediens*	Stomach content	601	73.33
Spring 2	*A. transgrediens*	eDNA	328	90.00
Spring 2	*G. affinis*	eDNA	383	93.33
Spring 3	*A. transgrediens*	Stomach content	133	80.00
Spring 3	*A. transgrediens*	Stomach content	328	73.33
Spring 3	*A. transgrediens*	Stomach content	601	70.00
Spring 3	*A. transgrediens*	eDNA	328	73.33
Spring 3	*G. affinis*	eDNA	383	90.00
Spring 4	*A. transgrediens*	Stomach content	NA	NA
Spring 4	*A. transgrediens*	Stomach content	NA	NA
Spring 4	*A. transgrediens*	Stomach content	NA	NA
Spring 4	*A. transgrediens*	eDNA	328	70.00
Spring 4	*G. affinis*	eDNA	383	83.33
Spring 5	*A. transgrediens*	Stomach content	133	86.67
Spring 5	*A. transgrediens*	Stomach content	328	80.00
Spring 5	*A. transgrediens*	Stomach content	601	66.67
Spring 5	*A. transgrediens*	eDNA	328	86.67
Spring 5	*G. affinis*	eDNA	383	96.67
Spring 6	*A. transgrediens*	Stomach content	133	80.00
Spring 6	*A. transgrediens*	Stomach content	328	70.00
Spring 6	*A. transgrediens*	Stomach content	601	70.00
Spring 6	*A. transgrediens*	eDNA	328	66.67
Spring 6	*G. affinis*	eDNA	383	80.00

NA: data not available.

the lack of amplification is not by chance. eDNA-based occurrence percentage of target species was calculated as 91.67%, whereas it was 66.67% for visual-based occurrence.

Direct DNA sequencing produced 791 readable sequences from a total of 810 PCR products. Readable sequences were also acquired for the remaining 19 PCR products only after a single repeat. All of the sequences were analyzed with BLAST after the alignment and confirmed as being from target species only (identification rate $\geq 98\%$). There were no deletions, insertions or stop codons in any of the sequence analyzed. Sequences were all long enough to make identifications at species level. No intraspecific variation was observed between samples of different stations or convergent parts of fragments in different lengths. Therefore, sequences were submitted as haplotypes to GenBank under accession numbers KJ599869–KJ599873.

Discussion

Alteration of aquatic ecosystems through introduction of invasive species have serious long-lasting consequences for trophic interactions (Baerwald et al., 2012). This is the first study presenting both eDNA and molecular detection of stomach content data from freshwater fish species, especially from an invasive species (*G. affinis*) and a Critically Endangered endemic species (*A. transgrediens*) which becomes the prey in its own ecosystem. In this study, presence of endemic native fish species, *A. transgrediens* and non-native *G. affinis* which was predicted to be the main reason behind the decay in *A. transgrediens* populations inhabiting Lake Acı, was revealed without any sampling of both species. Instead, water samples from springs around the Lake Acı were taken as environmental genetic material indicating the existence of both species. eDNA samples extracted from these water samples were used in molecular identification of target species. Then, predation of *A. transgrediens* by invasive *G. affinis* was revealed using the DNA extracted from complex stomach contents of *G. affinis* specimens.

Molecular identification at species level is receiving more attention in recent ecological studies on prey–predation interaction as the data collection is not limited with undigested remnants of the prey and other solid parts remaining (Deagle et al., 2005). To identify different species or one particular species from a mixture such as the stomach content, species-specific primers that allow only the amplification of the target species could be used. One of the factors that was limiting the applicability of this approach is that identification of the target species relies on a comparison with a reference DNA sequence previously submitted to nucleotide databanks such as GenBank or BOLD. But today, with the rapid increase of available reference DNA sequence data, molecular identification at species level, especially for freshwater fish species, becomes more possible for most of the species. *NADH1* sequences generated in this study was paired off with reference sequences with identity ratios of >98%. It will be useful to search for available sequences of target species on GenBank and specify the gene which will be amplified according to both suitability of conserved regions and existence of comparable sequences, before designing the molecular phase of the study.

It is known that detection and identification of semi-digested fish species from the stomach contents of predators is difficult with morphological methods (Deagle et al., 2005). Dietary samples such as stomach contents of freshwater fish species are complex sources of DNA. Stomach content DNA include genetic material of the species from which they originate, intestinal parasites, symbionts and its prey (Jarman, 2004). In this case, our phylogenetic range of interest is only restricted with one species. This reduction in the target diversity allows to design and use group/species-specific primers which will eventually simplify the analyses. Usage of group/species-specific primers for PCR is the most significant factor in order to exceed the wide range of species diversity in a complex DNA source. Targeting only one prey and predator species in this study makes the molecular process easier to conduct and prevent all kind of false positive amplifications by restricting the PCR with species-specific primers.

Group/species-specific primers have many advantages over widely used universal primer sets. First of all, universal primer sets were mostly designed for widely used mitochondrial genes such as *16S rRNA*, *12S rRNA*, *cytochrome b* and *cytochrome c oxidase I*. Main reason behind this tendency is the available reference nucleotide sequences. The usage of mitochondrial *NADH1* gene was mandatory for this study because there is no other nucleotide sequence to compare in GenBank for molecular identification of *A. transgrediens*. Also, other genes were not suitable markers for species level identification of *A. transgrediens* in terms of sequence lengths shorter than 600 base pairs, like in this study. Results of this study clearly indicate an increasing success rate at PCR amplification as the target fragment gets shorter. Results with the best amplification rates were gathered from the shortest fragment of 133 base pairs long. Another advantage of group/species-specific primer over universal primers is the tendency of universal primers to miss rare templates in complex DNA at the earlier stages of the PCR (Jarman, 2004). Boom et al. (2002) compared the bacterial community diversity data based on universal and group specific PCR primers. Eventually, group specific primers have detected more of the bacterial diversity than the universal primers.

Semi-digested biological materials like the DNA extracted from the stomach contents were only able to produce short

fragments of PCR yield. Generally, PCR amplification from degraded DNA is available only if the target amplicon is shorter than 200 base pairs (Valdez Moreno et al., 2012). It is hard to find a part of a mitochondrial gene that is at most 200 base pairs long and conserved enough to be used as a specific marker for only one species. This fact motivates studies to be designed as targeting longer fragments with universal primers, as it is hard to find the suitable fragment and its unique primer. However, universal primers are not literally ''universal'' as they have a great tendency to amplify DNA from taxa with exact or more likely complimentary nucleotide sequences (Blankenship & Yayanos, 2005). In our study we used complete *NADH1* and *NADH2* gene sequences of our target species and find fragments that can be used in species level molecular identification of the species. Eventually, short fragments of *NADH2* gene were found to be not variable enough to make identification at species level. Choosing mitochondrial genes was also important as they are known to evolve faster than nuclear genes and also each cell has many copies of mtDNA which allows a greater chance to amplify fragments from degraded samples such as stomach content. All the primers used were designed for this study and only works with target species, and confirmed by the PCR results of positive control samples. Primer binding sites were selected from highly conserved binding sites for *A. transgrediens* and *G. affinis*. Primers were not only designed as having highly conserved binding sites, but also targeting three different fragment lengths (133, 328 and 601 base pairs long) in order to determine the PCR success based on the size of the amplicon. Results indicated a higher amplification percentage as the target amplicon gets shorter. The shortest fragment of 133 base pairs long was 7% and 17% more successful in PCR than the fragments of 328 and 601 base pairs long, respectively.

Molecular identification of prey using DNA extracted from stomach content of the predator with primers targeting short sequences allowing species level identification could be employed regardless of the life stage of the prey. Another aspect of this study is to detect both the prey and the predator species through eDNA, without even a visual detection. Detection success percentages of the target species in this study clearly indicate that eDNA approach gives 20% higher results. This amount of difference in detection sensitivity is considered significant in ecological studies of cryptic, endemic and invasive species. A detection rate difference of 20% could divert to a result of the absence of target species in studies with very low abundance of the target species. eDNA results of *A. transgrediens* for the springs 3, 4 and 6 provide critical data for the occurrence of the species, in which traditional surveys failed to reveal existence. Besides the more accurate detection rates, traditional samplings cost two to three times more in terms of expenditure and time (Michelin et al., 2011).

Conclusions

In this study, predation of Critically Endangered and endemic freshwater fish species, *A. transgrediens*, by invasive mosquitofish, *G. affinis,* was revealed by molecular identification from stomach contents. Moreover, existence of *A. transgrediens* in six springs around Lake Acı was recorded using eDNA samples extracted from water samples, while no *A. transgrediens* specimens were found using traditional surveys. Molecular analyses were conducted using species-specific primers designed in this study and all controls (negatives and positives) indicated no signs of contamination during the sampling process and no false positive amplification is available with the primers designed in this study. Despite the known limitations (degraded DNA, false positives, and contaminations) of molecular identification from stomach content, this approach was found to be very effective in determination of predator–prey interactions, especially among specific species. Predation studies on species level interactions based on molecular data will ease collection of precise information on food-web characterizations, trophic relationships and enable us to model ecological food-webs from wider taxon ranges. With the current expansion of DNA databases, molecular-based approaches will become increasingly useful and more effective in studying ecological relationships of both aquatic and terrestrial organisms.

Declaration of interest

The author reports no conflicts of interest. The author is responsible for the content and writing of the paper.

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