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Original Article

Combined mitochondrial DNA analysis of the Mesopotamian spiny eel, *Mastacembelus mastacembelus* (Banks & Solander 1794), and its phylogenetic position

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Abstract: Nucleotide sequences of the 12S rRNA, 16S rRNA, $tRNA^{Phe}$ and $tRNA^{Val}$ genes of mtDNA of Mesopotamian spiny eel, *M. mastacembelus*, was determined for the first time. The comparison of the three populations of Mesopotamian spiny eel from Turkish part of the Tigris basin based on the combined mitochondrial DNA was performed. Based on the results, no differences were determined and the identity found to be 100% among three populations. Furthermore, the obtained results from molecular methods were compared with morphological findings to validate the position of the studied populations of *M. mastacembelus*. In addition, the phylogenetic position of the Mesopotamian spiny eel was examined among the Mastacembelidae and Synbranchioformes based on 12S rRNA and 16S rRNA. The constructed phylogenetic relationship between *M. mastacembelus* and some other members of Synbranchioformes order supported their taxonomic hierarchy.

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Introduction

The order Synbranchioformes includes 120 species in three families, including Chaudhuridae (10 species), Synbranchidae (23 species) and Mastacembelidae (87 species) (Froose and Pauly, 2014). The members of the family Mastacembelidae, known as spiny eels, are found in freshwaters and distributed in tropical and subtropical Africa, the Middle East, South-East Asia and North of China (Coad, 2015). This family consists of three genera, including *Mastacembelus* (64 species), *Macrognathus* (22 species) and *Sinobdella* (1 species) (Vreven, 2005a; Froose and Pauly, 2014). Nine species of the genus *Mastacembelus* inhabit Asian inland waters, whereas 52 species occur in African inland waters and all members of the genus *Macrognathus* were recognized in Asian inland waters (Froose and Pauly, 2014).

Mastacembelids can attain a maximum length of about 1 m. They are eel-like fishes having a long series of well-separated dorsal spines and a short series of anal spines. They have no pelvic girdle and fins (Vreven, 2005b). More than 70 species of spiny eels are consumed as food fishes (Britz, 2007).

Mastacembelus mastacembelus occurs in the river basins of the Tigris and Euphrates in the Middle East; Turkey, Syria, Iraq and Iran (Coad, 1996; Froose and Pauly, 2014) and is known as Mesopotamian spiny eel referring to its inhabiting area. This taxon is a typical species of the Mastacembelidae and contains all the characteristics of the family (Coad, 2015).

The phylogenetic structure of the family Mastacembelidae is under debate and its classification has mainly been based on meristic and morphometric characters (Travers, 1984; Kottelat, 1991; Johnson and Patterson, 1993; Britz, 1996; Vreven and Teugles, 1996; Vreven, 2004; Vreven, 2005a; Vreven, 2005b; Britz, 2007; Çakmak and Alp, 2010; Plamoottil and Abraham, 2013). Although, the majority of the mastacembelids were morphologically described, a few species such as

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Mastacembelus aculeatus, *M. erythrotenia*, *M. armatus*, *Macrognathus aculeatus* and *M. pancalus* were described based on molecular data (Miya et al., 2001; Chen et al., 2003; Smith and Wheeler, 2006). However, there is no molecular information for *M. mastacembelus* and many mastacembelid species.

The mitochondrial DNA (mtDNA) is a circular and small molecule, self-replicating and usually about 15-18 kb in length. Mitochondrial genome contains two ribosomal RNA genes, which play primary role in protein synthesis (12S rRNA and 16S rRNA), 13 protein-coding genes (ATPase 6, ATPase 8, COI–III, *Cytb*, ND1-6 and 4L), 22 transfer RNA genes and a non-coding control region (D-loop) in charge of its replication and transcription factor as other vertebrates (Ishıguro et al., 2001; Kartavtsev et al., 2007). The gene content and organization of complete vertebrate mtDNA are quite conserved (Boore, 1999). The mitochondrial DNAs have been widely used as a marker for identification of species and phylogenetic researches, since a lot of characteristics are attributed to the maternal inheritance, high copy numbers in each cell, lack of recombination and high evolution rate (Kartavtsev et al., 2007; Cui et al., 2009; Cawthorn et al., 2012). In addition, the complete mtDNA has been widely used in the phylogenetic researches, partial gene fragments such as *Cytb*, 12S rRNA, 16S rRNA and the control region has become also very useful molecular tools for mitochondrial analysis (Cruz-Agüero et al., 2012). Therefore, the mitochondrial DNA has been considered a popular marker in many areas including fisheries biology, management and aquaculture, especially for population and evolutionary studies (Avise, 1994; Okumuş and Çiftci, 2003; Galtier et al., 2009; Lin et al., 2014).

The aim of this study is to determine nucleotide sequences of the 12S rRNA, 16S rRNA, $tRNA^{Phe}$ and $tRNA^{Val}$ genes of the spiny eel, *M. mastacembelus*, and to determine its phylogenetic position among the mastacembelids and the members of Synbranchioformes. Furthermore, it is aimed to validate the obtained results from molecular methods with morphological findings for identification of this species. To my best knowledge, there is no report on molecular taxonomy of *M. mastacembelus*. This is the first report on molecular identification of this species. These findings can contribute to understanding of the evolution and phylogenetic characterization of the *M. mastacembelus* based on mtDNA.

Table 1. Denaturation, annealing and extension temperature and times in PCR.

Primer	Denaturation	Annealing	Extension
E1-E8	1 min in 94ºC	30s in 59ºC	2 min in 72ºC
E2	1 min in 94ºC	30s in 55ºC	2 min in 72ºC
E3-E6	1 min in 94ºC	30s in 58ºC	2 min in 72ºC
E4	1 min in 94ºC	30s in 64ºC	2 min in 72ºC
E5-E7	1 min in 94ºC	30s in 62ºC	2 min in 72ºC

Materials and Methods

Total DNA Extraction: A total of 57 individuals (36 of Karakaya Reservoir, 7 of Tohma Stream and 14 of Tigris River) of *M. mastacembelus* from three different locations at Tigris and Euphrates Rivers were sampled. Genomic DNA samples were obtained from ethanol preserved caudal fin tissues. Caudal fins of 20-30 mg were minced and 600 µL TEN (100 mM Tris, 10 mM EDTA and 250 mM NaCl), 40 µL 20% SDS (Sodium Dodecyl Sulphate) and 10 µL Proteinase K (10 mg/l) were added on the samples. They were incubated at 55°C for 24 hours. After the incubation, 10 µL RNase (5 mg/ml) was added and second incubation was applied at 55°C for 24 hours. The total DNA was purified by standard phenol:chloroform extraction and ethanol precipitation (Sambrook et al., 1989). Isolated DNA was inspected under UV light after 1% agarose gel electrophoresis.

PCR and Sequencing: PCR amplifications were performed in 50 µL tubes containing 5 µl 10X reaction buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton-X1-100), 0.5 µL 1mM dNTP (250 µm from each of nucleotides), 1 µl each of 20 pmol forward and reverse primers, 1 U Taq DNA polymerase, 1 µl DNA and 42 µl ddH_2O. Reaction mixtures were subjected to the following cycling protocol: Initial denaturation (94°C: 4 min), 30 cycles (94°C: 1 min;

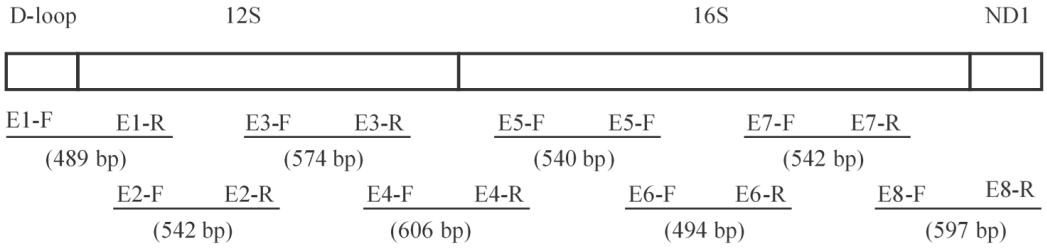

Figure 1. A diagram showing arrangement and position of all amplifying primers.

Table 2. Specific primers used for PCR amplification of target genes of *M. mastacembelus*.

			NC 003193 Location		
Primer	Sequence (5′ → 3′)	Length	Start	End	Tm (ºC)
E1-F	CCGGAAACAGGAAAACCTCT	20	24	43	64.10
E1-R	TAGCTTTCGTGGGGTCAGAA	20	565	582	64.56
E2-F	CTACGGCGTAAAGAGTGGTT	20	453	472	60.71
E2-R	CTTTAGAACCGGTTTCAGCA	20	976	995	61.72
E3-F	CAAACGTCAGGTCGAGGTGTA	21	843	863	64.97
E3-R	ATCATGATGCAAAAGGTACGAG	22	1396	1417	62.69
E4-F	TGCAAGTCGGATCACCCTGA	20	1172	1191	69.20
E4-R	CGCTTTCTATTGTGGTGGCTGC	22	1757	1778	69.61
E5-F	ATAGCTGGTTGCCCGAGAACTG	20	1570	1591	68.31
E5-R	GGTAAACAGGCGAGGCTTATAAGG	20	2087	2110	66.31
E6-F	GCCAACCTCTCTCCAAACAC	20	1894	1918	63.62
E6-R	GTGTCTAAAGCTCCACAGGG	20	2369	2388	61.27
E7-F	CCCCAAGGAAAGGCTGAAAG	21	2042	2061	66.76
E7-R	CTTGAAGGGGATTGCGCTG	22	2565	2584	67.93
E8-F	CGGGGATAACTCCATAAGAC	20	2310	2329	64.20
E8-R	GGATTTGAACCTCTGTGGTAAAGG	22	2903	2926	65.43

55°C: 30 s; 72°C: 2 min) and final extension (72°C: 5 min) (Table 1).

In order to amplify mitochondrial DNA with standard PCR techniques, the new primers were designed because mtDNA of *M. mastacembelus* has not been determined so far. In order to design PCR and sequencing primers for mtDNA genes, sequence for each gene were retrieved from the mitochondrial genome data of *M. favus* (Accession No. NC_003193). Sequence length of 12S rRNA gene of *M. favus* was 947 bp and 16S rRNA gene was 1671 bp (http://www.ncbi.nlm.gov). The target DNA fragment had 3036 bp and contains the region of D-loop (last 100 bp), $tRNA^{Phe}$, 12S rRNA, $tRNA^{Val}$, 16S rRNA, $tRNA^{Leu}$, and ND1 (initial 100 bp) (Fig. 1). The primers were designed on the alignments of these sequences. DNA fragment was divided into 8 sections because 400-600 bp were desired to sequencing. Forward and reverse primers were designed for each section. The length and temperature of these primers were given in Table 2. For sequence analyses, three samples from each population were used for sequence of mitochondrial 16S rRNA, 12S rRNA and tRNAs genes, which sequenced in Iontek (http://www.iontek.com.tr).

Data analysis and phylogenetic relationships: The 16S rRNA, 12S rRNA, $tRNA^{Phe}$ and $tRNA^{Val}$ sequences of nine samples of *M. mastacembelus*

Table 3. GenBank accession numbers and location of examined species for phylogenetic relationships

Order	Family	Species	Common names*	Distribution*	12S rRNA gene	16S rRNA gene
					GenBank accession no.	GenBank accession no.
Synbranchiformes	Mastacembelidae	*Mastacembelus mastacembelus*	Mesopotamian spiny eel	Asia: Tigris and Euphrates basin	GU174757	GU174759
	Mastacembelidae	*Mastacembelus armatus*	Zig zag eel	Asia: Pakistan to Viet Nam and Indonesia	AF508066	DQ532904
	Mastacembelidae	*Mastacembelus erythrotaenia*	Fire eel	Asia:Thailand and Cambodia to Indonesia	AY141349	AY141419
	Mastacembelidae	*Mastacembelus favus*	Tire track eel	Asia:Thailand to the Malay peninsula	NC_003193	NC_003193
	Synbranchidae	*Monopterus albus*	Swamp eel	Asia:India to China, Japan, Malaysia and Indonesia	NC_003192	NC_003192
	Synbranchidae	*Synbranchus marmoratus*	Marbled Swamp eel	Central and South America: Mexica to northern Argentine	AP004439	AP004439
Acipenseriformes	Acipenseridae	*Acipenser stellatus*	Starry sturgeon	Eurasia: Caspain, Balck, Azov and Aegean Seas	NC_005795	NC_005795

*Common names and distribution were taken from www.fishbase.org.

from three different locations were analyzed to determinate nucleotide composition by MEGA 5.2 software (Tamura et al., 2011). A blast search was performed on NCBI to compare the sequences of *M. mastacembelus* populations from Karakaya Reservoir, Tohma Stream and Tigris River, and its phylogenetic tree were constructed based on maximum likelihood model using MEGA 5.2 software (Tamura et al., 2011).

The 16S rRNA and 12S rRNA nucleotide sequences of seven species, including *M. mastacembelus* (in this study), *M. armatus*, *M. erythrotaenia*, *M. favus*, *Monopterus albus* (Synbranchidae) and *Synbranchus marmoratus* (Synbranchidae) from the order Synbranchioformes registered to GenBank as in-group and *Acipenser stellatus* as out-group was used to study the phylogenetic relationships of *M. mastacembelus* among the members of Synbranchioformes (Table 3). The 16S rRNA and 12S rRNA of these species were translated in different formats aligned using Clustal X (Thompson et al., 1997). The genes of $tRNA^{phe}$ and tRN^{Aval} were excluded from this step since there is no sequence knowledge found in public databases such as NCBI and EMBL. Phylogenetic trees were constructed using Maximum Likelihood (ML) (Felsenstein, 1981) and Neighbor Joining (NJ) (Saitou and Nei, 1987) methods using MEGA 5.2 software (Tamura et al., 2011). The robustness of the internal branches of trees was assessed by bootstrapping with 1000 replicates. Phylogenetic trees including nucleotide sequences of *M. mastacembelus* individuals and *M. favus* were similarly constructed using ML and NJ methods.

Results

In the present study, I provided complete sequences of the mitochondrial 16S rRNA, 12S rRNA and tRNA genes of *M. mastacembelus*. The total length of the 12S rRNA, 16S rRNA, $tRNA^{phe}$ and $tRNA^{val}$ genes of *M. mastacembelus* were found to be 947 bp, 1667 bp, 69 and 73 bp, respectively. The 12S rRNA, 16S rRNA, $tRNA^{Phe}$ and $tRNA^{Val}$ gene sequences were deposited in GenBank (with accession no GU174757, GU174759, KM211690 and GU174758, respectively). The nucleotide composition of

Table 4. Base compositions (% of total number) of target genes of *M. mastacembelus*.

Fragment	A (%)	C (%)	G (%)	T (%)	Total (bp)
$tRNA^{Phe}$	39.1	23.2	20.3	17.4	69
12S rRNA	32.4	26.7	19.6	21.2	947
$tRNA^{Val}$	30.1	31.5	21.9	16.4	73
16S rRNA	34.3	26.2	19.3	20.3	1667
Average	33.7	26.4	19.5	20.4	565.2

Figure 2. Phylogenetic trees constructed from combined target nucleotide sequences (12S rRNA, 16SrRNA and tRNAs genes) of different studied populations of *M. mastacembelus* and *M. favus* based on ML methods with bootstrap support values for each branch.

12S rRNA is A: 32.4%, C: 26.7%, G: 19.6% and T: 21.2%. The content of A+T (53.6%) is higher than that of C+G (46.3%). The nucleotide composition of 16S rRNA is A: 34.2%, C: 26.2%, G: 19.3% and T: 20.3%. The content of A+T (54.6%) is higher than that of C+G (45.5%). The base compositions of the 12S rRNA, 16S rRNA and tRNAs nucleotide sequences are given in Table 4.

The phylogenetic relationships of the studied *M. mastacembelus* populations i.e. the Karakaya Reservoir, Tohma Stream and Tigris River populations were investigated using combined mitochondrial DNA sequence. For this purpose, their combined identified sequences were compared using NCBI blast software and based on the results no differences were determined between populations and the identity found to be 100% among all three populations. Furthermore, based on the combined target sequences i.e. 12S rRNA, 16S rRNA and tRNA genes of these populations and *M. favus*, the ML tree was constructed. In this tree, the members

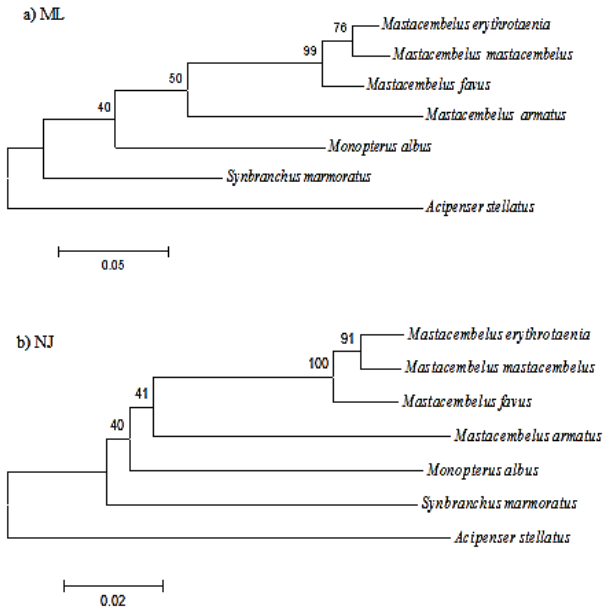

Figure 3. Phylogenetic trees based on ML and NJ methods of 12S rRNA genes with bootstrap support values for each branch. *Acipenser stellatus* was used as out-group.

of three studied population were clustered together with *M. favus* in another branch (Fig. 2).

The phylogenetic position of *M. mastacembelus* among the mastacembelids and members of Synbranchioformes that their 16S rRNA and 12S rRNA nucleotide sequences were available in GenBank was constructed (Figs. 3 and 4). Both ML and NJ phylogenetic trees showed two main branches viz. the members of Synbranchiformes as in-group and *A. stellatus* as out-group showing monophyly of the Synbranchiformes with the families Mastacembelidae and Synbranchidae in discrete clade as sister groups. *Mastacembelus favus*, *M. erythrotaenia* and *M. mastacembelus* formed a monophyletic group with high bootstrap value. *Mastacembelus mastacembelus* diverged with high bootstrap value from *M. erythrotaenia* in trees based on 12 rRNA (Fig. 3) whereas, phylogenetic trees of 16S rRNA showed that *M. mastacembelus* and *M. armatus* are the closest (Fig. 4).

Discussion

Identification of fish species is traditionally based on morphological methods i.e. morphometric, meristic and anatomical features. However, there are major

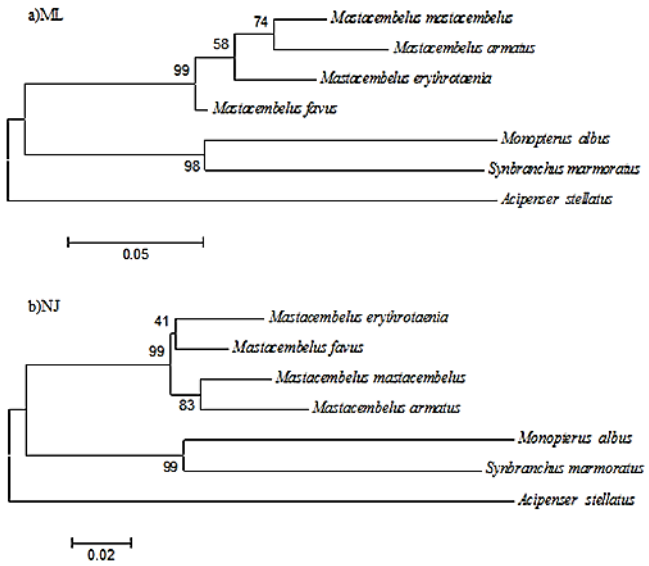

Figure 4. Phylogenetic trees based on ML and NJ methods of 16S rRNA genes with bootstrap support values for each branch. *Acipenser stellatus* was used as out-group.

problems to identify the fish species solely based on the morphological characters due to different ecological conditions, which are lead to morphological variations (Lakra et al., 2009; Teletchea, 2009; Chen et al., 2012). Therefore, molecular methods especially based on mtDNA are used as alternative for their identifications. These methods are highly specific, sensitive and simple compared with morphological methods (Comesana et al., 2003).

Based on the results, the sequences of 12S rRNA, 16S rRNA, $tRNA^{Phe}$ and $tRNA^{Val}$ genes in *M. mastacembelus* from different studied locations showed no differences. However, in a previous study significant differences among these populations were observed in terms of morphometric characters (Çakmak and Alp, 2010). Based on Çakmak and Alp (2010), the Karakaya Reservoir population was morphologically different than two other populations. Such condition was reported in sea-bass, *Dicentrarchus labrax* by Turan and Erguven (2005). They noted that molecular techniques have great potential to support the detected phenotypic differentiation. Furthermore, 12S rRNA and 16S rRNA genes are highly conserved in of animal kingdom (Cawthorn et al., 2012). These genes have been proven to be the powerful phylogenetic tools (Cruz-Agüero et al., 2012). The 12S rRNA has been used to higher categorical levels such as in phyla and 16S rRNA often used for studies at middle categorical levels such as families or genera (Arif and Khan, 2009). Therefore, the results also showed that this genes are not proper to study the genetic population of the genus *Mastacembelus* as well.

The 12S rRNA and 16S rRNA genes were respectively bordered by the $tRNA^{Phe}$ and $tRNA^{Val}$ genes and by the $tRNA^{Val}$ and $tRNA^{Leu}$ genes as in other vertebrates (Nagase et al., 2005). The location of these genes are conserved in vertebrates (Chang et al., 1994; Cui et al., 2009). The results of this study were supported by location of these genes. The 12S rRNA and 16S rRNA genes of *M. mastacembelus* exhibit A+T rich-content like as other bony fishes (Chang et al., 1994).

Phylogenetic trees based on ML and NJ method using 12S rRNA and 16S rRNA gene sequences showed that members of the same genus have been clustered together confirming the current taxonomic classifications of the studied fish species (Vreven, 2005a; Froose and Pauly, 2014). Although little nucleotide sequences of the members of Synbranchioformes were available in GenBank.

This study is the first attempt to identify phylogenetic position of Mesopotamian spiny eel, *M. mastacembelus* based on mitochondrial sequences. The sequences of Mesopotamian spiny eel, *M. mastacembelus*, generated that were previously unavailable in GenBank. These sequences will be valuable for future molecular studies and phylogenetic researches in mastacembelid species and the order of Synbranchioformes.

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چکیده فارسی

ترکیب DNA میتوکندیای ماراهی خاردار بین‌النهرین، *Mastacembelus mastacembelus* و جایگاه تبارزایی آن

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چکیده:
توالی نوکلئوتیدی ژن‌های 12S rRNA، 16S rRNA، $tRNA^{phe}$ و $tRNA^{val}$ مینوکندریایی ماراهی خاردار بین‌النهرین، *M. mastacembelus*، برای اولین بار تعیین گردید. مقایسه سه جمعیت ماراهی خاردار بین‌النهرین بخش حوضه تیگریس ترکیه براساس ترکیب DNA میتوکندریایی حاصل انجام شد. براساس نتایج، تفاوتی بین جمعیت‌های مورد مطالعه *M. mastacembelus* یافت نشد و توالی آن‌ها به‌طور ۱۰۰ درصد یکسان بود. همچنین مقایسه نتایج مولکولی و ریختی به منظور اعتبارسنجی جایگاه آرایه‌شناختی جمعیت‌های مورد مطالعه *M. mastacembelus* انجام شد. به علاوه جایگاه تبارزایی ماراهی خاردار بین‌النهرین در بین اعضای خانواده Mastacembelidae و راسته Synbranchioformes براساس 12S Rrna و 16S rRNA مورد بررسی قرار گرفت. روابط تبارزایی حاصل بین *M. mastacembelus* و برخی دیگر اعضای راسته Synbranchioformes جایگاه آرایه‌شناختی آنها را مورد تایید قرار داد.
کلمات کلیدی: *Mastacembelus*، 16S rRNA، 12S rRNA، $tRNA^{Phe}$، $tRNA^{Val}$، روابط تبارزایی.