Natural and Engineering Sciences

Volume 1, No. 3, 66-77, 2016

- RESEARCH ARTICLE -

Genetic barcoding and preliminary phylogenetic analysis of Serranidae species from Maltese coastal waters, with a perspective on their Mediterranean phylogeography

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Abstract

Ten species of the Serranidae Family sampled from the Mediterranean, including two non-native species, were identified using molecular genetic tools. Two mitochondrial genes, cytochrome c oxidase subunit I (COI) and Cytochrome b (Cyt b) genes were studied covering a total of 980 bp of which 360 bp exhibited genetic differences. Within species the members of the genus *Serranus* exhibited the highest haplotypic diversity, while the genera of larger grouper taxa have shown low haplotypic and nucleotide diversity indices with these genetic markers. Each sequence was also checked against BOLD and GenBank databases to compare species categorization. COI data on *S. cabrilla* and *S. scriba* were used in a preliminary phylogeographic analyses for these two species. Results show significant differences between certain sampling locations, indicating localized populations within the Mediterranean.

Keywords:
Serranidae; DNA barcoding; phylogenetics; phylogeography; COI; Cyt b

Article history:
Received 16 December 2016, Accepted 19 December 2016, Available online 21 December 2016

Introduction

In the Mediterranean, the Family Serranidae is represented by eleven native species from the subfamilies Anthiinae, Epinephelinae and Serraninae (Heemstra & Randall 1993; Abdul Malak

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et al., 2011; Froese & Pauly 2016). In addition to these, there have been increasing records of non-native serranids from the subfamily Epinephelinae (Heemstra & Randall 1993; Ben-Tuvia & Lourie 1969; Heemstra & Golani 1993; Lelong 2005; Bariche & Heemstra 2012; Dulčić & Dragičević, 2013; Golani et al., 2015; Rothman et al., 2016; Vella et al., 2016), with *Epinephelus coioides* (Ben-Tuvia & Lourie, 1969; Parenti & Bressi, 2001; Gokoglu & Ozvaol, 2015) and *C. taeniops* (Ben Abdallah et al., 2007; Guidetti et al., 2010; Vella et al., 2016) showing up multiple of times in landing records possibly indicating multiple introductions or population establishment in the region.

The Family Serranidae encompasses a number of economically important species, that are commonly caught by commercial, artisanal and recreational fishermen. Therefore, the correct species identification is an important basic requirement in monitoring for sustainable fisheries management and identifying the species specific conservation needs of this Family's biodiversity throughout the Mediterranean region (Vella, 2009). FAO data for the GFCM area indicates that in 2014 at least 5.7% of the marine fish landings were classified as unidentified, while several others, including some species of groupers, are classified down to high taxonomic levels such as *Ephinephelus* spp. (FAO, 2016). Moreover, there are several instances where due to untrained personnel or due to unpronounced and overlapping morphological characters, species are misidentified and placed in the wrong species category (Vella, 2009; personal observations by authors). Illegal, unreported and unregulated (IUU) fisheries activities which impact on species of this Family are difficult to quantify, especially where sport spearfishing is also practiced, with the fish being sold privately or used for personal consumption and not recorded through the national fish-market. Most of these species are coastal and exposed to unrecorded catches by recreational fishermen (personal observation by authors). These factors affect the actual landings, exploitation and their accurate records for species of the Family Serranidae.

Direct exploitation has been the main cause leading to the inclusion of *Ephinephelus marginatus* as an Endangered species within the IUCN Red List of Threatened Species both at global (Cornish & Hermelin-Vivien, 2004) and at Mediterranean level (Cornish & Hermelin-Vivien, 2011) due to serious declines in its population. However environmental changes in the marine habitats due to climate change, anthropogenic activities and increasing presence of alien species (Coll et al., 2010; Occhipinti-Ambrogi & Galil, 2010; Vella et al., 2015 a & b; Vella et al., 2016 a & b) are posing additional threats to the Serranidae species in the central Mediterranean Sea. Therefore, to ensure effective conservation measures, the genetic identity and phylogenetics of the species around the Maltese Islands are useful to comparing these with similar species found elsewhere within and outside the Mediterranean region. Accurate genetic identification through the analyses of multiple genes can be used as molecular tools to assess the genetic identity and phylogenetic relationships between species, while subtle genetic differences found within each species can provide preliminary understanding on its phylogeographic distribution, thus identifying any distinct stocks or limited gene flow.

Materials and Methods

In this study, 43 specimens representing 10 species of the Family Serranidae (including *Anthias anthias*; *Cephalopolis taeniops*; *C. nigri*; *Epinephelus costae*; *E. marginatus*; *Hyporthodus haifensis*; *Serranus atricauda*; *S. cabrilla*; *S. hepatus*; and *S. scriba*) were collected from fisheries landings and from recreational fishermen catches. Specimens were identified down to the species level using morphological identification keys (Heemstra & Randall, 1993; Froese

& Pauly, 2016). DNA was extracted from 5 mg caudal fin clips using GF-1 Tissue DNA Extraction Kit (Vivantis Technologies) following the manufacturer's protocol. The two sequences analysed in this study were the partial cytochrome c oxidase subunit I (COI) gene which is homologous to the conventional gene used in DNA barcoding and the 5' end of cytochrome b (Cyt b) gene. The COI gene was amplified using the FISH-F1 and FISH-R1 primers as described in Ward et al., (2005), while Cyt b gene using primers A and C for all specimens except for *Anthias anthias* which was amplified through the use of A and D primers as described in Martin and Palumbi (1993). PCR products were sequenced in both directions using ABI3730XL and the forward and reverse sequence of each PCR product were assembled using Geneious R10 (http://www.geneious.com, Kearse et al., 2012). The resulting sequences of the native Serranidae species were deposited in GenBank under accession number KX925317-98.

The sequences were aligned using ClustalW within Geneious R10. Phylogenetic analyses were conducted via MEGA v7 (Kumar et al., 2016) using Kimura-2-parameter distance model (Kimura, 1980), with Maximum Likelihood and 1500 bootstraps were carried out for the COI, Cyt b and for the combined data. Molecular diversity indices (haplotype and nucleotide diversity) were estimated through the use of Arlequin v3.5. (Excoffier & Lischer, 2010).

COI sequences of *S. cabrilla* and *S. scriba* were mined from GenBank and BOLD, and used for phylogeographic analyses. Sixty-seven *S. cabrilla* and 26 *S. scriba* barcodes of known sampling locations were collected from these genetic databases. The sequences were integrated with data collected from current study to make a total of 79 *S. cabrilla* and 31 *S. scriba* barcodes. The length of the sequences considered for this part of the study was reduced to 455 bp, to match the smallest homologous sequence. Pairwise ϕ_{ST} values (100000 permutations using 0.05 level of significance) utilizing the K2P model were estimated using Arlequin v3.5. (Excoffier & Lischer, 2010). A Median Joining Network (Bandelt et al., 1999) was constructed using PopART (Leigh & Bryant, 2015) to analyse the association between the various haplotypes.

Results

Sequence-based classification

In this study, 602bp from COI and 378bp from Cyt b representing 200 amino acids and 126 amino acids respectively, were sequenced for each specimen. No insertions, deletions or stop codons were observed, consistent with functional protein coding genes, while all Cyt b sequences had the start codon ATG at position one. The 43 analysed specimens yielded 18 and 21 haplotypes for COI and Cyt b genes respectively, while a total of 24 different haplotypes were noted for the concatenated sequence of these two genes.

Analysis of the concatenated sequence of these two genes led to the identification of a distinct set of haplotypes for each native species (Figure 1). One haplotype being recorded for *E. costae*, *H. haifensis* and *S. scriba*; 2 haplotypes for *A. anthias*; 3 haplotypes for *E. marginatus* and *S. atricauda*; 4 haplotypes for *S. hepatus*; and 7 haplotypes for *S. cabrilla*. The nucleotide diversity individuals within species ranged from 0 in species exhibiting one haplotype to 0.0081 (±0.0046) in *S. cabrilla*. *S. hepatus* exhibited the highest degree of haplotypic diversity (Table 1).

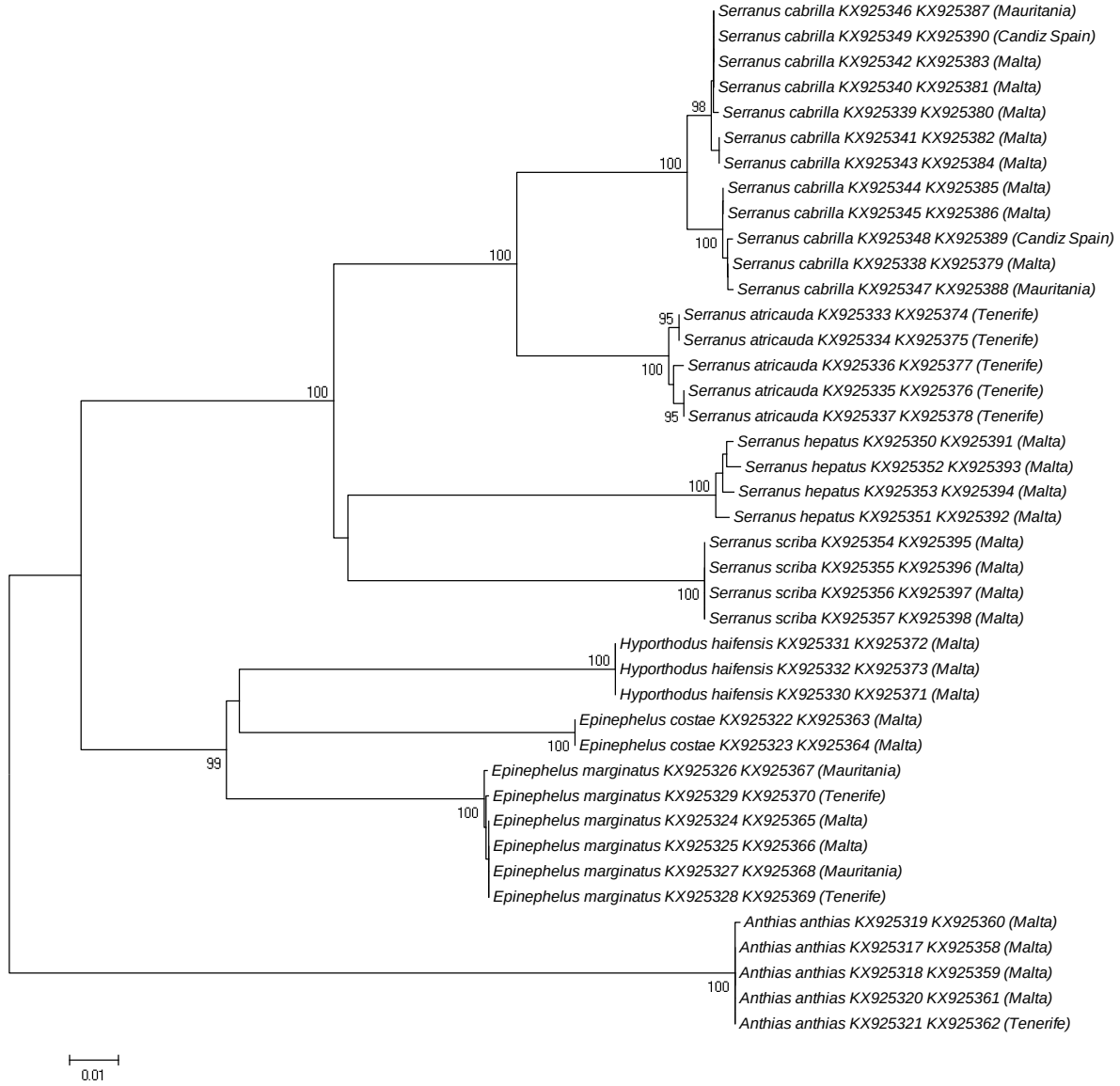

Figure 1. Maximum Likelihood tree of K2P distances of DNA barcodes from the 41 specimens representing the 8 native Serranidae species analysed in this study, using the concatenated data of the COI and Cyt b genes. The GenBank accession numbers represent COI and Cyt b sequences respectively. Bootstrap values ≥95% together with a K2P distance scale bar are included in the diagram.

Table 1. Molecular indices as estimated for the 43 analysed specimens. [N, number of specimens; *H*, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity; SE, standard error using the K2P distance model]

Species	Sampling site	N	coi			Cyt b			coi + Cyt b		
			H	*h*	π	*H*	*h*	π	*H*	*h*	π
ANTHIINAE											
Anthias anthias	Malta	4	1	0.000	0.00000	2	0.400 ±0.237	0.00106 ±0.00135	2	0.400 ±0.237	0.00041 ±0.00052
	Tenerife	1									
EPINEPHELINAE											
Epinephelus costae	Malta	2	1	0.000	0.00000	1	0.000	0.00000	1	0.000	0.00000
Epinephelus marginatus	Malta	2	2	0.333 ±0.215	0.00055 ±0.00073	2	0.333 ±0.215	0.00088 ±0.00116	3	0.600 ±0.215	0.00068 ±0.00069
	Mauritania	2									
	Tenerife	2									
Hyporthodus haifiensis	Malta	3	1	0.000	0.0000	1	0.000	0.0000	1	0.000	0.0000
Cephalopholis taeniops*	Malta	1	1	-	-	1	-	-	1	-	-
Cephalopholis nigri*	Malta	1	1	-	-	1	-	-	1	-	-
SERRANINAE											
Serranus atricauda	Tenerife	5	2	0.600 ±0.175	0.00199 ±0.00176	3	0.800 ±0.164	0.00688 ±0.00514	3	0.800 ±0.164	0.00388 ±0.00274
Serranus cabrilla	Malta	4	4	0.742 ±0.084	0.00783 ±0.00464	6	0.864 ±0.072	0.00838 ±0.00525	7	0.879 ±0.075	0.00813 ±0.00458
	Malta - offshore	4									
	Mauritania	2									
	Candiz	2									
Serranus hepatus	Malta	4	4	1.000 ±0.177	0.00443 ±0.00353	3	0.833 ±0.222	0.00706 ±0.00562	4	1.000 ±0.177	0.00544 ±0.00396
Serranus scriba	Malta	4	1	0.000	0.0000	1	0.000	0.00000	1	0.000	0.0000

* non-native Epinephelinae

Phylogeographic analyses of COI barcodes

The 79 *S. cabrilla* COI sequences coded for 18 different haplotypes, with 26 positions (out of the 455 bp analysed) showing genetic differences. A total of 22 sites exhibited transitions, while 5 sites exhibited transversions. One of the position exhibited both a transition and a transversion. Only four mutations led to differences in the amino acid sequences. The overall haplotype diversity was 0.775 ±0.063, with a nucleotide diversity of 0.0089 ±0.0053. The Median Joining Network has shown that the analysed haplotypes fall into two major clades (Figure 2). Clade 1 was predominantly found to contain specimens from Turkey, while specimens from other locations were more evenly distributed amongst the various haplotypes (Figure 2).

Pairwise analyses have shown significant ϕ_{ST} value differences between Turkish specimens and the other locations (Table 2). The major noted difference was observed between specimens from Turkey and those from the central Mediterranean that is Malta and Sicily, with the ϕ_{ST} between Turkey and Malta being 0.4868 ($P < 0.0001$), and the ϕ_{ST} between Turkey and Sicily being 0.4309 ($P = 0.0004$).

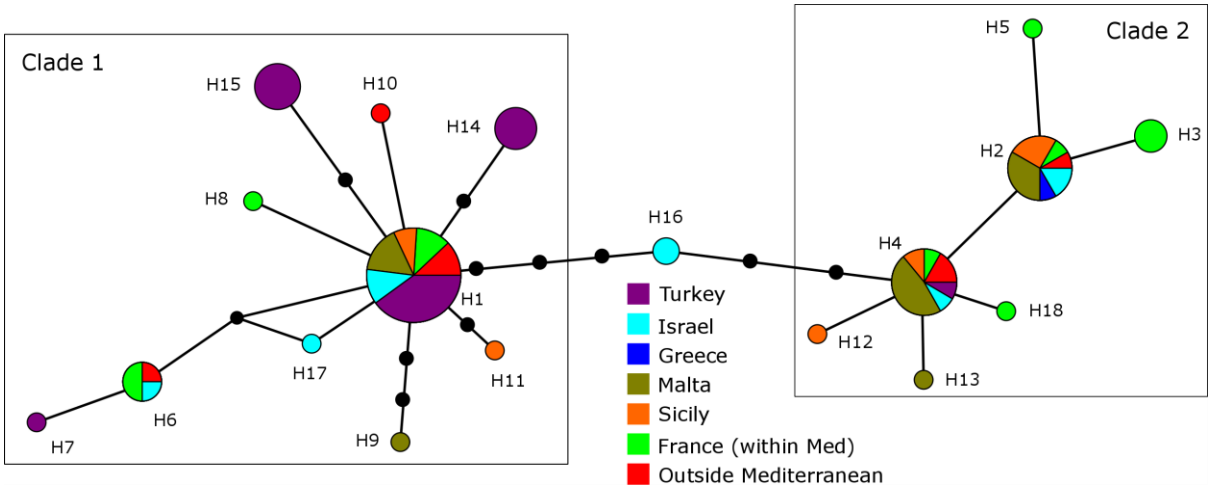

Figure 2. A Median Joining Network of 18 haplotypes of *Serranus cabrilla* using 455bp COI sequences. [Branches indicate one substitution even each. The black unlabelled circles represent inferred putative haplotypes that were not observed in this study.]

Table 2. Pairwise ϕ_{ST} values (below diagonal line) and P-vales (above diagonal line) for the ϕ-statistics of *Serranus cabrilla* between the various Mediterranean regions using the K2P distance model. The bold indicate combinations which exhibit significant differences using 0.05 level of significance.

Locations	Turkey *(N = 23)*	Israel *(N = 11)*	Malta *(N = 16)*	Sicily *(N = 8)*	France *(N = 12)*
Turkey *(5 haplotypes)*		**0.0009**	**< 0.0001**	**0.0004**	**0.0008**
Israel *(7 haplotypes)*	**0.2948**		0.2151	0.4689	0.8984
Malta *(5 haplotypes)*	**0.4868**	0.0356		0.8006	0.2790
Sicily *(5 haplotypes)*	**0.4309**	-0.0414	-0.0685		0.5451
France *(8 haplotypes)*	**0.2971**	-0.0721	0.0107	-0.0576	

A similar scenario was noted on *S. scriba*, where the 31 COI sequences analysed represented 5 haplotypes. Six sites exhibited genetic differences, with only one of them leading to an amino acid difference. Phylogeographic analyses of the data collected for this species has shown that three haplotypes were represented only by specimens from Turkey (H3, H4 and H5), while the other two haplotypes (H1 and H2) are predominantly represented by specimens

of central and western Mediterranean origin (Figure 3). A significant ϕ_{ST} value (ϕ_{ST} = 0.4703; *P*< 0.0001) was found through K2P pairwise analyses between the specimens from Turkey and those from central and western Mediterranean Sea (Table 3).

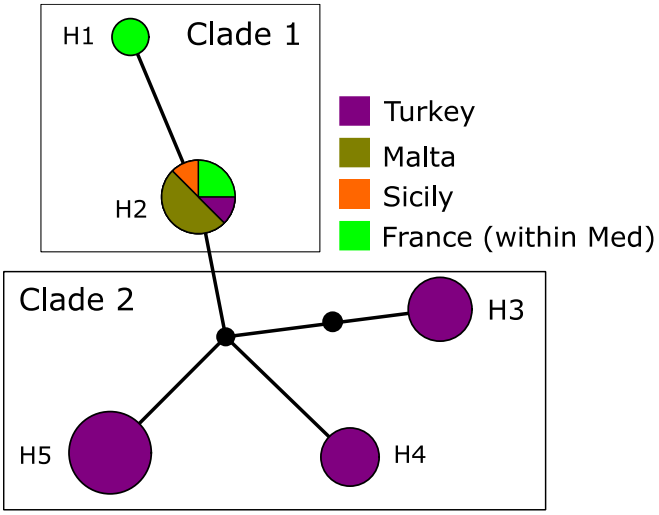

Figure 3. A Median Joining Network of 5 haplotypes of *Serranus scriba* using 455bp COI sequences [each hatch mark representing one mutation].

Table 3. Pairwise ϕ_{ST} values (below diagonal line) and *P* values (above diagonal line) for the ϕ-statistics of *Serranus scriba* between Turkey and the central & western Mediterranean Sea (CW Med representing specimens from Malta, Sicily and France) using the K2P distance model. The bold indicate that there is significant difference using 0.05 level of significance.

Locations	Turkey *(N = 22)*	CW Med *(N = 9)*
Turkey *(4 haplotypes)*		< **0.0001**
CW Med *(2 haplotypes)*	**0.4703**	

Discussion

This study has shown that each serranid species studied has a unique set of haplotypes and without exceptions, all species could be clearly discriminated with genetic differences within a species being less than 1%. This further confirmed the efficacy of COI and Cyt b in distinguishing species genetically. In addition to this, the phylogeographic analyses also gave first insights on the genetic population structure of *S. cabrilla* and *S. scriba* in the Mediterranean.

A Mediterranean-wide effort to scientifically monitor and protect all these species should be implemented for their conservation. Currently, in most countries, the main research focus is placed on the species, *E. marginatus*, due to its declining stocks and conservation status (Cornish and Hermelin-Vivien, 2011). *E. marginatus* is also considered a flagship species for

marine biodiversity conservation, including the protection of the other large grouper species. In the EU Council Regulation - EC 1967/2006, there is a minimum hook sizes for bottom long-lines and minimum mesh sizes for bottom-set gillnets, limiting the capture of small individuals by professional fishermen, while the 45cm minimum body length limit of this regulation also protects young groupers (Chapter V, Article 15, EUR-Lex, 2016). Additionally, in Malta through the same regulation in Article 8, there is the prohibition of the use of spear-guns by divers using underwater breathing apparatus (aqualung) or at night from sunset to dawn (Chapter IV, Article 8). Nonetheless, there are still threats due to the lack of continuous monitoring, especially IUU catches by hobby-fishermen, most of whom do not know or follow local regulations. Efforts to promote awareness of the regulations and the conservation needs of these ecologically important species are necessary. Moreover, other anthropogenic activities such as coastal habitat modification and pollution impose extra pressures on the populations of these species. Consequently, to effectively investigate and mitigate the threats, accurate species-specific data collection is essential.

Different species have different life-histories, survival requirements and vulnerabilities. Furthermore, species' populations or stocks may have unique necessities for their effective conservation and sustainable management. This preliminary set of results point toward evolving diversification that may also be the result of shrinking populations and fragmentation due to different levels of survival of the serranid species within and between marine protected areas in the Mediterranean (Andrello et al., 2013). For this reason, the current research work will expand to involve more serranid species and population studies for the various serranid species, such as the population genetic study undertaken for *E. marginatus* in Maltese waters by Buchholz-Sørensen and Vella (2016).

Acknowledgements

The research disclosed in this publication is funded by the University of Malta research grant, ERDF and the REACH HIGH Scholars Programme-Post Doctoral Grants. The latter may be part-financed by the European Union, Operational Programme II - Cohesion Policy 2014 - 2020 "Investing in human capital to create more opportunities and promote the well being of society" - European Social Fund. We would also like to acknowledge the assistance of Ms. C. Costa during the sampling work in Tenerife.

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Supplementary material

S1 – The COI sequences used for the phylogeographic analyses of *Serranus cabrilla* and *Serranus scriba*. [Codes mined from genetic databases (*italics*) and those from current study (**bold**)]

Serranus cabrilla

Record ID / Accession Number	Database
5279492	*BOLD*
5282535	*BOLD*
5283388	*BOLD*
5283389	*BOLD*
5283403	*BOLD*
5284371	*BOLD*
5288018	*BOLD*
FN688968	*GenBank*
FN689015	*GenBank*
FN689054	*GenBank*
FN689277	*GenBank*
FN689278	*GenBank*
FN689279	*GenBank*
FN689280	*GenBank*
FN689281	*GenBank*
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FN689288	*GenBank*
JQ623994	*GenBank*
KC501453	*GenBank*
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KC501461	*GenBank*
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KC501468	*GenBank*
KC501469	*GenBank*
KC501470	*GenBank*
KC501471	*GenBank*
KC501472	*GenBank*
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KJ709627	*GenBank*
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KJ709629	*GenBank*
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Record ID / Accession Number	Database
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FN689017	*GenBank*
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