Zoologica Scripta

KUNGL. VETENSKAPS-AKADEMIEN
THE ROYAL SWEDISH ACADEMY OF SCIENCES

Cryptic diversity and species assignment of large lantern sharks of the *Etmopterus spinax* clade from the Southern Hemisphere (Squaliformes, Etmopteridae)

Nicolas Straube, Jürgen Kriwet & Ulrich Kurt Schliewen

Submitted: 30 April 2010
Accepted: 12 September 2010
doi:10.1111/j.1463-6409.2010.00455.x

Straube, N., Kriwet, J. & Schliewen, U. K. (2010). Cryptic diversity and species assignment of large lantern sharks of the *Etmopterus spinax* clade from the Southern Hemisphere (Squaliformes, Etmopteridae). — *Zoologica Scripta*, *00*, 000–000.

Many species of the speciose deep-sea shark family Etmopteridae (lantern sharks) are a regular by-catch component of deepwater trawl and longline commercial fisheries. As for many elasmobranchs, the low fecundity, late sexual maturation and extreme longevity of the lantern sharks increase their susceptibility to overfishing. However, the taxonomic uncertainty within etmopterids and the poorly known patterns of dispersal of these shark species hampers the establishment of reasonable monitoring efforts. Here, we present the first molecular approach to clarify the taxonomy and distribution of a morphologically uniform group of lantern sharks comprising *Etmopterus granulosus* and closely related congeners by using nucleotide sequence data from the mitochondrial DNA cytochrome oxidase I gene and amplified fragment length polymorphisms. Samples were collected from several locations in the Southern Hemisphere, where the species occur. Our analyses reveal a high level of cryptic diversity. *E. granulosus* is not endemic to Chile, but instead has a widespread distribution in the Southern Hemisphere being synonymous to New Zealand *Etmopterus baxteri*. Conversely, specimens previously assigned to *E. baxteri* from off South Africa apparently represent a distinct species. Our results provide the basis for the redescription of *E. granulosus* and *E. baxteri* which will help in the establishment of useful monitoring and management strategies.

Corresponding author: *Nicolas Straube*, *Zoologische Staatssammlung München*, *Sektion Ichthyologie*, *Münchhausenstrasse 21*, *81247 München*, *Germany*. *E-mail: straube@zsm.mwn.de*
Jürgen Kriwet, *Department of Paleontology*, *University of Vienna*, *Geozentrum*, *Althanstrasse 14*, *1090 Vienna*, *Austria*. *E-mail: juergen.kriwet@univie.ac.at*
Ulrich Kurt Schliewen, *Zoologische Staatssammlung München*, *Sektion Ichthyologie*, *Münchhausenstr. 21*, *81247 Munich*, *Germany*. *E-mail: schliewen@zsm.mwn.de*

Introduction

Deep-sea fishes in general, and deep-sea sharks in particular, are suspected to be highly vulnerable to recently expanding commercial deep-sea fisheries due to their extreme longevity, slow growth, late maturation and small litter sizes (Devine *et al.* 2006; Forrest & Walters 2009). Unfortunately, assessment of species-specific conservation needs is difficult as very little is known about the distribution and population genetics of deep-sea sharks, and because commercial fisheries and conservation efforts are usually focused on more valuable and productive teleost fishes (Bonfil 1994; Forrest & Walters 2009). The problem is made worse by the taxonomic uncertainty that often does not allow for the collection of accurate species-specific catch data. A recent study by Iglésias *et al.* (2009) has highlighted problems arising from the lack of accurate species identification of the commercially targeted skate species *Dipturus batis* and *Dipturus oxyrinchus*, whose landings data in fact comprises five distinct species. Mislabelling of specimens and hence incorrect monitoring data resulted in a dramatic decline of once common species increasing the risk of extinction (Iglésias *et al.* 2009). Deep-sea luminescent sharks of the squaliform genus *Etmopterus* are not directly targeted by commercial fisheries, but are a significant by-catch component of deep-sea fisheries (Clarke *et al.* 2005; Compagno *et al.* 2005; Jakobsdottir 2001; Wetherbee 1996, 2000). Despite being caught 'only' as by-catch, benthic and bentho-pelagic etmopterids are likely strongly affected by deep-sea fisheries targeting other species. Several lantern sharks are locally endemic to

small areas and hence may be especially vulnerable to overfishing. Another factor that has been shown to increase susceptibility to overfishing in the deep sea is that species are long-lived and late reproducing (Devine *et al.* 2006). Preliminary age estimates suggest *Etmopterus baxteri* to reach maturity between 10 and 20 years for males and 11.5 to 30 years for females (Irvine *et al.* 2006). In addition, several species are known to form sex and size specific aggregations (Jakobsdottir 2001; Wetherbee 1996). Some lantern sharks are only found regionally while others are distributed worldwide. For instance, the world's smallest shark species, *Etmopterus perryi* and *Etmopterus carteri*, are both considered endemic to a narrow stripe of the Caribbean coast of Colombia (Springer & Burgess 1985). In contrast, *Etmopterus pusillus* and *Etmopterus lucifer* are distributed almost circumglobally (Compagno *et al.* 2005). Contrary to highly migratory elasmobranchs such as *Isurus oxyrinchus* (Schrey & Heist 2003), *Rhincodon typus* (Castro *et al.* 2007), *Carcharodon carcharias* (Bonfil *et al.* 2005; Boustany *et al.* 2002) or the more closely related *Squalus acanthias* (McFarlane & King 2003; Verrissimo *et al.* 2010), lantern sharks are not known to undergo large scale migrations. However, migrations may occur to distinct spawning and mating grounds as indicated by the presence of size-related and sex-related aggregations (Forrest & Walters 2009; Jakobsdottir 2001; Wetherbee 1996). Consequently, assessment of by-catch impact for narrow endemics vs. wide spread and potentially migrating taxa need reliable data for correct species identification, which in turn allow to asses conservation relevant issues of their life history, ecology and distribution.

Among lantern sharks that are potentially most affected by deep-sea fisheries, the alpha-level taxonomy of the *Etmopterus spinax* clade (Straube *et al.* 2010) is particularly difficult. Species of this clade are distributed worldwide and comprise *E. spinax*, *Etmopterus princeps*, *Etmopterus dianthus*, *Etmopterus unicolor*, *Etmopterus granulosus*, and *E. baxteri*. Straube *et al.* (2010) further suggested the inclusion of *Etmopterus hillianus* and *Etmopterus litvinovi* as well as the undescribed *Etmopterus* sp. B. Although some species of the clade are morphologically distinguishable using the shape of bioluminescent flank markings such as *E. spinax*, and *E. dianthus*, others are not (e.g. *E. granulosus*, *E. unicolor*, *E. princeps*, and *E. baxteri*). The taxonomy and distribution of the Southern lantern shark, *E. granulosus* (Günther 1880), is controversial. The species is listed in the IUCN (2010) Red List of Threatened species as endemic to Chile. However, a very similar species described from New Zealand, *E. baxteri* (Garrick 1957), was synonymized with *E. granulosus* based on morphological data (Tachikawa *et al.* 1989). Despite Tachikawa *et al.* (1989) study, taxonomic uncertainty about the species status of different populations of lantern sharks broadly referable to either *E. granulosus* or *E. baxteri* has remained, as reflected in the inconsistent usage of both species names in the most recent taxonomic shark literature. For instance, *E. baxteri* and *E. granulosus* are either accepted as two distinct species (Compagno *et al.* 2005; Last & Stevens 2009) or mentioned as *E. granulosus* comprising different populations (Forrest & Walters 2009; Wetherbee 1996, 2000). Both species are considered as 'least concern' in the IUCN (2010) Red List of Threatened Species.

Catch records of *E. granulosus*-like specimens from off South Africa, South America, Australasia, New Zealand and the Kerguelen Plateau are doubtful with regard to correct species assignment, as cryptic diversity has not been analysed in detail so far (IUCN Red List 2010). Phylogenetic analyses based on nuclear and mitochondrial DNA sequences including several *E. granulosus*-like specimens from Chile, the Tasman Sea, New Zealand, South Africa and the Kerguelen Plateau did not provide a fine-grained resolution to the species status problem, but highlighted the paraphyly and cryptic diversity within the *E. spinax* clade (Straube *et al.* 2010).

Here, we provide the first phylo- and population-genetic analysis investigation of the cryptic diversity among a group of deep-sea sharks with a still unresolved taxonomic background that is potentially affected by fisheries targeting shrimp and Orange Roughy (Wetherbee 1996; IUCN 2010). We included all available *E. granulosus/E. baxteri*-like specimens from the Southern Hemisphere to critically test for sympatric and allopatric diversity among specimens recorded as *E. granulosus* or *E. baxteri*. We tested for assignation of all individuals to discernable genetic clusters, i.e. potential species or populations. The results are used to provide information on population structure of *E. granulosus*, which is the basis for adequate conservation measures and estimating the cryptic diversity among specimens assigned previously to *E. granulosus* and *E. baxteri*, respectively. We further used our data to re-analyse the phylogenetic interrelationships of the *E. spinax* clade for establishing an improved resolution of the clade.

Material and methods

Sampling

Tissue samples from fresh or frozen specimens from the Southern Hemisphere of *E. granulosus sensu* Compagno *et al.* 2005 (n = 13, Chile), *E. baxteri sensu* Last & Stevens 2009 (n = 24, New Zealand), *E. baxteri sensu* Compagno *et al.* (1991) (n = 8, South Africa), *E.* cf. *baxteri* (n = 1, Amsterdam Island), *E.* sp. B *sensu* Last & Stevens 1994 (n = 6, Norfolk Ridge), *E. granulosus* (n = 1, NE of the Kerguelen Plateau), and *E.* cf. *granulosus sensu* Duhamel *et al.* 2005 (n = 9, New Zealand and Kerguelen Plateau),

were preserved in 96% ethanol. In addition, cytochrome oxidase I (COI) sequences from Genbank [n = 5 specimens of *E.* cf. *unicolor sensu* Ward *et al.* 2008 from off Indonesia (accession numbers EU398778, EU398779, EU398780, EU398781, EU398782)] and n = 2 specimens of *E. granulosus sensu* (Ward *et al.* 2008) from the Tasman Sea (accession numbers DQ108226, DQ108216) were included. Further, samples from the Northern Hemisphere were analysed in order to test for refined phylogenetic resolution of the entire *E. spinax* clade *sensu* Straube *et al.* (2010), i.e. specimens of *E. unicolor* (n = 3, North-West Pacific), *E. princeps* (n = 3, North-East Atlantic), *E. spinax* (n = 3, North-East Atlantic), and *Etmopterus brachyurus* (n = 3, Japan, North-West Pacific). *E. brachyurus* was chosen as outgroup as it is the most closely related taxon to the *E. spinax* clade (Straube *et al.* 2010), for which high quality DNA was available. For a summary of all specimens analysed in this study see Supporting Information S1, for sampling locations see Fig. 1.

DNA extraction, sequencing and phylogenetics

Total genomic DNA was extracted from muscle tissues using the QIAmp tissue kit (Qiagen®, Valencia, CA, USA). The mitochondrial COI gene was sequenced (655 bp) as it is a well-established gene fragment for identification of shark species (Ward *et al.* 2005, 2007). The COI sequences were amplified using primers S0156 (5′-TAGCTGATGAATCTGACCGTGAAAC-3′) and R0084 (5′-TGAACGCCAGATTTCATAGCGTTC-3′) following the PCR protocol of Iglésias *et al.* (2005). The PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen®) following the manufacturer's protocol. Cycle sequencing was performed at the sequencing service of the Department of Biology of the Ludwig Maximilian University (Munich), using ABI Big Dye 3.1 chemistry (PE Applied Biosystems®, Foster City, CA. USA).

Sequences were edited using the BioEdit software version 7.0.9 (Hall 1999) and aligned with MUSCLE v3.6 (Edgar 2004). Check of COI sequences against nuclear pseudogene status was done by searching for stop codons and by translating sequences into amino acids. Ambiguous sites in nucleotide sequences, attributed to double peaks in the electropherogram, were coded referring to IUB symbols. The software NETWORK v4.5.1.6 (fluxus-engineering.com) was applied to the smallest resulting sequenced fragments homologous to all taxa. The final alignment had 659 bp and was used as the basis to reconstruct most parsimonious phylogenetic networks (Bandelt *et al.* 1999). The network was calculated using the median joining algorithm (allowing for multistate data) under default settings (weights = 10, epsilon = 0).

Genotyping and subsequent analyses

We genotyped amplified fragment length polymorphisms (AFLPs), (Vos *et al.* 1995; Meudt & Clarke 2007) as a basis for model based clustering methods and assignment of individuals to genotypic clusters. The AFLP dataset differs from the mtDNA data by the exclusion of 16 specimens [*E.* cf. *granulosus* (n = 3), *E.* cf. *baxteri* (n = 1), *E. granulosus* (n = 3), *E.* sp. B (n = 1), *E. brachyurus* (n = 3), *E. unicolor* (n = 1), *E.* cf. *unicolor* (n = 5)], which could not be amplified or for which highly genomic DNA was not available.

Methods for AFLP genotyping (restriction/ligation/primary amplification) follow Herder *et al.* (2008). The following restrictive primer combinations, based on the core sequences provided in Vos *et al.* (1995) (EcoRI: 5′-CTCGTAGACTGCGTACC; MseI: 5′-GAC

Fig. 1 Sampling sites of specimens used in this study.

GATGAGTCCTGAG), were used: EcoRI-AGG/MseI-CTG, EcoRI-ACA/MseI-CAA, EcoRI-ACA/MseI-CTG, EcoRI-ACT/MseI-CAA, EcoRI-AGG/MseI-CTC, EcoRI-ACC/MseI-CTA, EcoRI-ACT/MseI-CAG, EcoRI-ACC/MseI-CAT, EcoRI-AGG/MseI-CTA, EcoRI-ACA/MseI-CAT, EcoRI-ACT/MseI-CTG, EcoRI-ACC/MseI-CAG, EcoRI-ACT/MseI-CTT, EcoRI-AGC/MseI-CTC, EcoRI-AGG/MseI-CAA, EcoRI-AGC/MseI-CAC, EcoRI-AGG/MseI-CTT, EcoRI-AGC/MseI-CAG, EcoRI-ACT/MseI-CAC, EcoRI-ACC/MseI-CTC.

Capillary electrophoresis was conducted on an ABI 3130 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) with an internal size standard (ROX 500 XL). Binary character matrices were produced from each primer combination using automated peak scoring (binning) in the GeneMapper® Software v4.0 (PE Applied Biosystems, Foster City, CA, USA). Quality of runs were checked by eye and repeated if necessary. For each primer, a range of 50–499.5 bp was analysed. For optimizing automated AFLP scoring, peak height threshold was set to 50 relative fluorescent units (RFU), and bin width was set to 0.75 bp. The option of 'light' smoothing was chosen, the Local Southern Method was evaluated as size calling method and common alleles were deleted from the matrix. Each run included six replicate samples to detect and delete inconsistently produced fragments. Each single matrix resulting from the 20 different primer combinations was further corrected by removing all pairs of neighbouring bins in which the minimum distance between them was less than 0.25 bps, as well as those bins containing fragments differing by more than 0.65 bps in size (Albertson *et al.* 1999). For comparison with the mtDNA sequence data, a neighbor-joining network was calculated using the software Splitstree4 v4.10 (Huson & Bryant 2006). PAST v1.94b (Hammer *et al.* 2001) allowed visual inspection of principal components after principal component analysis (PCA) of the combined data set. For phylogenetic inferences based on neighbor-joining distances of AFLP data we used the Link *et al.* (1995) algorithm as implemented in the software package TreeCon v1.3b (Van de Peer & De Wachter 1994) with a subsequent bootstrap analysis comprising 2000 replicates. The algorithm by Link *et al.* (1995) uses shared and present bands only, while absent bands are not included in analyses. This is important for AFLP data because the absence of a band in the final data matrix may have more reasons as compared with the presence of a band.

Adopting results of previous analyses and hence accepting *E. granulosus* being a synonym to New Zealand *E. baxteri*, the software package Arlequin v3.5 (Excoffier & Schneider 2005) was employed to conduct analyses of molecular variance (AMOVA) to evaluate the amount of population genetic structure of *E. granulosus* between the two sampling locations New Zealand and Chile and to estimate pairwise F_{ST} values. The AFLP data set of *E. granulosus* was further analysed with BAYESCAN (Foll & Gaggiotti 2008) to identify loci which are under selection and are therefore strongly affecting population structuring. Subsequently, the AMOVA was re-run without the loci identified by BAYESCAN as contributing the most for the population structure to test for changes in the percentage variation and pairwise F_{ST}. For comparison, pairwise Φ_{ST} values were computed in Arlequin for the mtDNA (COI) sequence data including two separate groupings to explore differentiation of *E. granulosus* from Chile and specimens from New Zealand with 10 000 permutations and a significance level of 0.01 using haplotype frequencies only.

STRUCTURE v2.2.3 (Pritchard *et al.* 2000; Falush *et al.* 2003) was used to calculate model based genotypic clusters and to assign individuals to genotypic clusters (populations). We treated AFLP loci as either being present (i.e. di-allelic), or as missing as recommended by Falush *et al.* (2007) for dominant markers. To detect population structure according to a hierarchical model, we followed methodologically Evanno *et al.* (2005), testing numbers of populations from $K = 1$ to $K = 12$. Each test was performed 15 times with a burn-in of 75 000 generations and following 2 00 000 Markov chain Monte Carlo (MCMC) generations, respectively after exploratory preruns to estimate convergence of likelihoods with different burn-ins and MCMC generations. The allelic frequency was set to 1. We applied the admixture model and the allele frequency model assuming correlated allelic frequencies as recommended in the user's manual. The mean ln of likelihoods of 15 runs for each K was used to estimate the true number of K by computing ΔK following Evanno *et al.* (2005). A second analysis focused on a smaller dataset including only specimens assigned to *E. granulosus* from Chile and *E. baxteri* from New Zealand as no population structure was detected between the two sampling locations within the full dataset (as e.g. in Warnock *et al.* 2009). The smaller dataset removes part of the variance of the full dataset which may reveal subtle population structure. STRUCTURE v2.3.1 runs were repeated twice, excluding and including prior location information as informative prior settings (Hubisz *et al.* 2009).

Due to the high morphometric similarity of *E. granulosus* specimens with those previously assigned to *E. baxteri* sampled off South Africa and due to a potential Northern Hemisphere origin of the Southern Hemisphere *E. granulosus*, STRUCTURE v2.3.2 *beta* was used to test for a mixed ancestry of *E. princeps*, *E. granulosus* and specimens assigned to *E. baxteri* sampled off South Africa. To test for patterns of mixed ancestry among individuals of the three

groups, we used the program option using putative prior information on population origin and a defined number of past generations (GENSBACK subpackage of STRUCTURE). In our case, the implemented model translates into the assumption that the largest part of individuals assigned to *E. baxteri* from South Africa is genotypically differentiable and that a small portion of individuals may have a mixed ancestry of the species specific genotypes of *E. granulosus* and/or *E. princeps* from the North Atlantic (Falush *et al.* 2007). We did so by using settings of GENSBACK between two and four past generations and a fixed number of $K = 3$ as derived from our prior analyses, i.e. representing *E. granulosus* from off Chile and New Zealand, *E. princeps* from the North Atlantic, and specimens assigned to *E. baxteri* sampled off South Africa. MIGPRIOR was set to 0.001 using the admixture model as suggested by Falush *et al.* 2007, and 1 50 000 MCMC generations with a burnin of 50 000 generations for each run were performed.

Results

Phylogenetics

mt DNA. The COI alignment has 541 constant characters plus 17 variable characters, which are parsimony-uninformative and 101 characters which are parsimony-informative. Base frequencies are equally distributed in all positions (chi-square test: $\chi^2 = 34.42$, d.f. = 201, $P = 1.0$). Empirical base frequencies are 0.26 for A, 0.25 for C, 0.18 for G, and 0.31 for T. Altogether 63 haplotypes were

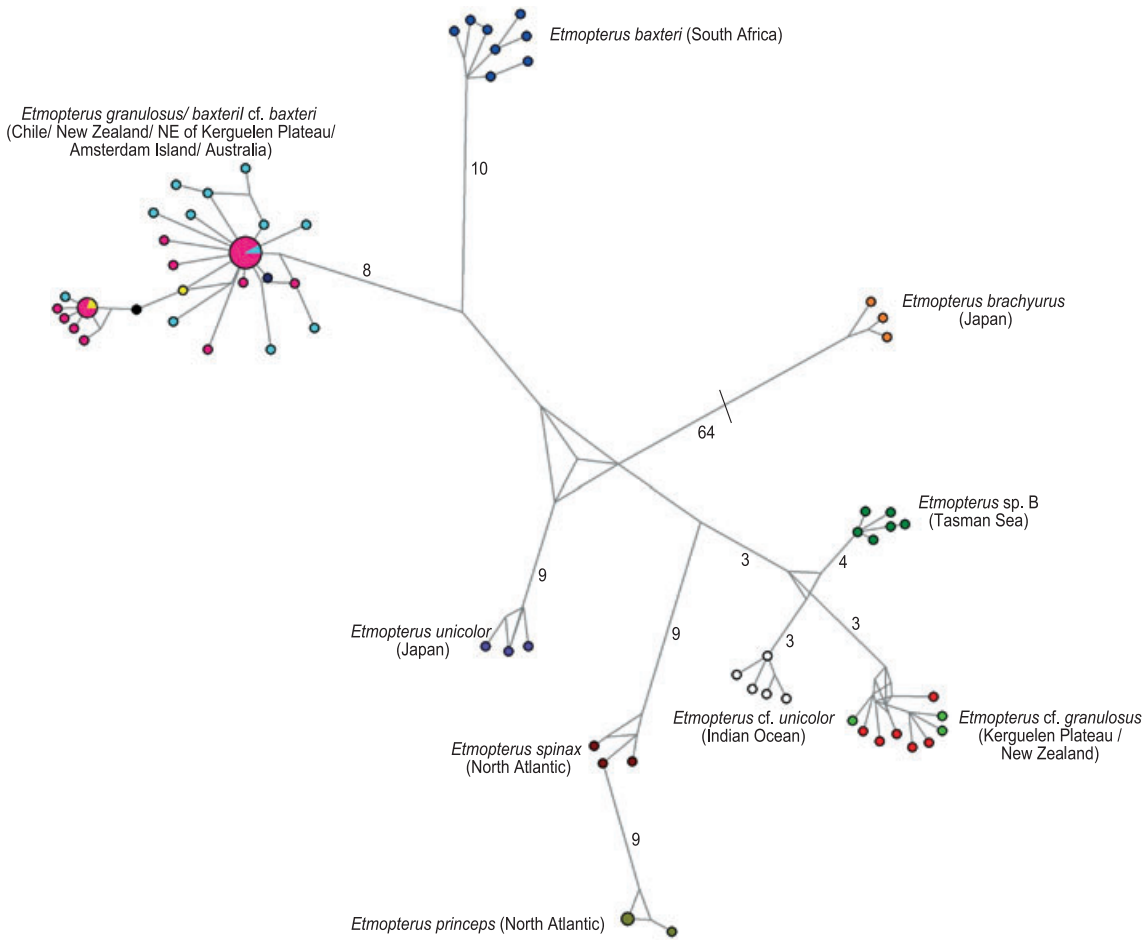

Fig. 2 Most parsimonious haplotype network structure attained from cytochrome oxidase I sequences (mitochondrial DNA). Numerals above branches indicate the number of mutated positions. Branches without numbers show two or less mutated positions. Pink = *Etmopterus baxteri* (New Zealand). Turquoise = *Etmopterus granulosus* (Chile). Yellow = *E. granulosus* (Tasman Sea). Black = *E.* cf. *baxteri* (Amsterdam Island). Blue = *E. baxteri* (South Africa). Purple = *Etmopterus unicolor* (Japan). Orange = *Etmopterus brachyurus* (Japan). Dark red = *Etmopterus spinax* (North Atlantic). Olive = *Etmopterus princeps* (North Atlantic). Dark green = *Etmopterus* sp. B (Norfolk Ridge). Red = *E.* cf. *granulosus* (New Zealand). Green = *E.* cf. *granulosus* (Kerguelen Plateau). White = *E.* cf. *unicolor* (Indian Ocean). Dark blue = *E. granulosus* (NE of Kerguelen Plateau).

detected, and the estimate for mutations steps for the shortest network is 328. The most parsimonious network identifies nine major monophyletic clusters, i.e. *E. spinax* (NE Atlantic), *E. princeps* (NE Atlantic), *E.* cf. *granulosus* (*sensu* Duhamel *et al.* 2005; Kerguelen Plateau & New Zealand), *E.* sp. B (*sensu* Last & Stevens 1994; Norfolk Ridge), *E. unicolor* (Japan), *E. brachyurus* (Japan), *E. baxteri* (*sensu* Compagno *et al.* 2005; South Africa), *E.* cf. *unicolor* (Ward *et al.* 2008; Indonesia), and *E. granulosus–E. baxteri* (Chile and New Zealand). Within the latter cluster there is no apparent lineage sorting between *E. granulosus* from Chile (close to the type locality of *E. granulosus*) and *E. baxteri* from New Zealand (close to the type locality of *E. baxteri*) according to location or preliminary species assignment. In contrast, specimens of *E. baxteri* sampled off South Africa form a distinct cluster (Fig. 2).

AFLP data. The AFLP scoring resulted in a binary matrix comprising 2655 loci in 68 specimens.

A neighbor-joining network calculation based on AFLP data (Fig. 3) identified the same eight major clusters retrieved by the network using mtDNA data (Fig. 2). The *E. baxteri* (New Zealand) and *E. granulosus* (Chile) cluster together. The *E. baxteri* (South Africa) forms a distinct cluster along with *E. spinax*, *E. princeps*, *E.* cf. *granulosus*, *E.* sp. B, *E. unicolor* and *E. brachyurus*.

For phylogenetic inferences of the *E. spinax* clade, a neighbor-joining tree was calculated from AFLP data. All specimens sampled in the Southern Hemisphere constitute a monophyletic group (Fig. 4). Its basal sister clade comprises specimens of *E. princeps* from the North Atlantic. *E. princeps* (NE Atlantic) and the Southern Hemisphere species are sister to *E. spinax* (NE Atlantic). The monophyletic lineage is sister to *E. unicolor* (NE Pacific). Again, there is no species delimitation between *E. baxteri* sampled off New Zealand and *E. granulosus* sampled off Chile, which are sister to *E.* cf. *granulosus* and *E.* sp. B. Specimens assigned to *E. baxteri* from South Africa form a distinct clade sister to a clade comprising *E. granulosus* (Chile)/*E. baxteri* (New Zealand), *E.* sp. B and *E.* cf. *granulosus*. Bootstrap support is high for all clades, lower bootstrap support values are found at nodes explaining the interrelationships of the Southern Hemisphere clade.

Population genetics

PCA. The PCA computed from the AFLP dataset reveals five clusters when plotting principal component (PC) 1 against PC2 (Fig. 5A), i.e. one for *E. granulosus* (Chile) and *E. baxteri* (New Zealand) and one for *E.* cf. *granulosus* and *E.* sp. B. Specimens assigned to *E. baxteri* from South Africa form a third cluster. Finally, the two specimens of *E. unicolor* from Japan and the *E. baxteri* specimens from South Africa each plot as separate but neighbouring groupings. *Etmopterus spinax* (NE Atlantic) and *E. princeps* (NE Atlantic) form additional clusters (Fig. 5A). The PCs 1 and 2 explain 22.9% of the total variance, the variance evenly decreases with increasing PCs. Plotting PCs 1 and 3, *E.* cf. *granulosus* and *E.* sp. B form distinct cluster (20.3% of variance explained, Fig. 5B). The same applies to comparison of PCs 2 and 3 (8.78% of variance explained), whereas *E. granulosus* and *E. baxteri* always broadly overlap independent of PC comparison. Subsequent plotting of PCs 1 & 2 (11.52% of total variance), 1 & 3 (10.77% of total variance), and 2 & 3 (9.49% of total variance) using AFLP data

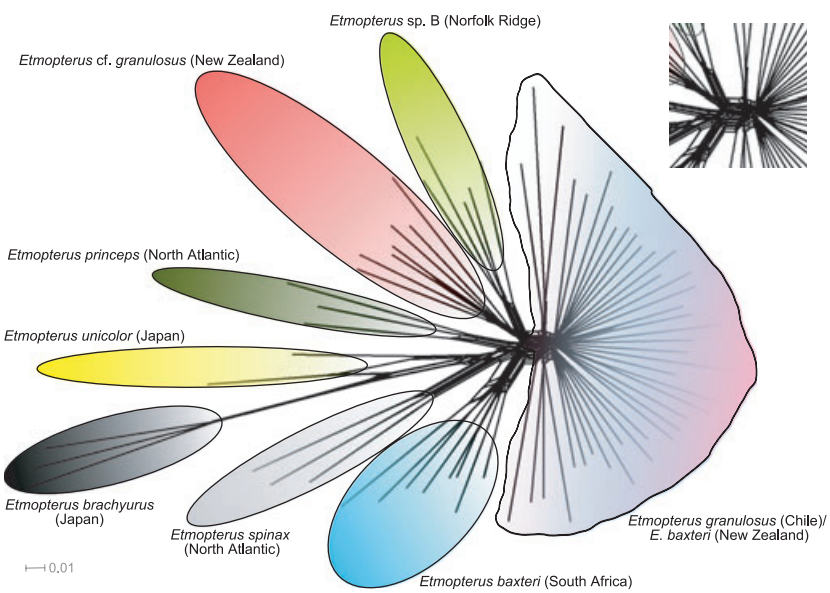

Fig. 3 Neighbor network structure attained from amplified fragment length polymorphism genotyping based on the algorithm by Link *et al.* (1995). Conflicting phylogenetic signal in the centre magnified top right.

of *E. granulosus* and *E. baxteri* showed again strong overlap of both species. Conversely, plotting PCs 1 and 2 (explaining 30.71% of total variance) of *E.* cf. *granulosus* against *E.* sp. B, specimens form distinct clusters (data not shown). There are no differences of *E. granulosus* (Chile) and *E. baxteri* (New Zealand), whereas specimens assigned to *E. baxteri* from South Africa form a distinct cluster, not overlapping with *E. baxteri* (New Zealand) and *E. granulosus* (Chile), respectively. The *E.* cf. *granulosus* and *E.* sp. B seem closely related, but form distinct clusters, if PCs 1 and 3 as well as PCs 2 and 3 are compared.

F-*statistics*. The F_{ST} value between *E. granulosus* (Chile) and *E. baxteri* (New Zealand) was estimated using AFLP data to assess the degree of genetic differentiation between the two groups. The percentage of variation is 2.43% among populations, whereas it is 97.57% within populations on a highly significant level ($P < 0.01$). Pairwise difference between both locations show a low but significant F_{ST} ($F_{ST} = 0.024$, $P < 0.01$) (Table 1).

BAYESCAN identified no loci as decisive factors for population structuring, assuming a posterior probability of 0.99 to 1.00 [Bayes factors (BF) = 99 and increasing] as threshold for identifying loci which are under strong selection and therefore cause population structuring. Decreasing the threshold to a posterior probability from of 0.99 to 0.72 (BF = 3) only reveals one locus as strongly selected. Further decreasing the posterior probability show a second locus at $P = 0.68$ ranging in the field of 'barely worth mentioning' loci under population shaping selection (Foll & Gaggiotti 2008). This indicates the absence of loci which account for population structure of the two locations New Zealand and Chile. Excluding those two loci and re-running an AMOVA in Arlequin slightly decreased the percentage of among population variation to 2.19%, and rose the variation within populations to 97.81%,

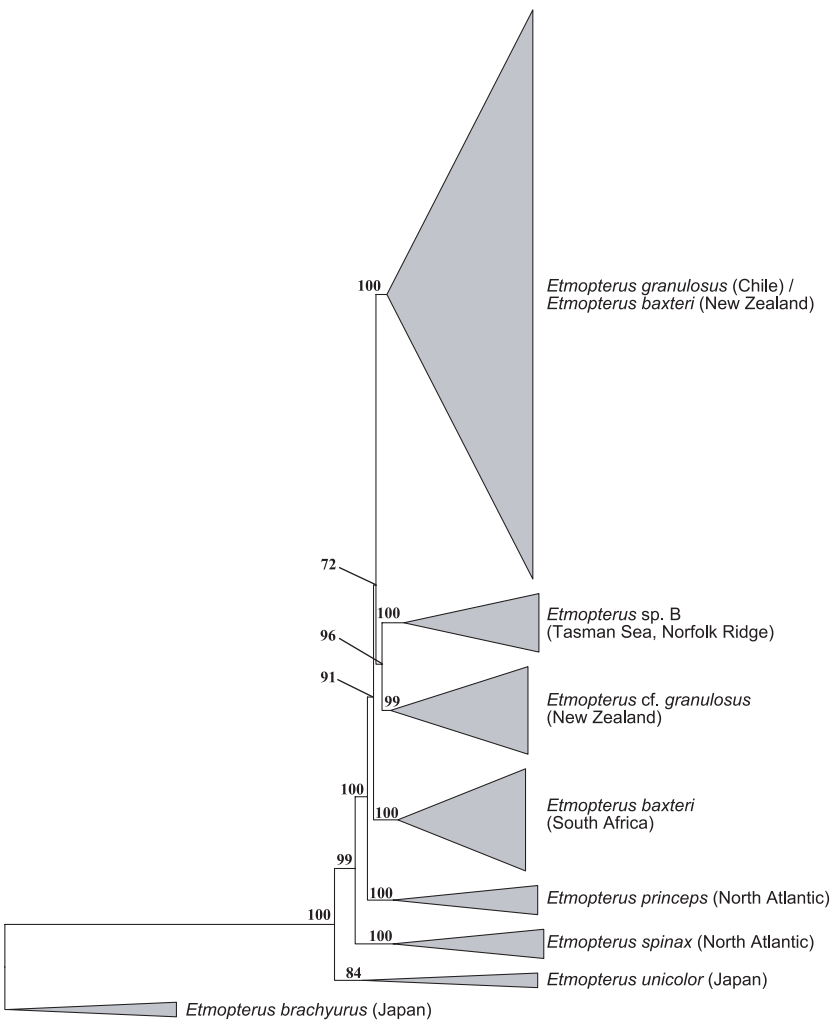

Fig. 4 Neighbor-joining tree calculated from amplified fragment length polymorphism data with bootstrap support values above nodes computed from 2000 bootstrap replicates. Main clusters are summarized to ease visualization.

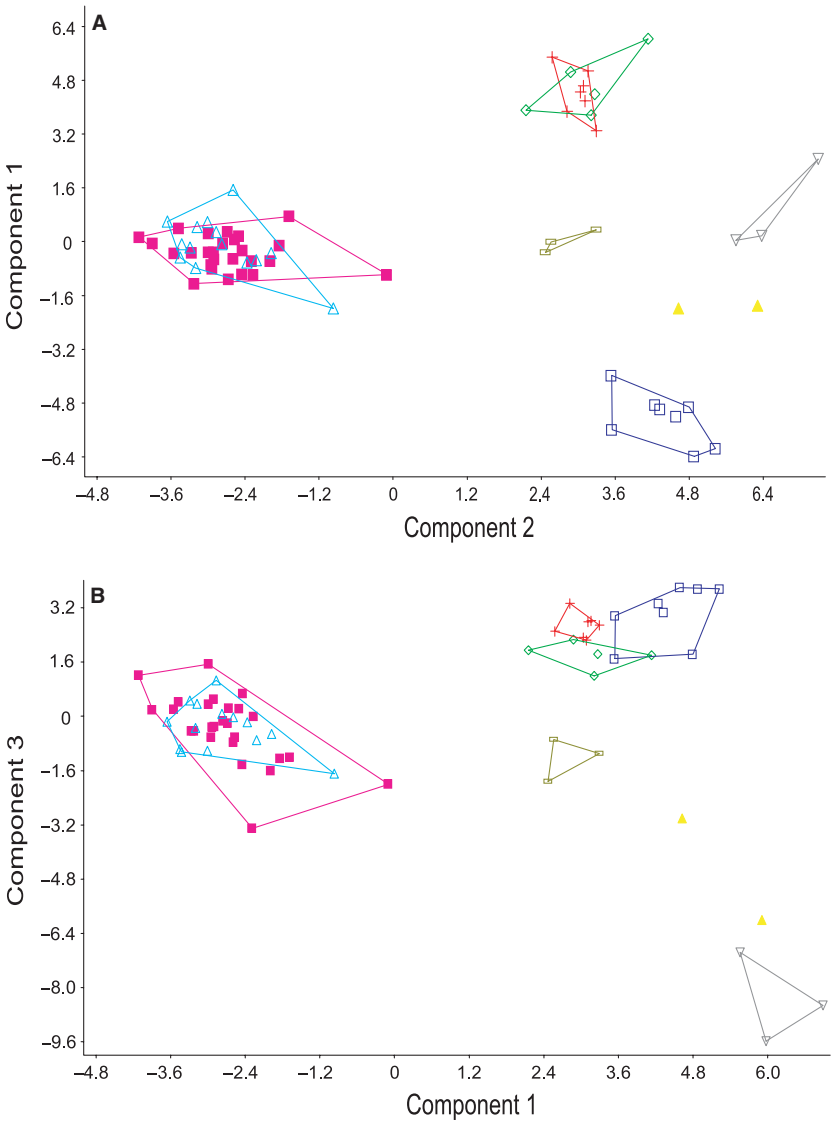

Fig. 5 Scatter plot from principal component (PC) analysis comparing PCs 1 & 2 (A) and PCs 1 & 3 (B) based on amplified fragment length polymorphism data. Filled squares = *Etmopterus baxteri* (New Zealand). Empty squares = *E. baxteri* (South Africa). Empty triangles = *Etmopterus granulosus* (Chile). Filled triangles = *Etmopterus unicolor* (Japan). Crosses = *E.* cf. *granulosus* (New Zealand). Diamonds = *Etmopterus* sp. B (Norfolk Ridge). Headstanding triangles = *Etmopterus spinax* (North Atlantic). Rectangles = *Etmopterus princeps* (North Atlantic).

Table 1 Percentage of molecular variation among (V_A) and within (V_W) two populations of *Etmopterus granulosus* from New Zealand and Chile and pairwise Φ_{ST} and F_{ST} estimates

	mtDNA	AFLP data	AFLP data after exclusion of population structuring loci
V_A	19.14	2.43	2.19
V_W	80.86	97.57	97.81
Φ_{ST}/F_{ST}	0.043*	0.024*	0.022*

*P-values highly significant ($P < 0.01$).
mtDNA, mitochondrial DNA; AFLP, amplified fragment length polymorphism.

which is in concordance with our expectations, since an exclusion of population structure giving loci should decrease the detected structuring of populations further indicating low to none population structure between sampling sites of *E. granulosus* in the SE (Chile) and SW Pacific Ocean (New Zealand).

The computed pairwise Φ_{ST} value for the two separate groupings *E. granulosus* (SW Pacific) and *E. baxteri* (SW Pacific) display a significant U_{ST} of 0.043 indicating the absence of population differences. For a summary of computed population variation and U_{ST}/F_{ST} estimates see Table 1.

Population assignment using STRUCTURE. Assignment of individuals to genotypic clusters primarily required an estimation of the true number of K populations. Estimates resulted in a proposed number of $K = 8$ ($\Delta K = 16.37$), i.e. referring broadly to the number of geographic groups,

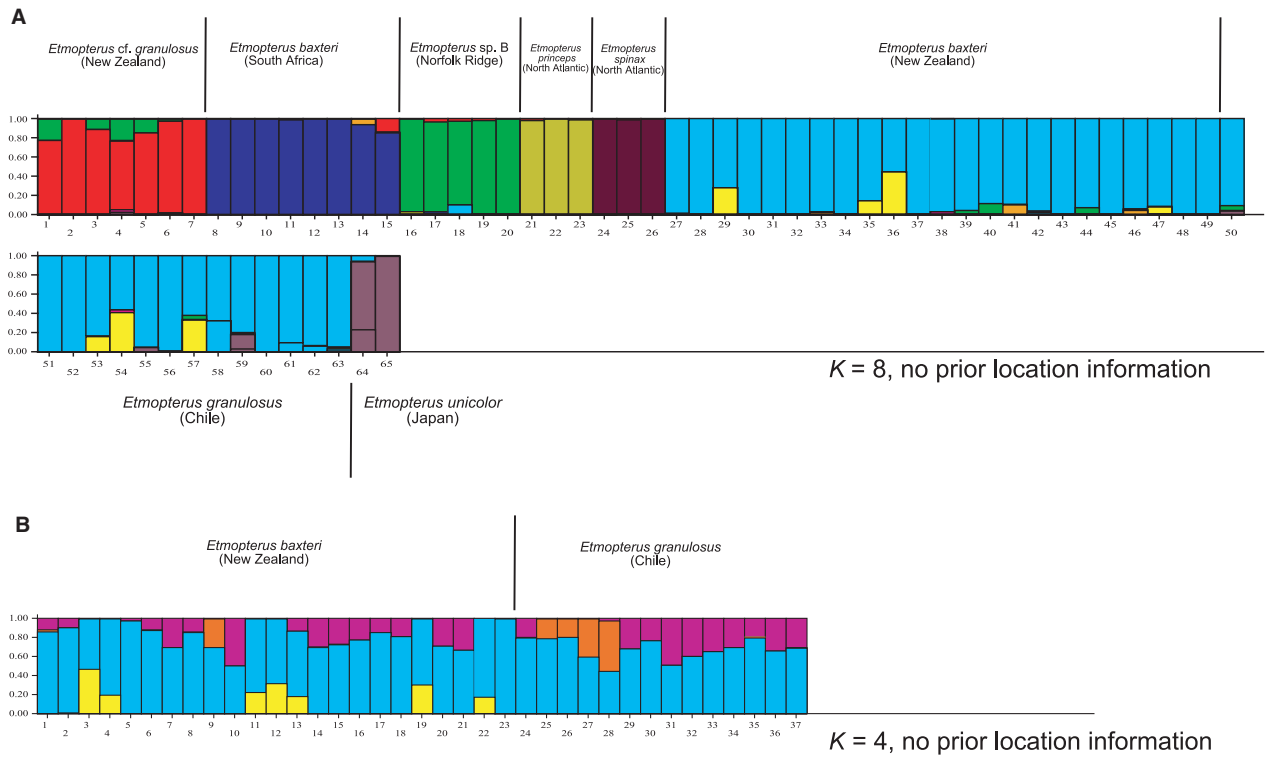

Fig. 6 Bar plots of hierarchical STRUCTURE analysis displaying population assignments for the full amplified fragment length polymorphism dataset (A) and a downsized dataset (B) focusing on sampling sites Chile (*Etmopterus granulosus*) and New Zealand (*Etmopterus baxteri*). Each bar represents an individual on the *x*-axis, the *y*-axis displays the likelihood of assignment for $K = 8$ (A) and $K = 4$ (B).

namely *E. granulosus* (Chile) plus *E. baxteri* (New Zealand), *E. baxteri* (South Africa), *E.* cf. *granulosus* (New Zealand), *E.* sp. B (Tasman Sea), *E. princeps* (NE Atlantic), *E. spinax* (NE Atlantic), and *E. unicolor* (NE Pacific). An eighth *K* was introduced due to variance within the largest cluster formed by *E. granulosus* (Chile) and *E. baxteri* (New Zealand) (Fig. 6A). As discussed by Evanno *et al.* (2005), $K = 2$ corresponds to the uppermost level of structuring ($\Delta K = 107.47$) (Supporting Information S2). The assignment test was run on the full dataset to test whether STRUCTURE detects differences between different species and to check for additional intraspecific population structure. Given no prior location information, STRUCTURE detected seven major clusters. There is no population structure for *E. granulosus* (Chile) and *E. baxteri* (New Zealand). Subsequent analyses including prior location information yielded no further structuring within the *E. granulosus* (Chile)/*E. baxteri* (New Zealand) cluster (Supporting Information S3).

For further investigation of population structuring, we analysed a smaller dataset including only samples of *E. granulosus* from Chile and New Zealand. Estimating the true number of *K* populations from specimens sampled at those two locations resulted in a proposed number of $K = 4$ ($\Delta K = 14.1$), given no prior population information. In this case, $K = 2$ ($\Delta K = 3.72$) does not correspond to the uppermost level of structuring. The proposed number of $K = 4$ shows no structuring referring to the two sampling locations New Zealand and Chile. Several individuals in the bar plot partially include different population information (Supporting Information S4).

Subsequently, we used prior location information to overcome the apparently weak information content of our dataset. Runs including prior location information also could not detect population structure. As in runs performed without any prior location information, increasing *K* increased the assignment of parts of single individuals as distinct populations, but did not reveal further population structure information which could be referred to the sampling locations New Zealand and Chile (Fig. 6B; Supporting Information S4). STRUCTURE detected the species assignment comparable to other applied methods to our AFLP dataset (Figs. 2–4). We could not detect population structure for the two sampling sites of *E. granulosus* in the SW (New Zealand) and SE Pacific (Chile).

Efforts to detect a mixed ancestry of *E. granulosus*, *E. princeps* and specimens assigned to *E. baxteri* from South Africa resulted in clear separation of the three clusters in all three analyzing runs differing in the assumed number of past generations. However, a fourth cluster including specimens of mixed ancestry was not detected (Supporting Information S5).

Discussion

Taxonomic confusion and conservation implications

Mitochondrial DNA-sequence ('barcoding') and high-resolution AFLP data presented herein demonstrate a complicated pattern of inter-specific and intraspecific relationships within etmopterid deep sea sharks (Figs. 2–6) that is not compatible with the current taxonomy. On the one hand, phylogenetic data strongly suggest that the taxon *E. baxteri* sampled off New Zealand is a synonym of *E. granulosus* sampled off Chile as suggested by Tachikawa *et al.* (1989). This argues in favour of a wide distribution in the Southern Hemisphere of *E. granulosus* and against an endemic distribution off southern South America (Fig. 6). On the other hand, specimens sampled off South Africa which have been tentatively assigned to *E. baxteri sensu* Compagno *et al.* (2005), as well as *E.* cf. *granulosus sensu* Duhamel *et al.* (2005) and *E.* sp. B *sensu* Last & Stevens (1994) form distinct clades representing most likely cryptic species. In combination, this strongly suggests the presence of two cryptic *E. granulosus*-like species in the Southern Hemisphere (Fig. 4). A third cryptic species of this Southern Hemisphere clade is *E.* sp. B, which according to our results branches as a distinct clade (Figs. 4 and 6A). Therefore, *E.* sp. B is not a synonym to *E. unicolor* from the NW Pacific as described in recent literature (Compagno *et al.* 2005; Last & Stevens 2009; Yano 1997).

This type of taxonomic confusion in combination with cryptic diversity may have profound effects on long term survival of species caught as by-catch of commercial fisheries. It is known from other shark genera, too, e.g. *Orectolobus* spp. off the Australian east coast, which exhibit also increased levels of cryptic diversity within a group of species with very similar morphological appearance (Corrigan *et al.* 2008).

However, there is still a limitation of available data on deep-sea sharks concerning behaviour (migration), spatial structuring of populations, taxonomy, and distribution. Considering that *E. granulosus* is widespread in the Southern Hemisphere, the species would require cooperative international efforts for conservation, whereas regional endemic species, such as specimens assigned to *E. baxteri* from off South Africa, need to be regionally managed (Ahonen *et al.* 2009). Forrest & Walters (2009) estimated the constant annual harvest rate (U_{MSY}) of several dogfish shark species including *E. granulosus* off Australia to be unsustainable indicating severe danger of overfishing if U_{MSY} is exceeded. Most likely, the same applies for the three cryptic species detected here, which inhabit the SW Pacific sympatrically with *E. granulosus*, and all of which are potential by-catch of increased deep-sea fisheries exploitation. Generally, there is a high level of unrecognized cryptic diversity among deep-sea sharks, which is also demonstrated by several recent publications on new species of deep-sea sharks especially within the order Squaliformes (e.g. Schaaf da Silva & Ebert 2006; Ward *et al.* 2005, 2007; White *et al.* 2008) and new information on patterns of dispersal of species (Nakaya *et al.* 2008; Oñate & Pequeño 2005; Reyes & Hüne 2006; Soto 2001). Results from our study reveal the existence of previously undescribed species and the problem of species misidentification in a group of sharks regularly caught as by-catch in commercial fisheries. Cryptic species need to be taxonomically described in order to make names and identification tools available for effective monitoring and conservation measures. Our study further highlights the necessity of taxonomically sound stock assessment analyses based on molecular data, not only for commercially targeted species but also for 'by-catch'.

Population structure and phylogeography of E. granulosus

For both sampling sites of *E. granulosus* (Chile and New Zealand) F_{ST} and Φ_{ST} values of the AFLP and mtDNA data, respectively, identify only extremely weak but nevertheless significant genetic differentiation of populations (Table 1). This is supported by AMOVA results indicating that the vast majority of nuclear variation resides among and not within the two samples (among population variation = 2.43%). A search for differentially segregating AFLP loci using the genome scan approach only yielded two candidate loci whose allele frequencies in the two samples might have been shaped by strong selection. However, removal of these two loci did only slightly affect population differentiation as measured by a lower but still significant pairwise F_{ST} value. Despite these low but significant values for population differentiation and despite an estimated number of populations within the *E. granulosus* sample of $K = 4$, STRUCTURE did not detect additional population structure between the two sampling locations. Instead, individuals within the New Zealand sample that were not unambiguously assignable to the large undifferentiated group of *E. granulosus*-like etmopterids, formed a separate cluster under $K = 4$ assumptions. In summary, the two sampling sites for *E. granulosus* (Chile and New Zealand) are separated by roughly 7000 km but show a very modest level of population differentiation only. The low level of population differentiation could either be indica-

tive for an isolation-by-distance scenario or by a very recent cessation of gene flow divergence of these populations. Isolation by distance would require the existence of intermediate populations allowing for connectivity between Chile and New Zealand. The few COI haplotypes of specimens identified as *E.* cf. *baxteri* (Amsterdam Island) and *E. granulosus* (NE of the Kerguelen Plateau) from the Indian Ocean and *E. granulosus* from off SE Australia [Tasman Sea, Genbank (accession numbers DQ108226, DQ108216)] fall into the *E. granulosus* network cluster (Fig. 2). This supports their identity as *E. granulosus* and the notion of close connectivity of populations separated by several thousands of kilometers along the subantarctic ecoregion. Such a connectivity may be facilitated by the circum-antarctic current passing all known sampling locations of *E. granulosus* (Fig. 1). A very recent separation of now reproductively isolated populations appears less likely given that overall regional diversity in the area has evolved into differentiated bathyal species ecoregions, i.e. New Zealand, Kermadec and Nazcaplatensis ecoregions are clearly discernable (UNESCO 2009). Genetic differentiation was already detected between pelagic Southern Australian dolphins (*Delphinus delphis*) over a distance of 1500 km, supporting the hypothesis of differentiated ecoregions in the Southern Hemisphere (Bilgmann *et al.* 2008). Nevertheless, the appropriate approach to test for these alternative hypotheses would be a classical tagging experiment allowing to track movements of individuals over large distances. So far, available data on migration behaviour of etmopterids in general is limited, because tagging studies do not exist (Forrest & Walters 2009).Yet another explanation for a subtle population differentiation between distant *E. granulosus* populations is a response to natural selection acting divergently between e.g. the New Zealand and Chile sample sites. Chilean *E. granulosus* occur in comparatively shallow depths from 200 to 637 m (IUCN 2010, and N. Straube personal observation), whereas the same species occurs off New Zealand on average much deeper between 850 and 1200 m (Bass *et al.* 1986; Garrick 1960; Wetherbee 1996; N. Straube personal observation). In this context, it must remain speculative, whether the two possible candidate loci identified in the AFLP genome scan relate to physiological characters under divergent selection for adaptations to different depths. However, the distribution range of *E. granulosus* is most likely circumglobally along the Southern Hemisphere, and reports off Sierra Leone (Golovan & Pukhorukov 1986) need confirmation.

Generally, our study supports the possibility of *E. granulosus* being a migratory rather than a resident species. Evidence for sex and size-related aggregations (Jakobsdottir 2001; Wetherbee 1996) might be related to socially induced migraton for mating or schooling purposes, too (Claes & Mallefet 2008, 2009).

Although the sample size of our study is limited, it represents the first population genetic approach applied to etmopterids, and it is based on a very large number of AFLP-loci, i.e. compensating partially unsatisfactory sample size by analyzing patterns of differentiation across the whole genome. Especially for comparatively low sample sizes, AFLPs are the appropriate method, because the AFLP technique often provides better resolution of population structure size than e.g. microsatellites (e.g. Cambpell *et al.* 2003; Evanno *et al.* 2005; Sønstebø *et al.* 2007). The robustness of analyses presented herein is supported by coherent results based on different analytical methods and on two different datasets (mtDNA and AFLPs). Obviously, future population genetic analyses of the *E. granulosus* species group should comprise additional samples of potentially existing intermediate populations especially with regard to validation of the hypothesis of migration versus isolation by distance. We anticipate that a larger sample size may further allow to confirm the presence of *E. granulosus* off South Africa.

Etmopterus spinax clade: biogeographic and alpha-level taxonomic implications

Results presented herein further resolve phylogenetic interrelationships of the *E. spinax* clade. Preliminary phylogenetic data of numerically limited samples had previously suggested the existence of hitherto undetected cryptic diversity and insufficient phylogenetic resolution among this morphologically uniform etmopterid group (*E. spinax* clade, *sensu* Straube *et al.* 2010). This study resulted in a polytomy displaying a weakly supported sister-relationship of NE Atlantic *E. spinax* and *E. princeps* to *E.* cf. *granulosus* and *E.* sp. B. The phylogenetic hypothesis based on AFLP data reveals *E. spinax* (NE Atlantic) as the basal taxon to a clade comprising morphologically similar large lantern sharks (*E. princeps*, *E. granulosus*, *E.* cf. *granulosus*, South African *E. baxteri*, and *E.* sp. B) with high bootstrap support. *E. princeps* (NE Atlantic) is the well-supported sister taxon to a clade comprising species from the Southern Hemisphere only (Fig. 4). This refined phylogenetic hypothesis suggests that the origin of the *E. spinax* clade is in the Atlantic, because both basal members of the clade are sampled in the North Atlantic and display its main distribution in the North Atlantic, whereas younger species are distributed in the Southern Hemisphere. Origin and subsequent Southern Hemisphere diversification of the *E. spinax* clade species occurred 36–22 Ma ago (Straube *et al.* 2010) and follow the Eocene/Oligocene climatic deterioration from greenhouse to icehouse condi-

tions (Eldrett *et al.* 2009; Lear *et al.* 2008). The climatic cooling and simultaneous ice sheet development on the Antarctic continent was connected to the final separation of Antarctica from the surrounding continents by opening of the Tasman and Drake passages. The development of these gateways initiated circumpolar circulations and the thermal isolation of the Antarctic continent and the Southern Ocean (Dingle & Lavelle 2000). Increased deepening of the Tasmanian and Drake passages at ca. 34 Ma resulted in enlarged Pacific throughflow and subsequent deeper Atlantic–Pacific connections close to the Eocene/Oligocene boundary (Sher & Martin 2006). Simultaneously, deep-sea temperatures decreased considerably from 12 to 4.5 °C (Zacho *et al.* 2001). We hypothesize that a species closely related to *E. princeps* dispersed into the Southern Hemisphere oceans through the deepend gateways and gave rise to the Pacific and Indian Ocean taxa. This interpretation also is supported by the fossil record of Southern Ocean sharks, which consists of a very diverse fauna prior to climatic cooling at the end of the Eocene including representatives of three squaliform sharks, *Centrophorus*, *Deania* and *Squalus*, but no etmopterid (Kriwet 2005). The gradual thermal isolation of the Southern Ocean, in which water temperatures finally dropped to below 0 °C barred sharks and most bony fishes from this hostile environment. The modern fish fauna is impoverished and striking in its low taxonomic diversity and sharks only sporadically intrude into the Southern Ocean (Long 1992a; Kriwet 2005). Only a few skates, which are assumed to have persisted since the Eocene, inhabit the Southern Ocean today (Long 1992b; Eastman 2005). Analogously, a recent study of the global population structure of another squaloid shark, the spiny dogfish, *Squalus acanthias*, identified a southward dispersal pathway from a putative Northern Hemisphere origin, which partially aligns with our results (Verrissimo *et al.* 2010).

Taxonomically useful information on the three cryptic molecular species identified herein is scarce. South African specimens assigned to *E. baxteri* by Compagno *et al.* (2005) and probably conspecific with our specimens assigned to *E. baxteri* sampled off South Africa, are reported to have a larger body size than *E. granulosus* (up to 85.5 cm in contrast to average 75 cm), but otherwise appear to be very similar to *E. granulosus sensu* lato (Ebert *et al.* 1992). Based on this similarity, we used the AFLP data set to test the hypothesis of mixed ancestry, that specimens assigned to *E. baxteri* from South Africa are of hybrid origin with *E. granulosus* (New Zealand & Chile) and *E. princeps* (NE Atlantic), but results did not indicate a hybrid origin of the specimens (Support Information S5). Haplotypes identified from mtDNA (COI) broadly refer to the different species, mixed haplotypes are only found among specimens assigned to *E. granulosus* from Chile, Australia, Amsterdam Island, NE of the Kerguelen Plateau and New Zealand.

Unfortunately, diagnostic morphological characters are still missing to separate the cryptic *E. baxteri* from South Africa from *E. granulosus*, but a DNA-barcoding approach would readily identify it. Monitoring etmopterid by-catch using DNA-barcoding would enable not only to study the distribution of this cryptic species, but might also allow to test for the existence of *E. granulosus* in waters off South Africa, which is not unlikely according to the presumed peri-antarctic distribution of this taxon as discussed.

Finally, specimens of *E.* cf. *granulosus* sensu Duhamel *et al.* (2005) from the Kerguelen Plateau and New Zealand appeared as a distinct clade in the AFLP and mtDNA phylogeny. Figure 2 reveals the species to be widespread as well, since specimens were sampled off New Zealand as well as in the Indian Ocean (Kerguelen Plateau). This species is not closely related to *E. granulosus* (as the name suggests), but is sister to the undescribed *E.* sp. B (Figs. 2–4), which was placed in synonymy with *E. unicolor* in recent publications (Last & Stevens 2009; Yano 1997). The results presented here and in a larger phylogenetic study of Etmoperidae (Straube *et al.* 2010) contradict a synonymy of *E.* sp. B with *E. unicolor*, because specimens of *E. unicolor* included in our sampling are clearly distinct from *E.* sp. B and unambiguously identified as *E. unicolor* using characters presented in the synonymisation of this species with Southern Hemisphere congeners (*E.* sp. B) by Yano (1997). In addition, our samples were collected in the NW Pacific (Japan) close to the type locality of *E. unicolor*. However, as in the previous species, diagnostic morphological characters for *E.* sp. B are still missing, rendering a barcoding approach to be promising for monitoring and conservation of cryptic members of the *E. unicolor* species complex. The mtDNA sequences further included in the analysis of specimens of *E.* cf. *unicolor* [Genbank (accession numbers EU398778, EU398779, EU398780, EU398781, EU398782)] from off Indonesia revealed a distinct clade as well, leading to the assumption of an even higher cryptic diversity among the *E. unicolor* species complex.

Acknowledgements

We would like to express our sincere thanks to S. Tanaka, T. Horie (Tokai University, Japan), G. Duhamel, Samuel Iglésias, Bernard Serét (Muséum National d'Histoire Naturelle), A. Loerz, K. Schnabel, D. Tracey, M. Watson, P. McMillan (NIWA, New Zealand); A. Stewart (Te Papa Tongarewa, New Zealand), D. Bray (Victoria Museum Melbourne, Australia), K. Matsuura, G. Shinohara (Museum of Nature and Science, Japan), G. Johnston (Marine Institute Rinville, Ireland), F. C. Toro (Universidad de Valparaíso, Chile), R. Leslie (Marine and Coastal Management, South

Africa); S. Socher, J. Schwarzer, A. Dunz, D. Neumann, M. Geiger and A. Verrissimo for useful comments on the manuscript. This study was financed by grants of the German Research Foundation DFG to J. Kriwet (KR 2307-4) and to U. Schliewen (SCHL 567-3). This research received further support from the SYNTHESYS Project http://www.synthesys.info/ which is financed by European Community Research Infrastructure Action under the FP6 "Structuring the European Research Area Programme."

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Support Information S1. Samples used in this study with location information and Genbank Accession numbers for cytochrome oxidase I sequences.

Support Information S2. Estimation criteria for the number of genetic clusters in the amplified fragment length polymorphism (AFLP) data set. **A:** Evanno's model choice criterion 'ΔK' for the uppermost level of genetic structure computed from the full AFLP dataset. **B**: Evanno's model choice criterion 'ΔK' for the uppermost level of genetic structure computed from the downsized AFLP dataset including specimens of *Etmopterus granulosus* from Chile & New Zealand only.

Support Information S3. Bar plots of STRUCTURE analyses showing population assignments for $K = 8$. Each bar represents an individual on the *x*-axis, the *y*-axis displays the likelihood of assignment for *K*. **A**: not including prior location information. **B**: including prior location information.

Support Information S4. Bar plots of STRUCTURE analyses of a smaller dataset including samples of *Etmopterus granulosus* from the SE Pacific and *Etmopterus baxteri* from the SW Pacific ($K = 4$). Each bar represents an individual on the *x*-axis, the *y*-axis displays the likelihood of assignment for *K*. **A**: not including prior location information. **B**: including prior location information.

Support Information S5. Bar plots of STRUCTURE analyses of potential mixed ancestry of *Etmopterus baxteri* (South Africa) with *Etmopterus granulosus* (Chile)/*E. baxteri* (New Zealand) and *Etmopterus princeps* (North Atlantic) ($K = 3$). Each bar represents an individual on the *x*-axis, the *y*-axis displays the likelihood of assignment for *K*.

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