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The secret of the mermaid's purse: Phylogenetic affinities within the Rajidae and the evolution of a novel reproductive strategy in skates

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Abstract

The systematics of the skates in the family Rajidae have been contentious for over 250 years, with most studies inferring relationships among geographically clustered species, and non-overlapping taxa and data sets. Rajid skates are oviparous, and lay egg capsules with a single embryo. However, two species exhibit a derived form of egg laying, with multiple embryos per egg capsule. We provide a molecular assessment of the phylogenetic relationships of skates within the family Rajidae based on three mitochondrial genes. The resulting topology supports monophyly the family. However the genus *Raja* is polyphyletic, and several species assemblages need to be revised. We proposed a new assemblage as the *Rostrorajini*, which organizes rajid species into three well-supported tribal lineages for the first time. Further, these data provide an independent assessment of monophyly for the two species exhibiting multiple embryos per egg capsule, supporting their status as the unique genus *Beringraja*. In addition, we find that among the different size classes of egg capsules, ranging from 1–8 embryos per capsule in this genus, there is variation in frequency and survivorship. In *Beringraja binoculata*, the strategy of having two embryos per egg capsule occurs in the highest frequency and has the highest survivorship.

Keywords

Rajidae; *Raja*; skates; multiple embryos; mitochondrial DNA

1. INTRODUCTION

The batoids (Superorder Batomorphii) are generally regarded as a monophyletic group of cartilaginous fishes based on morphological characteristics, which is now supported

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independently by molecular characters (Aschliman et al., 2012). The batoids are distinguished from typical “shark”-like fishes by their dorso-ventrally flattened bodies, and expanded pectoral fins that extend forward and are fused to the head. This group consists of the skates, thornback rays, electric rays, sawfishes, guitarfishes, and stingrays. Several opposing hypotheses have inferred the interrelationships within this group with varying degrees of resolution. However, a recent molecular phylogeny, based on the mitochondrial genome and two nuclear genes, has provided a well-resolved phylogeny of the batoids; indicating that batoids and sharks are reciprocally monophyletic, and further, that skates are the ancestral batoid group (Aschliman et al., 2012).

Both batoids and sharks exhibit multiple reproductive modes, including oviparity and viviparity. Several studies have made inferences about which of these reproductive modes is ancestral in elasmobranchs from topologies based on morphological and molecular characters (Dulvy and Reynolds, 1997; Musick and Ellis, 2005). Due to expanding data sets and different analytical algorithms used for phylogenetic analysis, the consensus on the ancestral condition in cartilaginous fishes and elasmobranchs has changed several times in the last three decades (Dulvy & Reynolds 1997; Music and Ellis 2005; Long et al 2008 and Wourms 1977). As the ancestral batoid taxon (Aschliman et al., 2012; Naylor et al., 2005); this topology does not clarify the issue. Rather the ancestral condition in elasmobranchs, and even the jawed vertebrates, remains equivocal. However, it is clear that viviparity arose early in the evolution of jawed vertebrates, based on recent evidence supporting viviparity in the Placoderm, *Ptyctontida* (Long et al., 2008); and an extinct holocephalan (Grogan and Lund, 2011). But because these groups are extinct, it is difficult to assess topologies based on different characters for inference of ancestral states. This demonstrates the importance of having the most reliable phylogenetic context possible to infer ancestral states and the evolution of derived traits.

Skates of the family Rajidae are oviparous, and lay egg capsules with a single embryo. However, two species exhibit a derived form of egg laying, with multiple embryos per egg capsule. These have recently been proposed as sister taxa based on this unique reproductive character and aspects of clasper morphology. Here, we independently assess the phylogenetic relationships of skates within the family Rajidae, and determine when this novel mode of oviparity arose in evolution.

1.1 Systematics and Phylogenetic affinities of skates in the family Rajidae

Skates are the most diverse order of cartilaginous fishes and are composed of 287 (Ebert and Winton, 2010) of the 1221 species (Naylor et al., 2012). The sub-order Rajoidei is divided into three families-the Rajidae, the Arhynchobatidae and Anacanthobatidae (Ebert and Compagno, 2007). The family Rajidae is considered monophyletic based on skeletal elements, appendage morphology, and electric organogenesis (McEachran and Dunn, 1998). Within the Rajidae, several “tribes” and species assemblages have been described (Table 1, Ebert and Compagno, 2007; McEachran and Dunn, 1998).

Rajid genera have been defined primarily by morphological characters (Last and Gledhill, 2007; Last and Yearsley, 2002; McEachran and Aschliman, 2004) including features associated with egg capsules as a unique tool for inferring phylogenetic relationships (Ebert

and Davis, 2007; Ishihara et al., 2012). Species assemblages have been proposed based on geographic region (Ebert and Compagno, 2007; Ebert and Winton, 2010). Most studies that evaluate the interrelationships within the family and generic level have been limited in taxonomic representation and geographic scale. Several studies call for a reliable phylogenetic hypothesis of the skates (Hyun Kyu Yoon, 2009), as well as members of the genus *Raja* (Tinti et al., 2003). While the family Rajidae is widely accepted as monophyletic, we know of no previous study that evaluates monophyly of the genus *Raja*, nor relationships within the Rajidae, based on molecular data. To date, two studies have proposed relevant phylogenetic hypotheses with representation of some rajid taxa: the first is based on a small number of taxa using one mitochondrial locus (16S, Turan, 2008), while a second employs a larger data set to evaluate higher level relationships within the Batoidea (Aschliman et al., 2012). While both of these studies included some rajid species, neither have the requisite taxonomic representation to address the phylogenetic affinities within the Rajidae. Collectively, these studies have proposed various topologies representing non-overlapping taxa. Most studies have focused on relationships within regional groups. Therefore, broad phylogenetic inferences have been hampered by sampling bias, historical, and geographic constraints. In this study, we use molecular markers to infer the phylogenetic affinities of rajid taxa from multiple geographic locations including Eastern/ Western Pacific, Eastern/Western Atlantic, and the Mediterranean and Black Seas.

In this study, we focus on the hardnose skates (family Rajidae). This study includes 13 of the 17 genera within the Rajidae—*Okamejei*, *Dipturus*, *Raja*, *Beringraja*, *Amblyraja*, *Leucoraja*, *Rajella*, *Rostroraja*, *Zearaja*, *Breviraja*, *Dactylobatus*, *Fenestraraja*, *Gurgesiella*, *Spiniraja*, *Dentiraja*, *Malacoraja*, *Neoraja* (see Ishihara et al., 2012; Last and Yearsley, 2002)—and evaluates the relationships of three species assemblages (with the generic name of *Raja*) proposed by McEachran and Dunn (1998) and Ebert and Compagno (2007, Table 1). The genus *Raja* has been confounded for over 250 years, when Linnaeus assigned every batoid to the genus *Raja* (see Linnaeus, 1758) and the genus is likely polyphyletic.

1.2 A novel reproductive tactic exhibited by the big skate and mottled skate

Rajid skates lay single or paired egg capsules, known colloquially as “mermaids purses”, which contain a single embryo, and exhibit protracted incubation times ranging from four to 15 months (Serra-Pereira et al., 2011). Egg capsules require a large maternal investment that is putatively associated with increased fitness—particularly in species exhibiting smaller body sizes (Musick and Ellis, 2005). However, the mottled skate and the big skate share a unique reproductive tactic of depositing multiple embryos per egg capsule: ranging from one to five embryos for mottled skate (*Beringraja pulchra*) and on to eight per capsule for big skate (*B. binoculata*) species (Ebert, 2003; Hitz, 1964; Ishiyama, 1958). Fitness advantages or tradeoffs of this tactic are currently unknown. However, captive big skate females can deposit over 350 capsules annually (Ebert et al., 2008), and up to 6000 over a reproductive life span. This implies that the big skate may be the most fecund of any extant elasmobranch species (Ebert et al., 2008). However, survivorship among classes of embryo density has not been previously characterized.

1.3 Phylogenetic affinities of the big skate and the mottled skate

Two species—big skate (*B. binocularata*) and the mottled skate (*B. pulchra*)—share a unique reproductive strategy (Ebert and Davis, 2007; Ebert et al., 2008), as well as a distinct clasper morphology (Ishihara et al., 2012). Based on these synapomorphies, Ishihara et al (2012) assigned these species, to the new genus *Beringraja* (formerly *Raja*). The mottled skate is endemic to the western North Pacific occurring around Japan, Korea and China at depths up to 850m, while the big skate is endemic to the eastern North Pacific occurring from Baja California to the eastern Bering Sea at depths up to 800m, albeit more commonly at depths less than 200m (Ebert et al., 2008; Ishiyama, 1958). In this study we independently evaluate the validity of the genus *Beringraja*, and infer whether this unique reproductive tactic shares a single evolutionary origin.

1.4 The objectives of this study are

1. Propose a phylogenetic framework, based on molecular data, of the family Rajidae for comparison with species assemblages defined by morphological characters
2. Independently evaluate the phylogenetic relationship between *B. binocularata* and *B. pulchra* to determine whether the unique characteristic of multiple embryos per egg capsule arose once in evolution, or twice independently.
3. Evaluate differential survivorship among egg capsule size classes to understand potential fitness tradeoffs.

2. MATERIALS AND METHODS

2.1 Samples and Data mining

Our study includes 53 species, topologies are based on individual loci (Table 1), but we did not have a uniform taxonomic representation. Tissue samples from ten species were sampled directly or obtained from aquarium or museum collections and we provide 30 new sequences. 42 additional taxa were used for verification of sequences downloaded from public databases (National Center for Biotechnological Information, Table 1). This is the first molecular study to include both *B. pulchra* and *B. binocularata* and we sequenced three individuals for each of these species. For all other taxa we included sequences from a single individual.

2.2. DNA Extraction and PCR amplification

Muscle or fin tissues were preserved in ethanol for DNA extraction. Total genomic DNA was extracted using Qiagen DNEasy blood and tissue kit (Qiagen Inc., Valencia, CA). We constructed a molecular phylogeny based on three mitochondrial loci: (*12S*, *16S*, *COI*). Sequences were amplified using primers constructed from published primers according to (Tinti et al., 2003) and (Spies et al., 2006) (Table 2). Amplifications were performed in 25 µl reactions consisting of 8.5 µl of deionized water, 12.5 µl of 1.1xTaq Reddy Mix (Thermo Scientific, Foster City, CA), 1 µl for each of the forward and reverse primer (20µM), and 2 µl of DNA. PCR amplifications occurred under the following conditions: 35 cycles of 95° C for 1 min, 53°C (12S and 16S) or 56°C (COI) for 30 s, and 72° C for 1 min. PCR products were visualized on a 1% agarose gel, stained with ethidium bromide and visualized under

UV light. The PCR product appeared as a single band and was purified using Qiagen PCR purification kit following the manufacture protocols. Sequences were deposited in GenBank under the following accession numbers (KF317707-KF317733, Table 1).

2.3 Sequence Alignment and Phylogenetic Analyses

Sequences were aligned using Sequencher 4.1.2 (GeneCodes Corp., Ann Arbor, MI), and Se-Al (v. 2.0a11 (Rambaut, 2002). Gene trees were constructed using Maximum parsimony (MP), and Neighbor joining (NJ) algorithms using in PAUP (Swofford, 2002). Bootstrap support for all nodes in NJ and MP was based on 2000 replicates. Bayesian inferences were performed in MrBayes 3.1.2 (Huelsenbeck, 2001) with model selection determined by the Akaike Information Criteria (AIC, see Table 2), as implemented in Model Test 3.7 (Posada and Crandall, 1998). Bayesian posterior probabilities (BPP) were estimated for 100,000 generations, and Log-likelihood scores were plotted to determine when stationarity was achieved. All trees preceeding stationarity were discarded, and multiple runs were executed from random trees to ensure that the optimum tree space had been explored, resulting in identical topologies. The Shimodaira–Hasegawa was used to test for congruence between datasets (implemented in PAUP), which indicated that 16s ad COI were congruent but 12S was not. However the 12S topology was not significantly different using the T₁-TPT test. Further, the topology based on the combined data set was more resolved with consistently higher support values. Therefore, we present a topology based on the concatenated data set. Finally, individual gene trees were often poorly resolved but were generally consistent with the tree from the concatenated analysis. There were no supported clades in any individual gene trees that disrupted any supported nodes in the topology inferred from the concatenated data set (see supplemental figures S1–S3).

2.4 Estimating survivorship of egg capsules in an Aquarium setting

Beringraja binoculara egg capsules were collected and tagged at Aquarium of Bay (Pier 39, San Francisco, CA). A total of 103 egg capsules randomly incubated in assigned bins with respect to the date they were laid. The bins were exposed to common garden conditions with recirculating filtered seawater, light regime and water temperature over the course of one year (from January 2012–January 2013). Temperatures varied according to ambient conditions in San Francisco Bay (for example 11°C in January 2012, 13°C in March, 15–16°C in August 2012, etc.). Egg capsules were checked weekly using an LED light to quantify the number of embryos per egg capsule.

3. RESULTS

3.1 Phylogenetic affinities within the Rajidae

Phylogenetic inferences from the concatenated data set were based on 1498 bp from three-mitochondrial loci-12S, 16S, and COI (Table 1–2). Of the three outgroup taxa, *Bathyraja kincaidii* (Arhynchobatidae) was the most closely related to the Rajidae, as expected. The family Rajidae is monophyletic in all analyses (100/99/100) for NJ/MP/BPP, as is the *Amblyrajini*, (100/94/100, following (McEachran and Dunn, 1998). However, the *Rajini*, which includes the genera *Raja*, *Okamejei*, *Dipturus* (plus three genera that were formerly associated with this genus, Table 1), and *Rostroraja*, according to McEachran and Dunn

(1998), is not monophyletic. *Rostroraja alba* is not associated with the remaining *Rajini*, but consistently groups with two *Raja* species-*R. texana* and *R. eglanteria* formerly described as part of the (AAA) Amphi-American assemblage (100/84/100, Figure 1). Interestingly, this clade is not closely associated with the remaining *Rajini* genera, but is interrupted by the *Amblyrajini* + *Rajini* clade (82/64/100).

These data clearly indicate that the AAA are not associated with the genus *Raja*, and should be renamed. And, we propose that *Rostroraja alba* plus the seven AAA species are part of a unique assemblage that we refer to as the *Rostrorajini*.

The (TRA) true *Raja* species assemblage group together with high support (99/76/100). The genera *Okamejei* and *Dipturus* are closely associated with the TRA (___/___/96, Figure 1). These genera are within the *Rajini*, following McEachran and Dunn (1998). The NPA, following Ebert and Compagno (2007), was not formally included in the *Rajini*, however we found a close association between *Okamejei* + *Dipturus* + *Raja* (100/88/100, Figure 1). Therefore we propose that the NPA should be considered part of the *Rajini*. Interestingly, the NPA was not recovered as a monophyletic clade in our analyses, but formed two distinct lineages. Importantly, *Beringraja binoculata* and *B. pulchra* are sister taxa (formerly *Raja* and NPA), supporting the new generic designation from molecular data for the first time, as an independent test of phylogenetic relationships.

3.2 Fitness advantages-Hatching frequency reveals an optimal strategy

The monophyly of the two *Beringraja* species indicates that the reproductive tactic of multiple embryos per egg capsule arose once in the skates. To understand fitness advantages associated with this reproductive strategy, we monitored 103 egg capsules of the big skate, *Beringraja binoculata*, deposited throughout a single observation year. Nine of these were non-fertile and were omitted from the study. Of the 95 remaining egg capsules, 21 hatched, 27 were still incubating at end of observation period, and 47 died. Egg capsules contain a range of 1–5 embryos. Egg capsules with two embryos occur with the highest frequency (56%), and one embryo per egg capsule is the second most common strategy (34%, Figure 2). Egg capsules with 3–5 embryos are rare (Figure 2). The average incubation is 172 days (~6 months). The incubation period for egg capsules with single embryos is the most variable, ranging between 138–207 days. Egg capsules with two embryos range from 164–187 days. The embryos in the egg capsule with five embryos emerged on two separate dates-ten days apart. Two emerged on day 155 and three emerged on day 166. Egg capsules with three and four embryos did not survive past 102 days.

Mortality was high in all egg capsule size classes. Interestingly, the size class with the lowest mortality during our observation period (42%, n=53), was the one that occurred with the highest frequency-two embryos per egg capsule. While the estimate of mortality for the three per capsule size class was close (43%, n=7), we had a much smaller sample size. Surprisingly, egg capsules with a single embryo exhibited the highest mortality (59%, n=32). Sample sizes for egg capsule size classes with three or more embryos were too small to estimate mortality (see supplemental figure-S4).

4. DISCUSSION

The genus *Raja* has been historically confounded and remains so to date. Phylogenetic affinities based on morphological characters have historically been confined to taxa that occur in specific geographic regions, making inferences of monophyly problematic. To our knowledge, this is the first attempt at inferring the phylogenetic affinities of “*Raja*” species based on molecular data with a sampling regime focused on the Rajidae. The genus *Raja* is clearly polyphyletic, and several taxa need revision.

According to McEachran and Dunn (1998) the *Rajini* tribe (Table 1) is defined by two morphological characteristics – (1) disc free of denticles, and (2) alar crowns with barbs. Our analyses support a monophyletic clade of four genera that share these characters-*Raja* (TRA+NPA, *sensu* Ebert and Compagno 2007), *Okameiji*, and *Dipturus*. However, neither *Rostroraja alba* nor the AAA are associated with this group, therefore these characters may have arisen convergently. We propose a revised *Rajini* tribe that excludes these taxa; and a new tribe, the *Rostrorajini* that joins them, resulting in a subdivision of rajid species into three well-supported tribal lineages for the first time.

We found no evidence to disrupt *Okameiji* or *Dipturus* as monophyletic assemblages. The TRA is monophyletic and excludes the “*Raja*” species associated with the NPA and the AAA. Our topology did not recover TRA and *Dipturus* as sister taxa, but phylogeographically they co-occur in the Mediterranean and Black sea. The close relationship between *Okameiji* + *Dipturus* + TRA indicates that all remaining taxa with the generic designation of “*Raja*” (not associated this clade) must be renamed. For example, species of the AAA are closely associated with the monotypic *Rostroraja alba*, and are not associated with the genus *Raja*, and this is consistent with the findings of Naylor et al. (2012). Together, we define these (formerly AAA + *Rostroraja*) as the *Rostrorajini* tribe. Likewise, the species that were formerly described as the NPA are not associated with the genus *Raja*, and need to be renamed as well. Our phylogenetic analysis supports the status of two species as *Beringraja* (Ishihara et al., 2012), and we note that the generic affinities of the remaining NPA species (Ebert and Compagno, 2007) need to be revised.

The “secret of the mermaid’s purse” reveals that the reproductive tactic of depositing multiple embryos per egg capsule arose once during the evolution of skates. This strategy is exhibited by only two species in the genus *Beringraja*, with two embryos per egg capsule as the most frequent, and optimal strategy. Because there is variation in fitness among the egg capsule size classes, the fecundity of *B. binoculata* is not necessarily equivalent to the number of embryos produced because mortality is high across all egg capsule size classes before hatching. Little is known about the total fecundity of *Beringraja* skates relative to other skates that lay a single embryo per egg capsule.

Both sharks and batoids exhibit various modes of reproduction including multiple conditions of maternally derived nutrition ranging from yolk-sac viviparity, histotrophy, to oophagy, to placentally derived nutrition. While a variety of viviparous conditions occur, the oviparous tactic is generally represented by a single embryo per egg capsule. Multiple embryos per egg capsule is a tactic that occurs in only two species within the Rajidae-the only batoid family

that exhibits oviparity. Oviparity is a common strategy among vertebrates, but the character of multiple embryos per egg capsule appears to be quite rare, and is therefore novel. While there have been reported cases of twin embryos in chicken eggs (Jeffrey et al., 1953), some reptiles (Marion, 1980), their frequency and survivorship is extremely low (eg. <1% survivorship, Jeffrey et al., 1953), and therefore could not be considered a reproductive strategy, nor a shared tactic within a lineage. It is unclear whether siblings within a capsule are clone mates, being derived from a single egg and sperm, or even if they are full siblings (i.e. with multiple sires). Regardless, cartilaginous fishes have captured the interest of evolutionary biologists, not only because they represent an ancestral vertebrate condition, but also because they exhibit many derived forms including a striking disparity of body plans and plasticity in reproductive modes. Understanding the evolution of such complex traits requires a robust phylogenetic context.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Aschliman NC, Nishida M, Miya M, Inoue GJ, Rosana KM, Naylor GJP. Body plan convergence in the evolution of skates and rays (Chondrichthyes: Batoidea). *Molecular Phylogenetics and Evolution*. 2012; 63:28–42. [PubMed: 22209858]
- Dulvy NK, Reynolds JD. Evolutionary transitions among egg-laying, live-bearing and maternal inputs in sharks and rays. *Proceedings of the Royal Society of London Series B*. 1997; 264:1309–1315.
- Ebert D, Compagno L. Biodiversity and systematics of skates (Chondrichthyes: Rajiformes: Rajoidei). *Environmental Biology of Fishes*. 2007; 80:111–124.
- Ebert, DA. *Sharks, Rays, and Chimaeras of California*. University of California Press; Berkeley: 2003.
- Ebert DA, Davis CD. Descriptions of skate egg cases (Chondrichthyes: Rajiformes: Rajoidei) from the eastern North Pacific. *Zootaxa*. 2007; 1393:1–18.
- Ebert DA, Smith WD, Cailliet GM. Reproductive biology of two commercially exploited skates, *Raja binoculata* and *R. rhina* in the western Gulf of Alaska. *Fisheries Research*. 2008; 94:48–57.
- Ebert, DA.; Winton, MV. Chondrichthyans of High Latitude Seas. In: Carrier, JC.; Musick, JA.; Heithaus, MJ., editors. *Sharks and their Relatives II-Biodiversity, Adaptive Physiology and Conservation*. CRC Press; Boca Raton: 2010. p. 116-158.
- Grogan ED, Lund R. Superfoetative viviparity in a Carboniferous chondrichthyan and reproduction in early gnathostomes. *Zoological Journal of the Linnean Society*. 2011; 161:587–594.
- Hitz CR. Observations on egg cases of the big skate (*Raja binoculata* Girard) found in Oregon coastal waters. *Journal of the Fisheries Board of Canada*. 1964; 21:851–854.
- Huelsenbeck JP, Fredrik Ronquist. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*. 2001; 17:754–755. [PubMed: 11524383]

- Hyun Kyu Yoon DJ, Chung Inhyuk, Jung Jin Wook, Kim Moon Ju Oh Sung, Lee Youn-Ho, Kim Choong-Gon, Hwang Seung Yong. Rapid Species Identification of Elasmobranch Fish (Skates and Rays) using Oligonucleotide Microarray. *Biochip Journal*. 2009; 3:87–96.
- Ishihara H, Treloar M, Bor P, Senou H, Jeong C. The comparative morphology of skate egg capsules (Chondrichthyes: Elasmobranchii: Rajiformes). *Bull Kanagawa Prefect Mus (Nat Sci)*. 2012; 41:9–25.
- Ishiyama R. Observations on the egg-capsules of skates of the family Rajidae, found in Japan and its adjacent waters. *Bulletin of The Museum of Comparative Zoology at Harvard College*. 1958
- Jeffrey FP, Fox TW, Smyth JR. Observations on double-yolked eggs from the domestic fowl. *J Hered*. 1953; 44:213–216.
- Last PR, Yearsley GK. Zoogeography and relationships of Australasian skates (Chondrichthyes: Rajidae). *Journal of Biogeography*. 2002; 29:1627–1641.
- Linnaeus, C. *Systema Naturae*. 10. Holmiae, Salvii: 1758.
- Long JA, Trinajstić K, Young GC, Senden T. Live birth in the Devonian period. *Nature*. 2008; 453:650–652. [PubMed: 18509443]
- Marion KR. One-egg twins in a snake, *Elaphe guttata guttata*. *Transactions of the Kansas Academy of Science (1903-)*. 1980; 83:98–100.
- McEachran JD, Dunn KA. Phylogenetic analysis of skates, a morphologically conservative clade of elasmobranchs (Chondrichthyes : Rajidae). *Copeia*. 1998:271–290.
- Musick, JA.; Ellis, JK. Reproductive evolution of chondrichthyans. In: Hamlett, WC., editor. *Reproductive Biology and Phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras*. Science Publishers, Inc; Enfield, NH: 2005. p. 45-79.
- Naylor GJ, Caira JN, Jensen K, Rosana K, White WT, Last P. A DNA sequence-based approach to the identification of shark and ray species and its implications for global elasmobranch diversity and parasitology. *Bulletin of the American Museum of Natural History*. 2012:1–262.
- Naylor GJ, Ryburn J, Fedrigo O, Lopez J. Phylogenetic relationships among the major lineages of modern elasmobranchs. *Reproductive Biology and Phylogeny*. 2005; 3:25.
- Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. *Bioinformatics*. 1998; 14:817–818. [PubMed: 9918953]
- Rambaut, A. SE-AL v. 2.0a11: sequence alignment program. 2002. (<http://tree.bio.ed.ac.uk/software/seal/>)
- Serra-Pereira B, Figueiredo I, Gordo LS. Maturation of the gonads and reproductive tracts of the thornback ray *Raja clavata*, with comments on the development of a standardized reproductive terminology for oviparous elasmobranchs. *Marine and Coastal Fisheries*. 2011; 3:160–175.
- Spies IB, Gaichas S, Stevenson DE, Orr JW, Canino MF. DNA-based identification of Alaska skates (*Amblyraja*, *Bathyraja* and *Raja* : Rajidae) using cytochrome c oxidase subunit I (coI) variation. *J Fish Biol*. 2006; 69:283–292.
- Swofford, DL. *Phylogenetic Analysis Using Parsimony (* and other methods)*. Sunderland MA: 2002.
- Tinti F, Ungaro N, Pasolini P, De Panfilis M, Garoia F, Guarniero I, Sabelli B, Marano G, Piccinetti C. Development of molecular and morphological markers to improve species-specific monitoring and systematics of Northeast Atlantic and Mediterranean skates (Rajiformes). *Journal of Experimental Marine Biology and Ecology*. 2003; 288:149–165.
- Turan C. Molecular systematic analyses of Mediterranean skates (Rajiformes). *Turkish Journal of Zoology*. 2008; 32:437–442.

Highlights

- We propose a more inclusive *Rajini* tribe that includes the NPA, *Okamejei*, *Raja* (TRA), and *Dipturus*; and a new tribe, the *Rostrorajini*, that results in a subdivision of Rajid species into three well-supported tribal lineages
- Reproductive tactic of depositing multiple embryos per egg capsule arose once during the evolution of skates.
- This strategy is exhibited by only two species in the genus *Beringraja*, with two embryos per egg capsule as the most frequent, and optimal strategy.

Figure 1.

Phylogenetic hypothesis of skates in the family Rajidae based on a concatenated data set from three mitochondrial genes (12s, 16s, COI, 1496 bp total). Support on nodes reflects NJ/MP/BPP that are greater than 50%. Black arrow indicates the family Rajidae. Blue lineage reflects a species assemblage previously described as the North Pacific Assemblage (NPA), and brown reflects the Amphi-American Assemblage (AAA). Gray clades highlight genera that interrupt the genus *Raja*. Black, gray, and red indicate clades where no nominal change is recommended. Blue and brown represents lineages in need of nominal revision. Stars indicate species that exhibit the character of multiple embryos per egg capsule. The bars on the right reflect distinct tribes within the Rajidae—black represents *Amblyrajini*; gray represents *Rajini* and brown represents the *Rostorajini*

Percent embryo survivorship among egg capsule size classes during a 1 year period (n=95)

Figure 2.

Observed survivorship for 95 *B. binocularata* egg capsules deposited within a single year, in an aquarium setting. There were five size classes of egg capsules with 1–5 embryos each. Green represents the proportion of egg capsules that hatched within each size class; blue indicates the proportion of capsules that were still incubating at the end of the observational period; and red indicates the percent that failed to hatch (i.e. died). Egg capsule size class with the highest frequency was two embryos per egg capsule. Egg capsule size class with a single embryo exhibited the second highest frequency. Sample sizes with 3–5 embryos were rare.

Table 1

Inventory of taxonomic sampling used in this study, including 35 taxa for 12S, 38 for 16S and 42 for COI.

Lineage	No. of Genera	No. of species	No. of species included	Scientific name	12S	16S	COI	CONCAT	Common name
Squaliformes				<i>Squalus Acanthias</i>	AY830766.1	EF119335.1	EF539290		Spiny Dogfish
Myliobatiformes				<i>Myliobatis californica</i>	KF317712	KF317716	KF317725		Bat Ray
Rajiformes									
Arhynchobatidae	12	89	1	<i>Bathyrhaja kincaidii</i>	KF317713	KF317717	KF317726		Sandpaper skate
Anacanthobatidae	2	18	0						
Rajidae	15	133	53						
<i>Amblyrajini</i> * 1	Amblyrajini	10	4	<i>Amblyraja badia</i>			FJ164276		Broad skate
				<i>Amblyraja doellojuradoi</i>			EU074312.1		Southern thorny skate
				<i>Amblyraja hyperborea</i>	EF100184.1	EF100184	JF895009		Arctic skate
				<i>Amblyraja radiata</i>	AF448012	AF106038	JF894832		Thorny skate
	Rajella	15	3	<i>Rajella bigelowi</i>			EU148301.1		Bigelow's skate
				<i>Rajella fyllae</i>	EF100182	EF100182	JF894978		Round skate
				<i>Rajella kukuevi</i>	EF100183	EF100183			Mid atlantic skate
	Leucoraja	12	4	<i>Leucoraja circularis</i>	EF100180	EF100180			Sandy skate
				<i>Leucoraja erinacea</i>	KCh22712	KCh22712	KCh22712		Little skate
				<i>Leucoraja fullonica</i>	EF100179	HM140439			Shagreen skate
				<i>Leucoraja naevus</i>	EF100181	EF100181	HM043212		Cuckoo skate
<i>Gurgesiellini</i> * 1	Malacoraja	4	1	<i>Malacoraja kreffii</i>	EF081262	EF081262			Kreffit's skate
<i>Rajini</i> * 1	Neoraja	4	1	<i>Neoraja caerulea</i>	EF100178	EF100178			Blue skate
	6 to 7	see <i>Rostroraja</i> below							
	Okamejei	12	4	<i>Okamejei acutispina</i>	AF448009	EF100189	EU334813.1		Sharpshin skate
				<i>Okamejei kenojei</i>	AY525783	AY525783	EU310804.1		Ocellate spot skate
				<i>Okamejei koreana</i>			EU339351.1		Korean skate
				<i>Okamejei meerdervoortii</i>			EU334814.1		Bigeye skate
	Dipturus	30	16	<i>Dipturus batis</i>	EF081278.1	EF081275	JQ774529		Blue skate
				<i>Dipturus canutus</i>			EU398776		Grey skate
				<i>Dipturus cerva</i>			DQ108189.1		White-spotted skate

Lineage	No. of Genera	No. of species	No. of species included	Scientific name	12S	16S	COI	CONCAT	Common name
True Raja Assemblage* 1,2 TRA				<i>Dipturus confusus</i>			EU398772		Longnose skate
				<i>Dipturus pullopunctatus</i>			GU805679		Slime skate
				<i>Dipturus campbelli</i>			GU804912		Blackspot skate
				<i>Dipturus gudgeri</i>			EU398764.1		Bight skate
				<i>Dipturus kwangtungensis</i>	AF448010		EU339346.1		Kwangtung skate
				<i>Dipturus macrocauda</i>	AF448011				Bigtail skate
				<i>Dipturus australis</i>			DQ108187.1		Sydney skate
				<i>Dipturus argentinensis</i>			EU074407		Argentine skate
				<i>Dipturus nidarosiensis</i>	EF081266	EF081268			Norwegian skate
				<i>Dipturus oxyrinchus</i>	EF081270	EU476893	GU805810.1		Long-nosed skate
				<i>Dipturus tenuis</i>	EF081265.1	EF081265			Acutenose skate
				<i>Dipturus springeri</i>			GU805029		Roughbelly skate
				<i>Dipturus laevis</i>			GU805788		Bamdoor skate
	formerly <i>Dipturus</i>	3	1	<i>Zearaja chilensis</i> *3			EU074404		Yellownose skate
	formerly <i>Dipturus</i>	1	1	<i>Spiriraja whitleyi</i> *4			DQ108181		Melbourne skate
	formerly <i>Dipturus</i>	2	1	<i>Dentiraja lenprien</i> *4		EU848453			Thornback skate
				<i>Raja asterias</i>		GU597962			Starry skate
		13	9	<i>Raja brachyura</i>	EF081263	EF081263	HM043202.1		Blonde skate
				<i>Raja clavata</i>	EF100186	EU476888	HM043195.1		Thornback ray
				<i>Raja microocellata</i>	EF081264.1	EF081264	HM043199		Small-eyed skate
				<i>Raja miraletus</i>		EU476885	HM043183		Twineyed skate
				<i>Raja montagui</i>	EF100188	EF100188	HM043209		Spotted skate
				<i>Raja polystigma</i>	EF100185	EF100185	GU805539		Speckled skate
				<i>Raja radula</i>		EU476896			Rough skate
				<i>Raja undulata</i>	EF100187	EF100187	HM043221		Undulate skate
North Pacific Assemblage* 1,2 NPA									
	1	6	5	<i>Raja inornata</i>	KF317707	KF317720	KF317729		California skate
				<i>Raja rhina</i>	KF317708	KF317722	KF317731		longnose skate
				<i>Raja stellulata</i>	KF317709	KF317723	KF317732		Starry skate

Lineage	No. of Genera	No. of species	No. of species included	Scientific name	12S	16S	COI	CONCAT	Common name
				<i>Beringraja binoculata</i> *5	KF317710	KF317718	KF317727		Big skate
				<i>Beringraja pulchra</i> *5	KF317711	KF317721	KF317730		Mottled skate
<i>Amphi-American Assemblage</i> *1,2									
AAA - <i>Rostrorajini</i> *6	1	7	2	<i>Raja eglanteria</i>	KF317714	KF317719	KF317728		Clearnose skate
				<i>Raja texana</i>	KF317715	KF317724	KF317733		Roundel skate
<i>Rostrorajini</i> *6	Formerly <i>Rajini</i>	1	1	<i>Rostroraja alba</i>	EF081261	EF081261	HM043192		Spearnose skate

Bold indicates 30 original sequences produced for this study. Nomenclature described by: *1 McEachran and Dunn 1998, *2 Ebert and Compagno 2007, *3 Last and Gledhill 2007, *4 Last and Yearsley 2002, *5 Ishihara et al 2012, *6 this study.

Table 2

Summary of characters analyzed and likelihood parameters for model selection with primers for three mitochondrial loci.

	12s	16s	COI	Concatenated
Number bp alignment informative sites	395	497	606	1498
Forward primer				
5'-3'	AAACTGGGATTAGA	CGCCTGTTTATCAAAAACAT	CCGCTTAACTCTCAGCCATC	
Reverse primer				
5'-3'	GAGGGTGACGGGCG	CCGGTCTGAACTCAGATCACG	TCAGGGTGACCAAAGAATCA	
Source	Tinti 2003	Tinti 2003	Spies et al. 2006	
MrModeltest				
hLRT-1	TrN+G	TrN+I+G	HKY+I+G	TrN+I+G
AIC	GTR+G	GTR+I+G	TrN+I+G	GTR+I+G
Model selected				
A	0.3341	0.3638	0.3171	0.3167
C	0.2371	0.2019	0.2658	0.2447
G	0.1874	0.1547	0.1439	0.1504
T	0.2414	0.2796	0.2732	0.2882
Ts/Tv				
pINVAR	0	0.2956	0.5736	0.5341
Gamma	0.1874	0.4351	0.9902	0.64

Bold represents the model selected based on the AIC in MrModeltest. ffff