Short Communication

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 in 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 propose a new assemblage, the Rostrajini, 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 to 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 most frequently and with the highest fitness.

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1. Introduction

The batoids (Superorder Batomorphii) are generally regarded as a monophyletic group of cartilaginous fishes based on morphological characteristics, which is now supported 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 and Reynolds, 1997; Musick and Ellis, 2005; Long et al, 2008; Wourms, 1977). The inferred topology indicating the Rajidae as the ancestral batoid taxon (Aschliman et al., 2012; Naylor et al., 2005); 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

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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), respectively. 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 at the 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 et al., 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 mitochrondrial 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. Here 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), including 13 of the 17 genera shown in bold – ***Okamejei, Dipturus, Raja, Beringraja, Ambylraja, Leucoraja, Rajella, Rostroraja, Zearaja,*** *Breviraja, Dactylobatus, Fenestraraja, Gurgesiella,* ***Spiniraja, Dentiraja, Malacoraja, Neoraja*** (see Ishihara et al., 2012; Last and Yearsley, 2002) – and evaluate the relationships of three species assemblages proposed by McEachran and Dunn (1998) and Ebert and Compagno (2007, Table 1) that include *Raja* species. 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 one to eight per capsule for big skate (*B. binoculata*) species (Ebert, 2003; Hitz, 1964; Ishiyama, 1958). Fitness advantages or tradeoffs associated with 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. binoculata*) 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 850 m, while the big skate is endemic to the eastern North Pacific occurring from Baja California to the eastern Bering Sea at depths up to 800 m, albeit more commonly at depths less than 200 m (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. binoculata* 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, but topologies based on individual loci (Table 1), did not have a uniform taxonomic representation, therefore our combined analysis includes 26 taxa with no missing data. Tissue samples from ten species were sampled directly or obtained from aquarium or museum collections and we provide 30 new sequences. 42 additional sequences were downloaded from public databases (National Center for Biotechnological Information, Table 1). This is the first molecular study to include both *B. pulchra* and *B. binoculata* 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 sample kit (Qiagen Inc., Valencia, CA). We constructed a

Table 1

Inventory of taxonomic sampling used in this study, including 35 taxa for *12S*, 38 for *16S* and 42 for *COI*. Bold indicates 30 original sequences produced for this study. TRA represents the True Raja Assemblage, NPA represents the North Pacific Assemblage, and AAA represents the Amphi-American Assemblage. 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.

Lineage	No. of genera	No. of species	No. of species included	Scientific name	12S	16S	CO1	CONCAT	Common name
Squaliformes				*Squalus Acanthias*	AY830766.1	EF119335.1	EF539290	√	Spiny Dogfish
Myliobatiformes				*Myliobatis californica*	**KF317712**	**KF317716**	**KF317725**	√	Bat Ray
Rajiformes									
Arhynchobatidae	12	89	1	*Bathyraja kincaidii*	**KF317713**	**KF317717**	**KF317726**	√	Sandpaper skate
Anacanthobatidae	2	18	0						
Rajidae	15	133	52						
Amblyrajini [*1]	*Amblyraja*	10	4	*Amblyraja badia*			FJ164276		Broad skate
				Amblyraja doellojuradoi		EU074312.1			Southern thorny skate
				Amblyraja hyperborea	EF100184.1	EF100184	JF895009	√	Arctic skate
				Amblyraja radiata	AF448012	AF106038	JF894832	√	Thorny skate
	Rajella	15	3	*Rajella bigelowi*			EU148301.1		Bigelow's skate
				Rajella fyllae	EF100182	EF100182	JF894978	√	Round skate
				Rajella kukujevi	EF100183	EF100183			Mid atlantic skate
	Leucoraja	12	4	*Leucoraja circularis*	EF100180	EF100180			Sandy skate
				Leucoraja erinacea	**KF561896**	**KF561897**	**KF561898**	√	Little skate
				Leucoraja fullonica	EF100179	HM140439			Shagreen skate
				Leucoraja naevus	EF100181	EF100181	HM043212	√	Cuckoo skate
Gurgesiellini [*1]	*Malacoraja*	4	1	*Malacoraja kreffti*	EF081262	EF081262			Krefft's skate
	Neoraja	4	1	*Neoraja caerulea*	EF100178	EF100178			Blue skate
Rajini [*1,6]	6–7	see Rostroraja below		*Okamejei acutispina*	AF448009	EF100189	EU334813.1	√	Sharpspine skate
	Okamejei	12	4	*Okamejei kenojei*	AY525783	AY525783	EU310804.1	√	Ocellate spot skate
				Okamejei koreana			EU339351.1		Korean skate
				Okamejei meerdervoortii		EU334814.1			Bigeye skate
	Dipturus	30	16	*Dipturus batis*	EF081278.1	EF081275	JQ774529	√	Blue skate
				Dipturus canutus			EU398776		Grey skate
				Dipturus cerva			DQ108189.1		White-spotted skate
				Dipturus confusus			EU398772		Longnose skate
				Dipturus pullopunctatus		GU805679			Slime skate
				Dipturus cambpelli			GU804912		Blackspot skate
				Dipturus gudgeri			EU398764.1		Bight skate
				Dipturus kwangtungensis	AF448010		EU339346.1		Kwangtung skate
				Dipturus macrocauda	AF448011				Bigtail skate
				Dipturus australis			DQ108187.1		Sydney skate
				Dipturus argentinensis		EU074407			Argentine skate
				Dipturus nidarosiensis	EF081266	EF081268			Norwegian skate
				Dipturus oxyrinchus	EF081270	EU476893	GU805810.1	√	Long-nosed skate
				Dipturus tengu	EF081265.1	EF081265			Acutenose skate
				Dipturus springeri			GU805029		Roughbelly skate
				Dipturus laevis			GU805788		Barndoor skate
	Formerly *Dipturus*	3	1	*Zearaja chilensis* [*3]			EUO74404		Yellownose skate
	Formerly *Dipturus*	1	1	*Spiniraja whitleyi* [*4]			DQ108181		Melbourne skate
	Formerly *Dipturus*	2	1	*Dentiraja lemprieri* [*4]		EU848453			Thornback skate
TRA [*1,2]	1	13	8	*Raja asterias*		GU597962			Starry skate
				Raja brachyura	EF081263	EF081263	HM043202.1	√	Blonde skate
				Raja clavata	EF100186	EU476888	HM043195.1	√	Thornback ray
				Raja microocellata	EF081264.1	EF081264	HM043199	√	Small-eyed skate
				Raja miraletus		EU476885	HM043183		Twineyed skate
				Raja montagui	EF100188	EF100188	HM043209	√	Spotted skate
				Raja polystigma	EF100185	EF100185	GU805539	√	Speckled skate
				Raja radula		EU476896			Rough skate
				Raja undulata	EF100187	EF100187	HM043221	√	Undulate skate
NPA [*1,2]	2	6	5	*Raja inornata*	**KF317707**	**KF317720**	**KF317729**	√	California skate

Table 1 (continued)

Lineage	No. of genera	No. of species	No. of species included	Scientific name	12S	16S	CO1	CONCAT	Common name
				Raja rhina	KF317708	KF317722	KF317731	✓	Longnose skate
				Raja stellulata	KF317709	KF317723	KF317732	✓	Starry skate
				Beringraja binoculata *5	KF317710	KF317718	KF317727	✓	Big skate
				Beringraja pulchra *5	KF317711	KF317721	KF317730	✓	Mottled skate
				Rostroraja alba	EF081261	EF081261	HM043192	✓	Spearnose skate
Rostrorajini *6	Formerly Rajini	1	1	Raja eglanteria	KF317714	KF317719	KF317728	✓	Clearnose skate
AAA – *1,2	1	7	2	Raja texana	KF317715	KF317724	KF317733	✓	Roundel skate
	Formerly Raja								
	Formerly Raja								

molecular phylogeny based on three mitochondrial loci: (*12S*, *16S*, *CO1*). 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 (20 μM), 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-KF561898, 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 and Ronquist, 2001) with model selection determined by the Akaike Information Criteria (AIC, see Table 2), as implemented in ModelTest 3.7 (Posada and Crandall, 1998). Bayesian posterier 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* and *COI* were congruent but *12S* was not. However the *12S* topology was not significantly different using the T-PTP 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 Figs. S1–S3).

2.4. Estimating survivorship of egg capsules in an aquarium setting

Beringraja binoculata 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 according 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 to 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

Table 2
Summary of characters analyzed and likelihood parameters for model selection with primers for three mitochondrial loci. Bold represents the model selected based on the AIC in MrModeltest. ffff.

	12S	*16S*	*COI*	Concatenated
Number bp alignment	395	497	606	1498
Informative sites				
Forward primer 5′–3′	AAACTGGGATTAGA	CGCCTGTTTATCAAAAACAT	CCGCTTAACTCTCAGCCATC	√
Reverse primer 5′–3′	GAGGGTGACGGGCG	CCGGTCTGAACTCAGATCACG	TCAGGGTGACCAAAGAATCA	√
Source	Tinti et al. (2003)	Tinti et al. (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
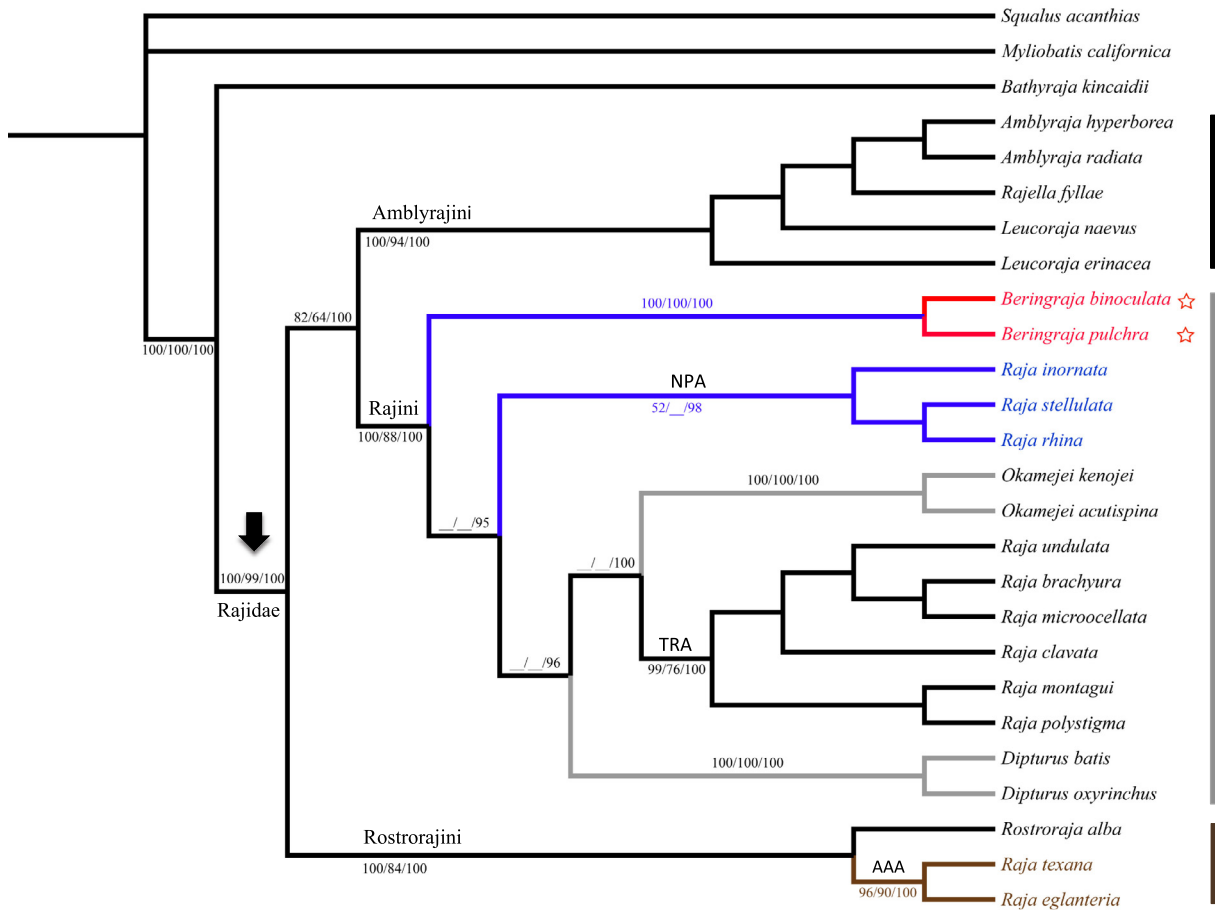
Fig. 1. Phylogenetic hypothesis of skates in the family Rajidae based on a concatenated data set from three mitochondrial genes (*12S*, *16S*, *COI*, 1496 bp total). Support on nodes reflects NJ/MP/BPP that are greater than 50%. Black arrow indicates the family Rajidae. Blue lineage reflects a species 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 Rostrajini.

COI (Tables 1 and 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 the genus *Dipturus*, 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 Amphi-American assemblage (AAA, 100/84/100, Fig. 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 is not associated with the genus *Raja*, and should be renamed. Further, we propose that *Rostroraja alba* plus the other AAA species are part of a unique assemblage that we refer to as the Rostrorajini.

Species assemblage the (TRA) true *Raja* form a monophyletic clade with high support (99/76/100). The genera *Okamejei* and *Dipturus* are closely associated with the TRA (__/__/96, Fig. 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* + TRA (100/88/100, Fig. 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 evolution of skates. To understand fitness advantages associated with this reproductive strategy, we monitored 104 egg capsules of the big skate, *Beringraja binoculata*, deposited throughout a single year. Nine of these were non-fertile and were omitted from the study. Of the 95 remaining egg capsules, a total of 21 hatched,
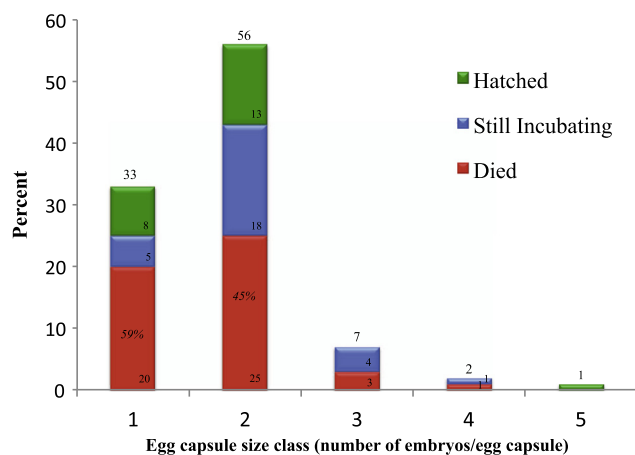
Fig. 2. Observed proportions of big skate (Beringraja binoculata) egg capsules of different size classes deposited within a single year, in an aquarium setting (n = 95). There were five egg capsule size classes with number of embryos varying from 1–5. Green represents the proportion of the total egg capsules that hatched during the observation period within each size class; blue indicates the proportion of capsules that were still incubating at the end of the observation period; and red indicates mortality, or the proportion that died during the observation period. Numbers on the top of each column represent the proportion for specific outcomes within size classes. The percent in italics represents mortality within that particular size class. Of the two most common size classes, two embryos per egg capsule occurred with the highest frequency and the lowest mortality. Egg capsules with greater than two embryos were relatively rare, resulting in small sample sizes.

27 were still incubating at end of observation period, and 47 died. In captivity, 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%, Fig. 2). Egg capsules with 3–5 embryos are rare (Fig. 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 and 207 days. Egg capsules with two embryos range from 164 to 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 were still incubating or did not survive past 102 days.

Mortality was high in all egg capsule size classes. Interestingly, of the two most common size classes, the lowest within class mortality during the observation period (45%, $n = 53$), 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%, $n = 7$), the sample size was too small for comparison. Surprisingly, egg capsules with a single embryo exhibited the highest within class mortality (59%, $n = 32$).

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*, *Dipturus*, and *Rostroraja*. However, neither *Rostroraja alba* nor the *Raja* species included in 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 *Okamejei* 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 *Okamejei* + *Dipturus* + TRA indicates that all remaining taxa with the generic designation of "*Raja*" (not associated with 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*. 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, at least

in an aquarium setting for *B. binoculata*. 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 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 if they are half 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.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.01.012.

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