Universidade Federal do Rio de Janeiro

Campus UFRJ-Macaé Professor Aloísio Teixeira

Programa de Pós-Graduação em Ciências Ambientais e Conservação

Diversidade, distribuição geográfica e relações filogenéticas das savelhas (gênero *Brevoortia*) do Atlântico Sul ocidental inferidas através de análises morfológicas e moleculares (Clupeomorpha, Clupeidae)

Allan Pierre Bonetti Pozzobon

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Ambientais e Conservação, *Campus* UFRJ-Macaé Professor Aloísio Teixeira, da Universidade Federal do Rio de Janeiro, como parte dos requisitos necessários à obtenção do título de Doutor em Ciências Ambientais e Conservação.

Orientador: Prof. Dr. Fabio Di Dario

Co-orientador: Prof. Dr. Pablo Rodrigues Gonçalves

Rio de Janeiro

12/2018

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Rio de Janeiro

12/2018

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RESUMO

Diversidade, distribuição geográfica e relações filogenéticas das savelhas (gênero *Brevoortia*) do Atlântico Sul ocidental inferidas através de análises morfológicas e moleculares (Clupeomorpha, Clupeidae).

Allan Pierre Bonetti Pozzobon
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Co-orientador: Prof. Dr. Pablo Rodrigues Gonçalves

Resumo da Tese de Doutorado submetida ao Programa de Pós-Graduação em Ciências Ambientais e Conservação, *Campus* UFRJ-Macaé Professor Aloísio Teixeira, da Universidade Federal do Rio de Janeiro, como parte dos requisitos necessários à obtenção do título de Doutor em Ciências Ambientais e Conservação.

Brevoortia é um gênero de Clupeidae (Teleostei) que inclui peixes marinhos, micrófagos e de tamanho moderado, distribuídos no norte e sul do Atlântico ocidental. Seis espécies são reconhecidas atualmente. Quatro delas ocorrem no Atlântico Norte, incluindo o Golfo do México: *B. tyranus* (Latrobe, 1802), *B. gunteri* Hildebrand, 1948, *B. patronus* Goode, 1878, e *B. smithi* Hildebrand, 1941. *Brevoortia aurea* (Spix & Agassiz 1829) e *B. pectinata* (Jenyns, 1842) são reportadas para o Atlântico Sul ocidental. Estudos focando nas relações filogenéticas entre todas as espécies de *Brevoorita* e na taxonomia das espécies do Atlântico Sul nunca foram desenvolvidos. O primeiro capítulo desta tese aborda as relações filogenéticas entre as espécies de *Brevoortia*, reconstruídas através de análises de três marcadores moleculares (dois mitocondriais e um nuclear), com um total de 113 espécimes analisados das seis espécies válidas do gênero. No Atlântico Norte, os resultados corroboram estudos anteriores que propuseram uma maior proximidade filogenética entre *B. tyrannus* e *B. patronus*, por um lado, e *B. smithi* e *B. gunteri*, por outro. *Brevoortia aurea* e *B. pectinata*, do Atlântico Sul, formam outro grupo monofilético, irmão do clado composto por *B. smithi* e *B. gunteri* (Atlântico Norte). O clado formado por *B. tyrannus* e *B. patronus*, por sua vez, é basal no gênero. Eventos cladogenéticos foram datados, e indicam que a origem de *Brevoortia* remonta ao Mioceno (há cerca de 15 m.a.). Cenários biogeográficos são propostos a partir das datações inferidas. O segundo capítulo trata da taxonomia e distribuição geográfica das espécies do gênero que ocorrem no Atlântico Sul, *B. aurea* e *B. pectinata*. A validade destas duas espécies têm sido questionada na literatura recente. Uma abordagem utilizando caracteres moleculares e de morfologia foi aplicada para buscar a resolução desta questão. Um total de 254 espécimes, provenientes de toda a distribuição geográfica do gênero no Atlântico Sul, foi analisado. Os caracteres morfométricos revelaram diferenças potenciais entre espécimes de regiões mais ao sul da distribuição *vs*. exemplares do estado do Rio de Janeiro. Entretanto, caracteres merísticos apresentaram uma sobreposição considerável entre todos os exemplares analisados. Além disso, observou-se um padrão de variação gradual relacionado à latitude nos caracteres merísticos, com exemplares dos extremos da distribuição geográfica diferentes entre si, mas com formas intermediárias entre estes extremos. Filogenias baseadas em dados moleculares, inferidas através de Parcimônia, Verossimilhança e Análises Bayesianas, não indicaram clados espécie-específicos, que também nao foram recuperados nas redes de haplótipos. Além disso, as divergências genéticas entre exemplares de diferentes localidades não foram maiores do que aquelas observadas entre espécimes da mesma região. Estes resultados indicam que apenas uma espécie de *Brevoortia* (*B. aurea*, o sinônimo sênior) deve ser reconhecida no Atlântico Sul ocidental.

Palavras-chave: Sistemática e taxonomia, dados merísticos e morfométricos, marcadores genéticos mitocondriais e nucleares.

ABSTRACT

Diversity, geographic distribution and phylogenetic relationships of menhadens (genus *Brevoortia*) from western South Atlantic, inferred through morphological and molecular analyses (Clupeomorpha, Clupeidae).

Allan Pierre Bonetti Pozzobon

Main advisor: Prof. Dr. Fabio Di Dario
Co-advisor: Prof. Dr. Pablo Rodrigues Gonçalves

Abstract of the Ph.D. Thesis submitted to the Graduate Program in Environmental Sciences and Conservation (PPG-CiAC), campus Macaé of the Federal University of Rio de Janeiro (UFRJ), as a requisite to obtaining the Ph.D. degree in Environmental Sciences and Conservation.

Brevoortia is a genus of the Clupeidae (Teleostei) that comprises moderately sized plankton-filtering marine coastal fishes distributed in the western north and south Atlantic. Six species are currently recognized. Four of them occur in the North Atlantic, including the Gulf of Mexico: *B. tyranus* (Latrobe, 1802), *B. gunteri* Hildebrand, 1948, *B. patronus* Goode, 1878, and *B. smithi* Hildebrand, 1941. *Brevoortia aurea* (Spix & Agassiz 1829) and *B. pectinata* (Jenyns, 1842) are reported from the western South Atlantic. Studies focusing on the phylogenetic relationships among all species of *Brevoortia* and in the taxonomy of the species from the South Atlantic were never conducted. The first chapter of this thesis addresses the phylogenetic relationships among all species of *Brevoortia*, reconstructed through the analyses of three molecular markers (two mitochondrial and one nuclear), in a total of 113 specimens representing the six valid species of the genus. In the North Atlantic, results corroborate previous studies that proposed a greater phylogenetic proximity between *B. tyrannus* and *B. patronus*, on the one hand, and *B. smithi* plus *B. gunteri*, on the other. *Brevoortia aurea* and *B. pectinata*, from the South Atlantic, form another monophyletic group, sister to *B. smithi* and *B. gunteri* (North Atlantic). The clade formed by *B. tyrannus* and *B. patronus*, in turn, is basal in the genus. Cladogenetic events were dated and indicate that the origin of *Brevoortia* lies in the Miocene (around 15 mya). Biogeographic scenarios are proposed based on the inferred dates. The second chapter deals with the taxonomy and geographic distribution of the species of the genus that occur in the South Atlantic, *B. aurea* and *B. pectinata*. The validity of both species has been questioned in recent literature. An approach based on morphological and molecular characters was applied in order to solve this question. A total of 254 specimens from all the geographic distribution of the genus in the South Atlantic was examined. Morphometric characters revealed potential differences between specimens from the more southern regions of the distribution *vs.* specimens from the Rio de Janeiro state. However, meristic characters presented a considerable overlap between all the examined specimens. In addition, a pattern of latitudinal gradual variation in the meristic characters was observed, with specimens of the extremes of geographic distribution different among themselves, but with intermediary forms between these extremes. Phylogenies based on molecular data, inferred through Parsimony, Maximum Likelihood and Bayesian Analyses, did not indicate species-specific clades, which were also not recovered in the haplotype networks. In addition, genetic divergences among specimens from different localities were not larger than values observed among specimens from the same region. Those results indicate that a single species of *Brevoortia* (*B. aurea*, the senior synonym) should be recognized in the western South Atlantic.

Key-words: Systematic and taxonomy, meristic and morphometric data, mitochondrial and nuclear genetic markers.

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Introdução geral

1. Relações em Clupeomorpha

Clupeomorpha é uma superordem de Teleostei, que inclui os peixes conhecidos popularmente como sardinhas e manjubas, distribuídos em cerca de 92 gêneros e 405 espécies recentes, além de 150 espécies fósseis (Grande, 1985; Nelson et al., 2016). Praticamente todas as espécies de Clupeomorpha possuem alguma relevância pesqueira, mesmo que seja em pequena escala (Whitehead, 1985; Whitehead et al., 1988). Das 25 espécies marinhas mais capturadas, entre 2005 e 2016, oito são representantes de Clupeomorpha (FAO Yearbook of Fishery Statistics, 2018). Uma das espécie do grupo, *Engraulis ringens* Jenyns, 1842 apresentou a maior média de desembarque, entre 2005 e 2014, 6.522.544 toneladas. *Brevoortia patronus* Goode, 1878, uma das espécies do gênero abordado nesse estudo, esta entre as 25 espécies marinhas com maior captura, apresentando uma média de desembarque, entre 2005 e 2014, igual a 464.165 toneladas, chegando a 618.719 toneladas em 2016 (FAO Yearbook of Fishery Statistics, 2018).

Os estudos mais recentes a lidarem com as relações entre os grandes grupos de Clupeomorpha baseados em morfologia são os de Grande (1985), Di Dario (2002, 2004, 2005, 2009), Di Dario e de Pinna (2006) e de Pinna e Di Dario (2010). A maioria dos estudos focalizados na resolução das relações filogenéticas em Clupeomorpha através da análise de dados moleculares são admitidamente preliminares (Li e Orti, 2007; Lavoué et al., 2007, 2008), embora nos últimos anos algumas tentativas mais amplas de recuperação da história evolutiva do grupo baseadas nessa técnica tenham surgido (Lavoué et al., 2013; Bloom & Lovejoy, 2014).

Lavoué et al. (2014) revisaram recentemente as hipóteses consensuais e contraditórias sobre as relações no grupo, considerando tanto estudos baseados em dados moleculares quanto aqueles oriundos da morfologia. Em linhas gerais, Clupeomorpha divide-se nas ordens Clupeiformes (com espécies modernas e fósseis) e Ellimmichthyiformes (apenas com espécies fósseis) (Chang & Maisey, 2003; Zaragueta-Bagils, 2004). Grande (1985) considerou que ✞*Erichalcis* era um representante basal de Clupeomorpha, possivelmente irmão dos clados restantes do grupo. Arratia (1997), por sua vez, retirou ✞*Erichalcis* de Clupeomorpha, a partir da hipótese de que esse gênero faz parte de Euteleostei. Nessa configuração, Clupeomorpha forma um grupo monofilético a partir de diversas sinapomorfias, sumarizadas por Lavoué et al. (2014), como a extensão da bexiga natatória dentro da caixa craniana se conectando ao ouvido interno, a redução do esqueleto caudal devido à

fusão do segundo hipural ao primeiro centro ural, e a presença de um ou mais escudos abdominais, que são elementos ósseos impares que estendem-se sequencialmente ao longo da linha mediana ventral do corpo.

O monofiletismo de Clupeiformes, a única ordem de Clupeomorpha com táxons modernos, é corroborado pela presença do *recessus lateralis* (estrutura associada com o reconhecimento espacial por pressão e vibrações), entre outros caracteres (Grande, 1985; Di Dario, 2004, 2005; Di Dario e de Pinna, 2006; Lavoué et al., 2014). Clupeiformes divide-se nas subordens Denticipitoidei e Clupeoidei. Denticipitoidei inclui apenas *Denticeps clupeoides* Clausen 1959, endêmico da Nigéria, Camarões e Benin e †*Paleodenticeps tanganikae*, do Terciário da Tanzânia (Greenwood, 1960; Di Dario e de Pinna, 2006; de Pinna e Di Dario, 2010).

Os primeiros estudos no paradigma cladista lidando com as relações filogenéticas em Clupeoidei são da década de 1970. Nelson (1970) dividiu Clupeoidei em quatro superfamílias: Chirocentroidea, Engrauloidea, Pristigasteroidea e Clupeoidea. Grande (1985) encontrou um caráter derivado compartilhado por Chirocentroidea e Clupeoidea (aumento na razão entre costelas pleurais e vértebras pré-urais), e agrupou ambos na superfamília Clupeoidea, como famílias Chirocentridae e Clupeidae. Grande (1985), Di Dario (2005) e Di Dario e de Pinna (2006) propuseram evidências adicionais corroborando o monofiletismo de Clupeoidei, indicando diversas sinapomorfias morfológicas adicionais para o grupo.

Di Dario (1999, 2002) encontrou evidências indicativas de uma relação de grupo irmão entre Clupeoidea e Engrauloidea. Em sua tese de Doutorado, Di Dario (2005) apresentou a hipótese filogenética mais ampla e recente para os grupos de Clupeiformes baseada na morfologia. Uma das conclusões desse estudo é que a maioria dos táxons supragenéricos de Clupeoidei tradicionalmente reconhecidos não são grupos monofiléticos, incluindo a família Clupeidae e suas subfamílias, com exceção de Dorosomatinae (Di Dario, 2005). Posteriormente, Di Dario (2009) e Malabarba e Di Dario (2017) ofereceram indícios de uma maior proximidade filogenética entre as famílias Engraulidae e Chirocentridae, relação que também foi recentemente corroborada através da análise de dados moleculares por Bloom & Lovejoy (2014). Resultados similares em relação ao não monofiletismo de Clupeoidea, de Clupeidae e de suas subfamílias também têm sido obtidos em outros estudos moleculares, como recentemente sumarizado por Lavoué et al. (2014), que concluíram que as relações entre

as famílias de Clupeoidei ainda não estão resolvidas e representam o maior desafio no que se refere às relações filogenéticas em Clupeiformes.

2. O gênero *Brevoortia*

Um dos gêneros tradicionalmente alocados em Clupeidae é *Brevoortia* Gill 1861, que inclui peixes pelágicos marinhos micrófagos e formadores de cardumes, com espécies distribuídas no norte e sul do Atlântico ocidental. Espécies de *Brevoortia* possuem porte relativamente grande (até 50 cm de CP) e, assim como outras espécies típicas de Clupeomorpha, possuem escudos abdominais ossificados que formam uma quilha ao longo da região ventral. Em Clupeidae, *Brevoortia* distingue-se dos outros gêneros pela seguinte combinação de caracteres: cabeça grande em relação ao corpo; maxila entalhada na região mediana; escamas pré-dorsais modificadas, formando uma crista; outras escamas do corpo sobrepondo-se umas às outras de maneira irregular, com suas porções posteriores serrilhadas ou pectinadas (Whitehead, 1985).

As espécies de *Brevoortia* alimentam-se principalmente de plâncton na fase adulta, ocupando uma guilda intermediária na teia trófica, sendo na maior parte do tempo consumidoras primárias e servindo como presa para uma série de consumidores secundários piscívoros de maior porte (Figueiredo & Menezes, 1978; Garcia et al., 2008). Whitehead (1985), que foi o último autor a trabalhar com a taxonomia de *Brevoortia* em nível mundial, reconheceu seis espécies, que atualmente são consideradas válidas (Fricke et al., 2018). Uma breve descrição das principais características destas espécies, de acordo com a literatura (Whitehead, 1985), é apresentada a seguir:

- *Brevoortia aurea* (Spix & Agassiz, 1829; Fig. 1): possui entre 48 e 56 escamas na série lateral e uma mancha escura após a abertura branquial. É frequentemente reportada na literatura como ocorrendo entre o de Rio de Janeiro (Brasil) e a foz do Rio da Prata (Argentina; Whitehead, 1985), embora sua localidade tipo tenha sido descrita como "Bahia". O maior espécime registrado possui 26 cm CP (Whitehead, 1985).

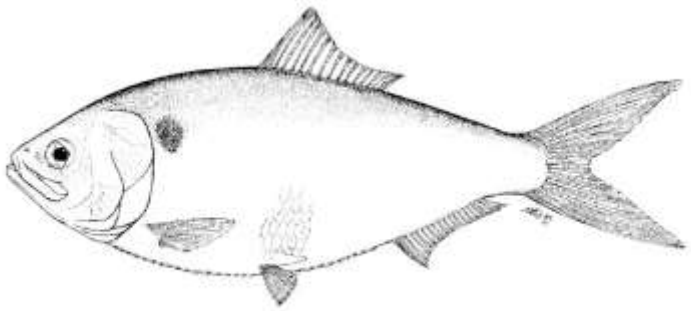

Figura 1. *Brevoortia aurea*, a partir de Whitehead (1985).

- *Brevoortia gunteri* Hildebrand, 1948 (Fig. 2): possui entre 60 e 77 escamas na série lateral e uma mancha escura após a abertura branquial. Ocorre no Golfo do México, Atlântico Norte. Atinge 26,4 cm CP (Whitehead, 1985).

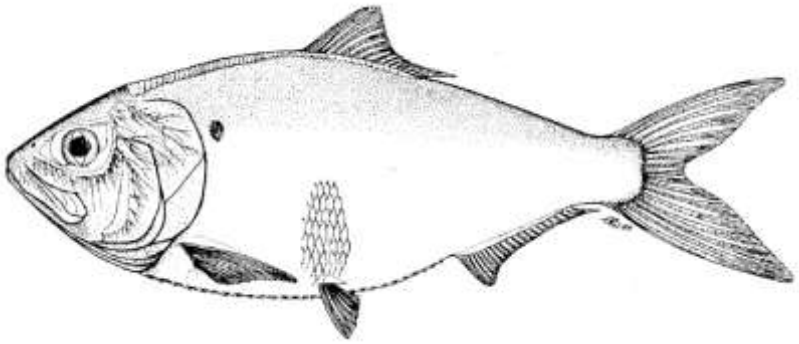

Figura 2. *Brevoortia gunteri*, a partir de Whitehead (1985).

- *Brevoortia patronus* (Goode, 1878; Fig. 3): possui entre 46 e 48 escamas na série lateral e uma mancha escura, mais desenvolvida, após a abertura branquial, seguida de uma série de manchas escuras menores ao longo da lateral no terço superior do corpo. Assim como *B. gunteri*, ocorre no Golfo do México, Atlântico Norte. Atinge 25 cm CP (Whitehead, 1985).

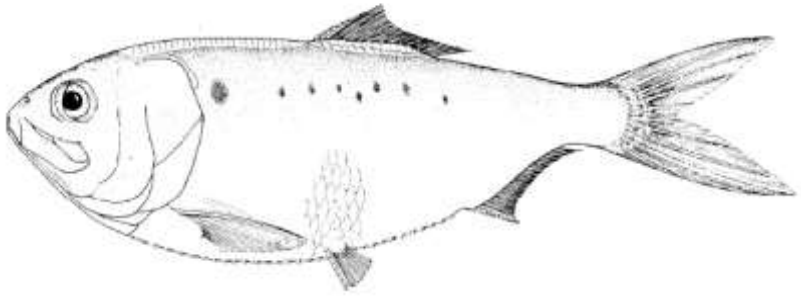

Figura 3. *Brevoortia patronus*, a partir de Whitehead (1985).

- *Brevoortia pectinata* (Jenyns, 1842; Fig. 4): possui entre 35 e 46 escamas na série lateral e apenas uma mancha escura após a abertura branquial. Ocorre entre São Paulo (Brasil) e a foz do Rio da Prata (Argentina). Atinge 30 cm CP (Whitehead, 1985).

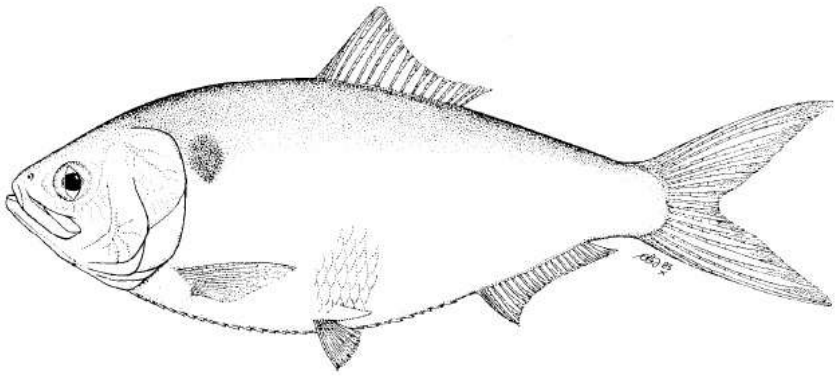

Figura 4. *Brevoortia pectinata*, a partir de Whitehead (1985).

- *Brevoortia smithi* Hildebrand, 1941 (Fig. 5): possui entre 54 e 80 escamas na série lateral (geralmente entre 60 e 70 escamas) e apenas uma mancha escura após a abertura branquial. Ocorre na costa atlântica dos EUA, entre a Carolina do Norte e Flórida, e no Golfo do México, entre a Flórida e Louisiana, Atlântico Norte. Atinge 29 cm CP (Whitehead, 1985).

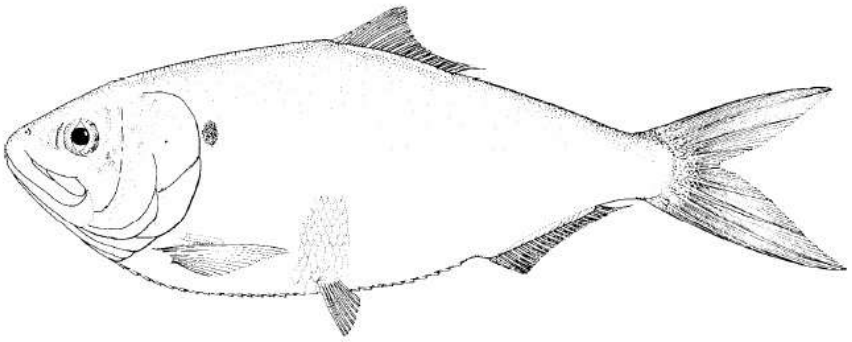

Figura 5. *Brevoortia smithi*, a partir de Whitehead (1985).

- *Brevoortia tyrannus* (Latrobe, 1802; Fig. 6): possui entre 40 e 58 escamas na série lateral (geralmente entre 45 e 52 escamas) e uma mancha escura mais desenvolvida após a abertura branquial, seguida de uma série de manchas escuras menores ao longo da lateral no terço superior do corpo. Ocorre na costa atlântica dos EUA e Canadá, entre

a Nova Escócia e a Flórida. É a maior espécie do gênero, com registros de até 50 cm CP (Whitehead, 1985).

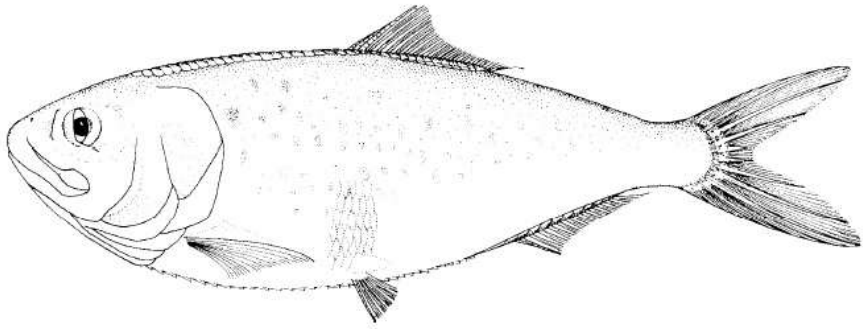

Figura 6. *Brevoortia tyrannus*, a partir de Whitehead (1985).

3. A pesca de "menhaden" nos Estados Unidos

Embora esse estudo não trate diretamente da pesca das espécies de *Brevoortia*, considerações sobre a exploração de algumas espécies do gênero em escala industrial no Atlântico Norte podem ser relevantes para ilustrar cenários que possam vir a ocorrer no Brasil no futuro. Essas considerações também ilustram de maneira prática a relevância das espécies do gênero na pesca industrial no Atlântico Norte.

Das quatro espécies de *Brevoortia* que ocorrem no Atlântico Norte ocidental, duas são reportadas em capturas na pesca industrial: *B. patronus*, com mais de 430 mil toneladas desembarcadas em 2010, e *B. tyrannus*, com desembarque de mais de 220 mil toneladas, também em 2010 (FAO Yearbook of Fishery Statistics, 2012). As outras duas espécies reportadas para aquela região (*B. smithi* e *B. gunteri*) são relativamente menores e menos capturadas pela pesca industrial. Franklin (2007) ofereceu um panorama bastante completo e pormenorizado de diversos aspectos históricos da pesca de *Brevoortia* nos Estados Unidos, onde as quatro espécies são comumente chamadas de "menhaden", desde a época da colonização europeia até os dias de hoje. De acordo com o autor, a pesca de "menhaden" constitui-se em uma das principais atividades industriais dos Estados Unidos, sendo gerida por uma elite formada nos primórdios do país e que controla essa atividade de forma pouco regulamentada, por conta de um forte "lobby" junto aos órgãos de fiscalização e manejo da pesca.

Ainda de acordo com Franklin (2007), a pesca de *Brevoortia* nos Estados Unidos começou como uma atividade artesanal costeira, adquirindo o "status" de pesca industrial ao substituir a atividade de caça às baleias no início do século 20, quando essa atividade faliu. Após a Segunda Guerra Mundial, com o advento de navios-fábricas e

tecnologias pesqueiras mais avançadas, como sonares e a utilização de aviões na busca dos cardumes, a pesca de “menhaden” tornou-se ainda mais lucrativa e ambientalmente agressiva. Hoje em dia, as populações de *Brevoortia* do Atlântico Norte foram dizimadas. Consequentemente, a maior parte dos estados Norte Americanos na região baniu as fábricas de processamento de “menhaden”, mas as consequências da pesca indiscriminada naquele país podem ser observadas até hoje. Um desses efeitos foi a diminuição na captura de outras espécies de peixes de maior porte apreciados na culinária e na pesca esportiva. Outro efeito foi o aumento descontrolado de fitoplâncton, que não mais consumido pelas espécies de *Brevoortia*, causam a eutrofização marinha, levando a uma mortalidade generalizada de espécies (Franklin, 2007).

4. Relações filogenéticas e filogeográficas entre as espécies de *Brevoortia*: o problema dos híbridos

Hipóteses recentes vêm destacando a hibridização como um importante mecanismo na radiação adaptativa e diversificação dos animais (Dowling & Secor, 1997; Seehausen, 2004). Acredita-se que eventos de hibridização sejam mais frequentes em peixes do que em outros grupos de vertebrados (Campton, 1987). Apesar disso, hibridizações entre espécies de peixes marinhos são geralmente entendidas como sendo eventos relativamente raros (Harrison, 1993). De acordo com Roques et al. (2001), a falta de caracteres diagnósticos confiáveis para a identificação de híbridos entre espécies marinhas deve-se ao modo de vida de grande parte dos organismos marinhos, que tipicamente formam grandes agrupamentos populacionais, com altas taxas de migração. Além disso, o ambiente marinho possui barreiras geográficas mais permissivas, fazendo com que o nível de diferenciação entre grupos seja baixo. Consequentemente, acessar divergências entre grupos próximos de peixes marinhos é mais difícil e requer ferramentas mais refinadas (Roques et al., 2001).

Embora híbridos possam contribuir com a variação genética das populações parentais, através de *backcrossing* e introgressão, a hibridização terá impacto limitado na estrutura populacional em grande escala das populações parentais, caso esses eventos sejam raros (Anderson & Karel, 2007). Dessa forma, estudos sobre *backcrossing* e introgressão de híbridos devem ser realizados para se compreender o modo como a diversidade genética de uma espécie se compôs e como pode ser alterada no futuro. Estudos que buscam compreender mecanismos de hibridização em peixes marinhos são

importantes no contexto do presente estudo, pois muitas vezes elucidam problemas relacionados aos resultados encontrados em estudos populacionais de sistemática e de taxonomia (Anderson & Karel, 2007).

Apesar de não existir nenhum relato de híbridos entre as espécies de *Brevoortia* do Atlântico Sul ocidental, esses eventos ocorrem com certa frequência entre espécies do Atlântico Norte ocidental, o que pode dificultar a interpretação dos resultados obtidos nesse estudo. A literatura indica registros de híbridos entre *B. smithi*, *B. tyrannus* e *B. patronus*. A partir da morfologia, Turner (1969), por exemplo, identificou híbridos entre *B. patronus* e *B. smithi* no sudoeste da Flórida, com exemplares apresentando formas morfologicamente intermediarias entre as duas espécies e uma predominância de aproximadamente 98% de machos entre os híbridos. Subsequentemente, Dahlberg (1970) reportou a existência de híbridos entre *B. tyrannus* e *B. smithi* na costa da Flórida, com praticamente todos os híbridos identificados sendo machos.

Anderson & Karel (2007) analisaram populações de *Brevoortia* de locais reconhecidos como zonas de hibridização entre as espécies do gênero, não encontrando, entretanto, formas híbridas entre *B. tyrannus* e *B. smithi*, como anteriormente reportado por Dahlberg (1970). Anderson & Karel (2007), por outro lado, apresentaram o primeiro possível relato de hibridização entre *B. patronus* e *B. tyrannus* na porção leste da península da Flórida. Devido a esses resultados, esses autores propuseram uma extensão da distribuição geográfica de *B. patronus* até a porção leste da península da Flórida, onde a espécie ainda não havia sido registrada. Anderson & Karel (2007) também relataram a ocorrência de híbridos entre *B. patronus* e *B. smithi* em ambos os lados da península da Flórida. Ainda de acordo com os autores, existe uma hibridização assimétrica entre essas duas espécies nessa região, pois todas as sequências de DNA mitocondrial (herança exclusivamente materna) dos híbridos se agruparam com sequências de *B. smithi*.

Anderson (2007) utilizou microssatélites e a região controle do DNA mitocondrial para propor um esquema de relações entre as quatro espécies da América do Norte. Foi encontrada uma maior proximidade entre *B. patronus* e *B. tyrannus*, de um lado, e entre *B. smithi* e *B. gunteri*, do outro. Essa proposta corroborou a percepção tradicional de que existem dois grupos morfologicamente distintos dessas espécies no

Atlântico Norte, um reconhecido pela presença de escamas grandes (*B. patronus* e *B. tyrannus*) e o outro por escamas pequenas (*B. smithi* e *B. gunteri*). Anderson (2007) também concluiu que esses dois grupos (escamas grandes e escamas pequenas) de *Brevoortia* da América do Norte seriam formados por linhagens irmãs que atualmente ocupariam o que seria a distribuição total de seu ancestral comum. No caso do grupo de escamas grandes, a linhagem ancestral ocorreria na costa do Atlântico Norte ocidental e no Golfo do México. Esse grupo ancestral teria originado *B. tyrannus* e *B. patronus* em suas respectivas distribuições geográficas atuais. No grupo de escamas pequenas, uma linhagem ancestral teria originado *B. gunteri* no oeste e *B. smithi* no leste do Golfo do México (Christmas & Gunter, 1960).

Ainda de acordo com os resultados de Anderson (2007), o grupo formado pelas espécies de *Brevoortia* de escamas grandes possui duas linhagens, uma delas contendo espécimes de *B. tyrannus* e *B. patronus* e uma segunda contendo apenas exemplares de *B. tyrannus*. Esses resultados parecem indicar um isolamento pretérito entre *B. patronus* e *B. tyrannus* seguido de um contato secundário entre as populações das duas espécies. Resultados similares foram reportados por Bowen & Avise (1990), que encontraram dois grupos divergentes de haplótipos para as espécies de escamas grandes de *Brevoortia*. Um destes grupos contém espécimes de *B. patronus* e *B. tyrannus*, ao passo que o outro possui apenas exemplares de *B. tyrannus*. Avise (1992) propôs que a explicação mais provável para esse resultado seria um evento de especiação que deu origem a *B. patronus* e *B. tyrannus*, em um primeiro momento, seguido de um contato secundário entre suas populações, com o restabelecimento de fluxo gênico entre elas.

Anderson (2007) também encontrou em sua rede de haplótipos um clado contendo exemplares de *B. smithi* e *B. gunteri* com alto suporte estatístico, o que pode indicar uma separação incompleta entre essas espécies. De acordo com Hildebrand (1963), características morfológicas de exemplares de *Brevoortia* ao oeste da península da Flórida sugerem que essas formas seriam intermediárias entre *B. gunteri* e *B. smithi* provenientes do leste dessa península. Anderson (2007) corroborou as suspeitas de Hildebrand (1963), encontrando uma divergência molecular entre populações de *B. smithi* do oeste e leste da Flórida maior do que a média encontrada entre populações das outras espécies.

5. Estrutura da Tese

Esta tese é dividida em dois capítulos, apresentados no formato de artigos redigidos na língua inglesa. O tema central do Capítulo 1 envolve as relações filogenéticas entre as espécies de *Brevoortia*, a variabilidade genética observada para os diferentes clados encontrados, assim como questões abordando a origem e dispersão do gênero, sendo datados os principais eventos de cladogenese observados. No Capítulo 2, por sua vez, são discutidas questões relacionadas à sistemática e taxonomia de um dos clados encontrados no primeiro capítulo, que abriga as espécies descritas no Atlântico Sul ocidental, sendo utilizadas abordagens morfológicas e moleculares para elucidar uma questão recorrente na literatura científica sobre o real número de espécies do gênero na região. Por último, é apresentada uma breve conclusão geral do estudo.

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Capítulo 1

Phylogenetic relationships and genetic diversity of menhadens, genus *Brevoortia* (Clupeiformes, Clupeidae) in the North and South Atlantic

Abstract

Brevoortia is a genus of the Clupeidae (Teleostei) that includes six species of the fishes commonly known as menhadens in North America and "savelhas" or "saracas" in southeastern South America. Species of *Brevoortia* have a major role in the marine food web of coastal ecosystems in the Atlantic, and also contribute significantly both to industrial and artisanal fisheries, with large catches reported mainly in the USA. In this study, the first phylogenetic hypothesis based on the six currently valid species of the genus *Brevoortia* is presented. A total of 113 specimens were analyzed using three molecular markers (two mitochondrial; COI and 16s; and one nuclear, RAG2). Maximum Parsimony, Maximum Likelihood and Bayesian Inference were employed for phylogenetic reconstructions. The results corroborated previous studies that indicate that the four species of the western North Atlantic are grouped in two distinct clades, one of them comprised by *B. tyrannus* and *B. patronus,* and the other by *B. smithi* and *B. gunteri*. The two species of *Brevoortia* from the western South Atlantic (*B. aurea* and *B. pectinata*) form a third clade, which according to results is sister to *B. smithi* and *B. gunteri*. Therefore, the clade formed by *B. tyrannus* and *B. patronus* is basal in the genus. Phylogenetic hypotheses obtained indicate that *Brevoortia* probably originated in the North Atlantic, during the Miocene (about 15 mya), with subsequent dispersion to the South Atlantic, possibly when the tropical Atlantic was generally colder compared to its modern temperatures. This relatively colder period was followed by a drastic warming of tropical waters during the middle Miocene, a situation that might have promoted the isolation between the northern and southern counterparts of the genus. Molecular analyses also failed to reveal support for the validity of all currently known species of *Brevoortia*, indicating that the taxonomy of all species of the genus is in need of revision.

Introduction

The genus *Brevoortia* Gill 1861 includes marine, coastal pelagic fishes that form large schools, distributed throughout the western portions of the North and South Atlantic, from Nova Scotia (Canada) southwards to northern Argentina, with the exception of the northern South America (Whitehead, 1985). These fishes have been a major component of the traditional fisheries in the more temperate and subtropical portions of the continent, with reports indicating that native Americans in the North Atlantic harvested menhaden before the European invasion of the continent (Franklin, 2007). In South America, traditional fishermen from Santa Catarina, Brazil, recognized "savelhas" as one of their main fisheries resources (Pinho, 2016). Species of the genus continue to sustain large commercial fisheries nowadays, especially in the western North Atlantic and the Gulf of Mexico, where hundreds of thousands of tons are still caught yearly (Franklin, 2007; FAO Yearbook of Fishery Statistics, 2018).

Six species of *Brevoortia* are currently recognized (Whitehead, 1985; Fricke et al., 2018). Four of them are distributed in the western North Atlantic: *Brevoortia tyrannus* (Latrobe 1802), *Brevoortia patronus* Goode 1878, *Brevoortia smithi* Hildebrand 1941 and *Brevoortia gunteri* Hildebrand 1948. Two species, in turn, are reported from the western South Atlantic: *Brevoortia aurea* (Spix and Agassiz 1829) and *Brevoortia pectinata* (Jenyns 1842).

Despite the economic and ecological relevance of menhadens, the number of valid species of *Brevoortia* is still a matter of controversy in the literature, hampering conservation efforts towards a more sustainable use of those fishes. This is especially true for the two supposedly valid species in the western South Atlantic, with can be hardly distinguished between themselves by morphology and also have a partially sympatric distribution (e.g., Whitehead, 1985; Figueiredo and Menezes, 1978; Menezes et al., 2003). As Whitehead (1985: 214) succinctly pointed out in the last global taxonomic revision of the genus "more work is needed to separate it [*B. pectinata*] from *B. aurea* and clarify if their [geographic] ranges really overlap." Indeed, recent morphological and molecular studies focused on South Atlantic menhaden failed in find evidence for the validity of the two species of *Brevoortia*. Cousseau and Diaz de Astarloa (1993), for instance, recognized the existence of a single species in the region between Argentina and Rio Grande do Sul (Brazil) according to morphology, where the

two species are supposed to be sympatric according to traditional literature. Recently, Garcia et al. (2008), analyzing the mitochondrial cytochrome *b* gene of specimens from the coasts of Uruguay, Argentina, and southern Brazil, also recognized the presence of only one species in the region. In the North Atlantic, Anderson (2007) provided the most comprehensive treatment of genetic variation among the species of *Brevoortia* so far, using both nuclear and mitochondrial DNA. Among the results recovered, Anderson (2007) found evidence for a closer relationship between *B. tyrannus* and *B. patronus*, and between *B. gunteri* and *B. smithi*, which were grouped in two distinct clades. Anderson (2007) also found evidence for recurrent events of hybridization between *B. smithi* and *B. patronus* and between *B. smithi* and *B. tyrannus*, suggesting that interspecies admixture might be a common process in the evolutionary history of the genus (Anderson and Karel, 2007). Despite the significance of those results, the phylogenetic relationships among all species of the genus have not been addressed so far.

In this study, the phylogenetic relationships and evolutionary divergence among the six currently valid species of *Brevoortia* are assessed based on the analyses of sequences of one nuclear and two mitochondrial genes under both supermatrix and multispecies coalescent approaches. The patterns recovered allowed inferences on the biogeography of the genus as well as on the extent of genetic exchange among species.

Material and methods

Sampling inventory

Tissue samples (fins and muscle) were obtained from 113 specimens of *Brevoortia* which were tentatively identified through the use of morphology. Sixteen of those specimens were identified as *B. tyrannus*, 17 as *B. gunteri*, 17 as *B. smithi*, 20 as *B. aurea*, 20 as *B. patronus*, and 23 as *B. pectinata* (Table 1). Samples of North American specimens were obtained through exchanges and donations of the Texas Parks and Wildlife Department/USA, and University of Kansas/USA. All samples from South Atlantic specimens are referenced to voucher-specimens deposited in the collections of the Núcleo em Ecologia e Desenvolvimento Socioambiental de Macaé, Universidade Federal do Rio de Janeiro (NPM), and the Coleção de Peixes,

Departamento de Zoologia, IB, Universidade Federal do Rio Grande do Sul (UFRGS). Sequences of 23 specimens of outgroup genera of the Clupeiformes were obtained from GenBank (Table 1). Outgroup taxa were selected to represent the five recognized families in the order (e.g., Di Dario, 2009; Miyashita, 2010; Lavoué et al., 2013). Those genera were chosen also to encompass reliable fossil calibration points. Representatives of the genera *Alosa*, *Sardinops*, and *Sardina*, which are genera closely related to *Brevoortia* according to previous molecular studies (Lavoué et al., 2013; Bloom and Lovejoy, 2014) were also included in the analyses.

DNA isolation and sequencing

Genomic DNA was extracted using a phenol-chloroform protocol (Sambrook et al., 1989). Sequences of the cytochrome oxidase subunit I (COI) and ribosomal subunit 16S (16S) from mitochondrial genome were amplified using the primers described by Ivanova et al. (2007) and Palumbi (1996), respectively. Partial sequences of the nuclear recombination activating gene 2 (RAG2) were amplified using the primers described by Li and Ortí (2007).

Polymerase Chain Reaction (PCR) profiles were the same for the COI and 16S fragments and consisted in 30 cycles of 94°C for 30 seconds, 50°C for 90 seconds and 72°C for 45 seconds. For the RAG2 fragment, the PCR profile consisted of 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds. The battery of cycles was preceded by a 5 min. denaturation at 94°C and succeeded by a 7 min. extension at 72°C. The PCR products were checked on 1.5% agarose gels and cleaned up with ExoSAP-IT (Affymetrix), prior to sequencing reactions, which were run in an Applied Biosystems 3130 Genetic Analyzer (ABI 3130). The primers used in sequencing reactions were the same used in amplification PCRs. COI and 16S sequences were obtained from all 113 tissue samples, but RAG2 sequences were amplified successfully in 59 of those specimens (nine specimens identified as *B. tyrannus* and 10 specimens of the other five species of *Brevoortia*). The sequences were eye checked and aligned on DAMBE 5.0.8 software (Xia and Xie, 2001) using the ClustalW algorithm (Thompson et al., 1997).

Phylogenetic analyses

The phylogenetic analyses were performed using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference (BY) through the softwares PAUP*v.4.0b10 (Swofford, 2002), RAxML-VI-HPC (Stamatakis, 2006) and MrBayes v.3.2 (Ronquist et al., 2011), respectively. The analyses were conducted for the genes isolated, divided into two datasets, a mitochondrial (COI+16S) and a nuclear (RAG2), and into a supermatrix with all the genes concatenated. MP trees were obtained under heuristic search with branch swapping (tree-bisection-reconnection) and starting trees constructed by 100 stepwise random additions of taxa. Only the topologies with the smallest number of steps were retained and the branches with zero lengths were collapsed. Statistical support for the nodes was assessed by 1000 *Bootstrap* replicates (Felsenstein, 1985). ML trees were assessed on the RAxML software (Stamatakis, 2006; http://www.trex.uqam.ca/index.php?action=raxml) using the GTRCAT substitution model and a rapid Hill-climbing algorithm to search for trees. Statistical support for the nodes was assessed by 1000 *Bootstrap* replicates (Felsenstein, 1985). For the Bayesian inference, the best-fit model was selected based on the Bayesian Information Criterion calculated by jModelTest 2 software (Darriba et al., 2012) and then implemented in MrBayes 3.2. Different models were selected for each one of the partitions. The model chosen for the supermatrix was the TIM2+I+G, for COI was HKY+I+G, for the 16S was TPM2uf+I+G and for the RAG2 gene, the TrN+G model was chosen. Posterior distributions of clades and model parameters were sampled every 100 generations across 1 million generations of four Monte Carlo Markov Chains run independently two times. The first 25% of samples were discarded as "burn-in" and the convergence of runs was assessed by examining the MCMC traces of parameters.

Genetic structure and gene flow estimates

The relationships among haplotypes and species were also depicted by haplotype networks constructed by the Median-joining method (Bandelt et al., 1999), using the software Network 5.0 (www.fluxus-engineering.com/sharenet.htm). Haplotype diversity, nucleotide diversity, genetic divergence in terms of pairwise differences between species, average number of pairwise differences (Theta) within species and the number of migrants per generation (Nm) were estimated using ARLEQUIN 3.5.1.2 (Excoffier and Lischer, 2010).

Molecular dating

Phylogenetic dating was conducted through a Multi-Species Coalescent analysis (*BEAST) on BEAST v2.4.3 (Bouckaert et al., 2014). This analysis estimates the species tree that is most probable given a set of gene trees. Here, the supermatrix was partitioned, each gene had their own site model implemented as indicated by Modeltest 3.06 (Posada and Crandall, 1998) and mentioned above. In turn, the "Clock Model" and the "Gene Tree" parameters were treated as two datasets (mitochondrial and nuclear). The "Lognormal relaxed clock (uncorrelated)" model was chosen for the molecular clock, applied to both the dataset. The "Yule model" of speciation was chosen to estimate final species tree.

Operational Taxonomic Units (OTUs), to which each sequence analyzed on BEAST supposedly belongs, were also predefined in the analyses. Species are generally assumed as monophyletic OTUs in the multispecies coalescent analysis, but when species monophyletism or identity are uncertain, as in the case of *Brevoortia*, this assumption becomes problematic and might bias inferences of topology and node dates. To circumvent this problem, OTUs were defined on the basis of haplotype sharing, i.e., specimens that shared the same mtDNA haplotype were grouped under the same OTU, irrespective of their morphological identification.

BEAST v2.4.3 was also used to infer divergence dates between lineages. Four fossil clupeiforms were used to assign minimum dates to specific nodes along the species tree. The Barremian †*Pseudoellimma gallae* (De Figueiredo 2009) is hypothesized as the fossil sister group of the Clupeoidei, therefore the minimum date of divergence between this group and the Denticipitoidei is between 129 - 125 millions of years ago (lognormal prior offset=110 mya; standard deviation=1.6 mya). A date of 18 mya, in turn, was assigned to the most recent common ancestor (MRCA) of the Alosinae (sensu Lavoué et al, 2013), based on †*Moldavichthys switshenskae* (Baykina and Schwarzhans, 2016; lognormal prior offset=17 mya; standard deviation=0.3 mya). A third fossil with reliable identification, †*Dorosoma petenense*, from between 2 and 3 mya (Miller, 1982), was also associated to the node of *Dorosoma* in the analyses (lognormal prior offset=1.3 mya; standard deviation=0.3). Finally, a fossil of *Sardinops melanostictus* (Yabumoto, 1988), found in a geological formation (Hayato Formation) dated around the end of middle Pleistocene (1.8 – 0.12 mya) was also associated with

the node of *Sardinops* included in the analyses (lognormal prior offset=-0.4 mya; standard deviation= 0.14).

The analysis on BEAST v2.4.3 was performed in two independent runs with 2,000,000,000 generations each and parameters sampled every 100,000 steps. The 200,000,000 initial samples were discarded (burn-in of 10%). Other samples were summarized, and the consensus tree with the divergence times was obtained using TreeAnnotator v1.7.2 (Drummond et al., 2012) and visualized in FigTree v1.4.2 (Rambaut, 2012). Tracer 1.6 (Rambaut et al., 2018) was employed to check if the convergence parameters were satisfactory.

Results

Genetic diversity of *Brevoortia*

Mitochondrial DNA:

A total of 80 haplotypes were recovered from the analyses of 113 specimens for the two mitochondrial genes. Overall haplotype diversity for the genus was h=0.707 and nucleotide diversity π=5.813. The genetic divergence in terms of pairwise differences between species ranges from 0.04 (between *B. patronus* and *B. tyrannus*) to 0.89 (between *B. aurea* and *B. patronus*). The average number of pairwise differences (Theta) within species ranged from 3.01 (*B. aurea*) to 12.5 (*B. tyrannus*) (Table 2).

The haplotype network of mitochondrial DNA revealed three groups with a pair of species in each one of them (*B. patronus* and *B. tyrannus*, *B. gunteri* and *B. smithi*, *B. aurea* and *B. pectinata*) (Figure 1). One mitochondrial haplotype is shared between *B. smithi* and *B. gunteri*, and another one is shared between *B. aurea* and *B. pectinata*. Remaining haplotypes are not shared among specimens of the other species tentatively identified by morphology. The number of migrants per generation between species ranged from 0.05 (between *B. patronus* and *B. pectinata*) to 9.76 (between *B. patronus* and *B. tyrannus*) (Table 3). Higher levels of admixture were observed between species that shared same haplotypes or clustered together on the haplotype network (*B. patronus* and *B. tyrannus*=9.76, *B. gunteri* and *B. smithi*=3.82, *B. aurea* and *B. pectinata*=2.2).

Nuclear DNA:

RAG2 sequences of 59 specimens were analyzed, resulting in a total of 40 haplotypes. The genetic divergence in terms of pairwise differences between species ranges from 0.07 (between *B. smithi* and *B. gunteri*) to 0.94 (between *B. aurea* and *B. tyrannus*). The average number of pairwise differences within species ranges from 1.37 (*B. pectinata*) to 5.15 (*B. patronus*) (Table 2).

The haplotype network of nuclear DNA revealed four groups. Two pairs of species are also grouped together (*B. gunteri+B. smithi* and *B. aurea+B. pectinata*), as in the mitochondrial haplotype network, with the difference that *B. patronus* and *B. tyrannus* were not grouped together in the haplotype network of nuclear DNA (Figure 2). One nuclear haplotype is shared between *B. smithi* and *B. gunteri*, and another one is shared between *B. aurea* and *B. pectinata*. Remaining haplotypes are not shared among specimens of the other species tentatively identified by morphology. Estimates of admixture between species according to RAG2 were considerably lower than those derived from mitochondrial markers, ranging from 0.015 (between *B. aurea* and *B. tyrannus*) to 2.91 (between *B. gunteri* and *B. smithi*) migrants per generation (Table 3). As in the mitochondrial data, higher levels of admixture were observed between species clustered together in the haplotype network (*B. gunteri* and *B. smithi*=2.91, *B. aurea* and *B. pectinata*=2.44). The number of migrants between *B. patronus* and *B. tyrannus* inferred by the RAG2 gene was considerably lower (0.065) than that inferred by mitochondrial genes.

Phylogenetic trees and dates of cladogenetic events

Phylogenetic trees obtained for each one of the genes were similar regardless of the optimality criteria employed for the reconstruction. Phylogenetic trees obtained with Parsimony, Bayesian and Likelihood methods are also almost identical when the concatenated matrix of the genes, with 2116 base pairs, is analyzed, except by some branches that were not recovered in the Parsimony analyses (Figure 3). In all trees, *Brevoortia* is monophyletic, with high values of statistical support (Figure 3). The sister group of *Brevoortia* among taxa included in the analyses is *Alosa*, and that assemblage, in turn, is sister to a clade composed of *Sardina* and *Sardinops*. *Brevoortia* is divided into three larger clades according to the results of all analyses (Figure 3). One of these larger clades is composed of specimens previously identified as *B. tyrannus* and *B.*

patronus. That clade is, in turn, sister to two further assemblages, one of them formed by specimens identified as *B. gunteri* and *B. smithi*, and the other by specimens identified as *B. aurea* and *B. pectinata* according to morphology. The phylogenetic structure within these three larger clades of *Brevoortia*, however, failed to reveal species-specific clades.

Similar results were also obtained in the Multispecies Coalescent trees, in terms of the monophyly of *Brevoortia*, the existence of three assemblages within the genus, and the lack of support for species-specific clades within these three main assemblages. The estimated dates of cladogenetic events are as follows (Figure 4): origin of *Brevoortia* is dated at approximately 15.05 mya (9.74–19.16 mya), the separation of the two main clades in the genus (((*B. tyrannus*+*B. patronus*) *vs*. ((*B. smithi*+*B. gunteri*) and (*B. pectinata*+*B. aurea*))) is dated at 10.97 mya (4.96–16.96 mya), with a further separation between *B. smithi*+*B. gunteri* and *B. pectinata*+*B. aurea* estimated at 6.36 mya (2.44–10.32 mya).

Discussion

General overview

The result obtained in the present study, the first phylogenetic study to include all currently recognized species of *Brevoortia*, overall indicate the genus as monophyletic and that three clades or groups of species should be recognized within the assemblage. In the last major review of the genus, Hildebrand (1948) proposed two possible groupings of species in *Brevoortia* based on anatomical features. According to him, when the size and arrangement of scales are considered, two groups of *Brevoortia* might be recognized. One of them includes the small-scaled menhadens, *B. smithi* and *B. gunteri*, which are distinct from the group formed by *B. tyrannus*, *B. patronus* and the South American *B. aurea* and *B. pectinata*, all of which have relatively large scales. When the shape of the pelvic fins is considered, in turn, Hildebrand (1948) concluded that a different arrangement of species might be recognized: one group would include *B. tyrannus* and *B. patronus*, which have “rounded fins”, i.e., the last pelvic fin-ray is not markedly shorter than the first ray, whereas the group formed by *B. smithi*, *B. gunteri*, *B. aurea*, and *B. pectinata* have nearly straight to oblique pelvic-fin margins since the

first pelvic-fin ray is markedly longer than the last ray. The results obtained herein might help to contextualize Hildebrand's (1948) observations in a proper phylogenetic context: both the "small scaled menhadens" (*B. smithi* and *B. gunteri*) and the "rounded pelvic-fin menhadens" (*B. tyrannus* and *B. patronus*) are distinct monophyletic assemblages within the genus. Anderson (2007) also concluded that the same two groups of *Brevoortia* should be recognized in the North Atlantic based on the analyses of the control region of mitochondrial DNA and microsatellites. However, Anderson (2007) did not include the South American species of *Brevoortia* in his analyses, therefore the monophyly of those groups was not properly tested. Circumstantial evidence supporting the same arrangement or groups of species in the North Atlantic is also found in the eggs, larvae, juveniles and adults (Dahlberg, 1970; Ahrenholz, 1991; Tolan and Newstead, 2004).

Evidence that the northern populations of *Brevoortia* might not constitute a single monophyletic assemblage irrespective of their close geographic proximity were previously reported by Anderson and McDonald (2007), who found a relatively large degree of morphological (meristic) and genetic (*F*st value) difference between *B. patronus* and *B. gunteri* in the Gulf of Mexico. More recently, Egan et al. (2018) found support for a sister group relationship between *B. aurea* and *B. smithi*, with *B. tyrannus* and *B. patronus* constituting a separate clade (*B. pectinata* and *B. gunteri* were not included in their analyses). Overall, these results and the ones obtained in this study strongly indicate that the North American species of *Brevoortia* indeed do not constitute a clade, a hypothesis that opens up a series of interesting questions in terms of biogeographic interpretations.

Cladogenetic and biogeographic events

The principal cladogenetic events identified herein in the case of *Brevoortia* seem to be restricted to the middle and late Miocene (Figure 4). The genus apparently diverged from the most recent common ancestor shared with *Alosa* in what is today recognized as the North Atlantic at around 15 mya. Similar dates for the separation between *Alosa* and *Brevoortia* were also recently obtained by Egan et al. (2018). It is interesting to note that species of both genera are restricted to the North Atlantic and the modern remnants of the western Tethys Ocean, including the Black and Caspian Seas, with the exceptions of *B. aurea* and *B. pectinata* (e.g., Whitehead, 1985), which are

discussed below. The dates of cladogenetic events uncovered herein and this pattern of distribution suggest that the distribution of the most recent common ancestor shared by *Alosa* and *Brevoortia* was already restricted to that portion of the Tethys Ocean before the closure of the Tethyan Seaway at around 13 mya, when the African/Arabian and Iranian/Eurasian plates collided in the upper Burdigalian (Reuter et al., 2007; Bowen et al., 2016). Recent evidence indicate that the continuous shallowing and closure of the Tethyan Seaway, since at least the early Burdigalian, generated an increasingly hypersaline environment which might have acted as a barrier for some components of the marine biota millions of years before the rise of the land bridge connecting Africa and Eurasia (Reuter et al., 2007). That condition might have prevented the dispersion of the ancestor shared by *Alosa* and *Brevoortia* from the western Tethys Ocean to the Proto Indo Pacific during that period, restricting the modern descendants of that form to the Atlantic Ocean and adjoining seas (e.g, the Caspian and the Black Seas) in its present configuration.

The phylogenetic relationships and dates of cladogenetic events hypothesized herein also indicate that the two main clades of *Brevoortia* (*B. tyrannus*+*B. patronus* vs. *B. smithi*+*B. gunteri* and *B. aurea*+*B. pectinata*) diverged in the proto-western North Atlantic during the middle Miocene, at approximately 11 mya (Figure 3; Ogg et al., 2016). In that scenario, it is presumed that the ancestor of the clade formed by *Brevoortia aurea* and *B. pectinata* secondarily dispersed to what is now recognized as the western South Atlantic from the north, as discussed below. During the Miocene, marine conditions fluctuated substantially in North America, and the Serravalian, in particular, was characterized by a cold climate with wildly fluctuating sea levels (Petuch 2004, 2012; Petuch and Drolshagen, 2009). In addition, evidence based mostly on the taxonomically diversified record of fossil marine gastropods indicate the existence of two coastal paleoprovinces in the eastern coast of North America from the late Oligocene (Chattian) to the Tortonian in the late Miocene, which were recognized as the paratropical Transmarian province in the north and the mostly eutropical Baitoan or Gatunian province in the south (Petuch, 2004; Landau et al., 2007). The relatively large Baitoan province of Petuch (2004) extended from South Carolina across the Gulf of Mexico to northern Brazil, and also included the region between southern California to northern Peru in the Pacific. Still according to Petuch (2004), fossil gastropods would further indicate that the Baitoan province was actually divided into several

subprovinces. The Onslowian, Chipolan and Agueguexquitean subprovinces of Petuch (2004), in particular, extended from modern-day North Carolina to the Gulf of Mexico. It is presumed that those provinces actually presented a latitudinal variation from a more paratropical in the north to a fully eutropical condition in the south (Petuch, 2004).

Petuch's (2004, 2013) biogeographic scheme has been criticized and further refined (e.g., Allmon, 2005; Landau et al., 2007; Petit, 2013), but it is interesting to note that the geographic range of the combined Transmarian province plus the Onslowian, Chipolan and Agueguexquitean subprovinces of Petuch (2004, 2013) is actually largely coincident with the modern-day distribution of *Brevoortia* in the North Atlantic. The specific conditions that promoted the subdivision of North America specimens of *Brevoortia* into its two main lineages cannot be determined at the moment, but it is suspected that this cladogenetic event is likely related to the complex paleo-biogeographic scenario of the coastal portion of that region, as evidenced by the fossil record of gastropods, in association with the aforementioned fluctuating climatic conditions experienced during the Miocene, in particular during the Serravalian. Fossil gastropods indicate that coastal paleoprovinces in eastern North America were again rearranged in the late Tortonian, and a single Caloosahatchian paratropical province resulting from the coalescence of the Agueguexquitean, Chipolan, and Onslowian subprovinces (Baitoan province) and of the Transmarian province can be recognized in the region between Nova Scotia in the north and Texas in the south, including the Gulf of Mexico (Petuch, 2004; Landau et al., 2007). The Caloosahatchian province is largely equivalent with the modern warm-temperate Carolina province of Briggs and Bowen (2012, 2013) or the northern province of Robertson and Cramer (2014), which essentially encompasses the distribution of *Brevoortia* in the North Atlantic.

The Carolina province of Briggs and Bowen (2012, 2013) is presumably divided into two parts [but see Robertson and Cramer (2014) for a different opinion]. The "Atlantic section" is located in the region between Cape Canaveral in the Florida peninsula and Cape Hatteras in North Carolina. The "Gulf section", in turn, would be located in the northern portion of the Gulf of Mexico between Cape Romano, Florida, and Cape Rojo in Mexico (Briggs and Bowen, 2012). If the paleo-biogeographic scenario proposed above for the subdivision of *Brevoortia* into its two North Atlantic lineages during the late Miocene (Tortonian; 12 mya) is correct, as the evidence indicated, it is interesting to note that this pattern of diversification contradicts results

obtained in a significant number of cases that indicate that population disjunctions between the Atlantic and the Gulf sections usually date to the late Pliocene or even the Pleistocene, as summarized by Bowen and Avise (1990).

One of the most interesting outcomes of the phylogenetic analyses presented herein is that the North Atlantic species of *Brevoortia* do not compose a monophyletic assemblage, as both the South American *B. aurea* and *B. pectinata* form a distinct clade that is sister to the northern *B. smithi* and *B. gunteri* (Figure 3). Indeed, as succinctly stated by Bowen et al. (2016: 7965), "species with disjunct distributions on both sides of the tropics provide fascinating subjects for phylogeographic study". In the Clupeiformes, for instance, *Sardinops* (Clupeidae) and *Engraulis* (Engraulidae) are represented by sister-pairs of antitropical species, whose biogeographic affinities have been recently studied (Bowen and Grant, 1997; Grant et al., 2005; Bowen et al., 2016). In both cases, evidence indicates that the splitting between the northern and southern species on each sister-pair occurred recently in geological terms, more specifically at approximately one mya in the case of *Sardinops caeruleus* (north) vs. *Sardinops sagax* (Bowen and Grant, 1997). Another particularly interesting antitropical pattern of distribution involving sister clades in the genus *Engraulis* is hypothesized as being the case of the eastern North Pacific *E. mordax* vs. the clade formed by the South American species of the genus, *E. ringens* (Pacific) and *E. anchoita* (Atlantic). Grant et al. (2005), for instance, concluded that the separation between the northern and southern forms of that assemblage occurred at around 7 to 10 mya, in the late Miocene, and might have been prompted by changes in climates and ocean circulation patterns.

Similarly, the cladogenetic event leading to the formation of the South American lineage of *Brevoortia* (*B. aurea+B. pectinata*) and the lineage including *B. smithi* and *B. gunteri* in the north is dated at approximately 6 mya, between the late Tortonian and early Messinian in the Miocene (Figure 3; Oggs et al., 2016). Haq (1980) identified four warming and cooling cycles of about 4 to 4.5 mya during the Miocene based on the paleo-biogeographic patterns of distribution of fossil calcareous nannoplankton in the North and South Atlantic, including the Caribbean and Gulf of Mexico. He identified a particularly strong "warm episode" in the Atlantic between 9 and 7.5 mya, a time frame that is largely coincident with the hypothesized date of separation between those two lineages of *Brevoortia*. Perhaps not coincidentally, during warm episodes in the middle and late Miocene the dominantly cold *Coccolithus pelagicus* nannoplankton

disappeared from the South Atlantic, being restricted to higher latitudes in the north, presumably due to the intensification of the equatorial thermal barrier. In addition, Haq (1980) also identified a peak of cold temperature in the Atlantic at around 10 mya.

We propose that this particularly strong peak of cold temperatures in the Atlantic at around 10 mya would have favored the dispersion of the ancestor of the clade composed by the South American species of *Brevoortia* plus *B. smithi* and *B. gunteri* into the South Atlantic. In that hypothesis, the relatively strong warming event that apparently occurred in the Atlantic between 9 and 7.5 mya would have caused a vicariant event leading to the isolation of the northern and southern populations of *Brevoortia* within that assemblage.

The colonization of western South Atlantic waters from the north has been debated extensively in the recent literature. Evidence generally indicates that the direction of dispersion between those compartments of the western Atlantic is primarily from north to south, as in the case hypothesized herein for *Brevoortia* (Robertson et al., 2006; Rocha et al., 2008; Caires, 2014; Briggs and Bowen, 2013; Reis et al., 2016; Pinheiro et al., 2018). For most groups of fishes examined so far, it is proposed that dispersion from north to south occurred in the Pliocene and Pleistocene (e.g., Rocha, 2003; Floeter et al., 2008; Pinheiro et al., 2018), but a few examples of more ancient migrations are also known. White (1986), for instance, proposed a complex pattern of colonization and vicariant events that would have shaped the relationships between members of the Atherinopsinae and Menidiinae in the American continent, with the divergence of the modern tribes of the Atherinopsine in the northern and southern hemispheres following climatic disruption in the Miocene. Burridge (2002), in turn, reviewed the phylogenies of 13 antitropical Pacific groups of fishes and concluded that three group-pairs in the Cliniidae (Myxodini), *Engraulis* (Engraulidae) and *Goniistius* (Latridae) also probably diverged during heating episodes in the middle Miocene.

Alpha-level taxonomy

Despite not being in the scope of this study, results obtained did not reveal species-specific clades within the three main assemblages of *Brevoortia* identified herein, indicating that the alpha-level taxonomy of the genus might be in need of

revision. Those results are also broadly congruent with previous studies that analyzed different subsets of populations of *Brevoortia* in different parts of the Atlantic. Garcia et al. (2008), for instance, employed the cytochrome b to analyze the population structure and phylogenetic relationships of *Brevoortia* in South America between Mar Chiquita lagoon (Argentina), Uruguay and Rio Grande do Sul (Brazil). They observed a complex population dynamic without a clear indication of the existence of different species-specific lineages in the region, supporting the idea that only one species of *Brevoortia* inhabit that portion of the South Atlantic. The levels of genetic divergence found for the mitochondrial and nuclear dataset in our study also supports the hypothesis that a single species of *Brevoortia* should be recognized in the western South Atlantic. The haplotype network and the high values (2.2 for the mitochondrial and 2.44 nuclear; Table 3) of Nm between the groups of specimens tentatively identified as *B. aurea* vs. *B. pectinata* through morphology also support the hypothesis that a single species of the genus should be recognized in the western South Atlantic. Nevertheless, further analyses of larger and geographically more representative samples are necessary in order to clearly settle this question. Similar results indicating the existence of a single species in the region based on morphology, which however also relied on a geographically restricted sample in the western South Atlantic, were previously reported by Reintjes (1969), Cousseau and Díaz de Astarloa (1993), and Segura and Díaz de Astarloa (2004).

In the North Atlantic, Anderson (2007) found a complex pattern of phylogeographic relationships among specimens in the clades composed by *B. tyrannus*+*B. patronus* and *B. gunteri*+*B. smithi*. Mitochondrial sequences did not indicate species-specific clades that are compatible with the currently accepted taxonomy of the species reported for the region. In the clade composed by specimens identified as *B. tyrannus* and *B. patronus* ("large-scaled menhadens"), for instance, Anderson (2007) found two separated lineages, one including only specimens identified as *B. tyrannus* and a second lineage including specimens identified as *B. tyrannus* and *B. patronus*. According to Anderson (2007), analyses of the mitochondrial sequences might indicate genetic isolation followed by secondary contact/hybridization between the two species that compose the clade of large-scaled menhadens, if these species are valid. Similar results were also reported by Bowen and Avise (1990) based on the analyses of mitochondrial DNA. Finally, Lynch et al. (2010) concluded that the

analyses of mitochondrial cytochrome c oxidase subunit I (COI) and of seven nuclear microsatellite loci indicated a pattern of relationship among specimens identified through morphology as *B. tyrannus* and *B. patronus* that is more characteristic of conspecific populations rather than that of separate species.

In the case of the clade formed by *B. gunteri* and *B. smithi* (the "small-scaled menhadens"), Anderson (2007) suggested that both putative species are derived from a single ancestral lineage with incomplete genetic divergence. Anderson (2007) also remarked on the high values of *F*st between two populations of *B. smithi* from the southern United States and the Gulf of Mexico, which are presumably separated by the Florida peninsula. Those values suggest a certain degree of isolation between those two populations, a hypothesis that could not be explored in this study since all sequences included in the analyses were originally obtained from specimens collected on the Gulf side of the Florida peninsula.

Therefore, we reinforce the necessity of the inclusion of a great number of specimens, scattered along the geographical distribution of each one of the three clades obtained in the present study, making possible fine-scale analyses about each one of the clades found here (*B. tyrannus*+*B. patronus*; *B. simithi*+*B. gunteri*; *B. aurea*+*B. pectinata*).

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Tables and Figures

Table 1: Tissue samples examined, with collection site/year and the Genbank code for each sample.

	Collection site/ dates/ previous code	Acronyms used in the present work	Genbank codes for COI	Genbank codes for 16S	Genbank codes for RAG2
Brevoortia tyrannus°*	*Chesapeake Bay- Virginia- USA/ 2002	BTY (01, 02, 04, 05, 07 to 11, 13 to 18)			
	°Atlantic Ocean- Virginia- USA/ ?/ KU:IT 1498	BTY (21)			
Brevoortia patronus°*	*Sabine lake- Texas- USA/ 2012	BPT (22 to 41)			
	°Off Brownsville- Texas- USA/ ?/ KU:IT 5103	BPT (21)			
*Brevoortia gunteri**	Sabine lake- Texas- USA/ 2004	BGU (01 to 17)			
*Brevoortia smithi**	Charlotte Harbor- Florida- USA/ 2003	BSM (01 to 18)			
Brevoortia aurea	Rio de Janeiro- RJ- Brazil/ 2015	BAU (01 to 20)			
Brevoortia pectinata^[#]	^Rio Grande- RS- Brazil/ 2015	BPE (01 to 15)			
	[#]Tramandaí- RS- Brazil/ 2013/ UFRGS 17689 (TEC3428 A/B), UFRGS 17685 (TEC 3424 A to F)	BPE (16 to 23)			
Alosa pseudoharengus		Alosa	NC_009576.1	NC_009576.1	DQ912149.1
Sardina pilchardus		Sardina	MFLPI128	EU419754	DQ912158.1
Ethmalosa fimbriata		Ethmalosa	AP009138.1	AP009138	
Tenualosa toli		Tenualosa	NF581	NC_016700.1	
Dorosoma cepedianum		Dcepedianum	JN025294.1	EU552748.1	DQ912132.1
Dorosoma petenense		Dpetenense	NC_009580.1	NC_009580.1	KJ158111.1
Opisthonema oglinum		Opisthonema	GU702358.1	EU552783.1	DQ912144.1
Sardinella aurita		Sardinella	KM538516.1	KR056175.1	KP325122.1
Harengula jaguana		Harengula	KF929959.1	EU552780.1	DQ912156.1
Pellonula leonensis		Pellonula	NC_009591.1	NC_009591.1	DQ912166.1
Hilsa kelle		Hilsa	AP011613.1	AP011613.1	
Nematalosa come		Nematalosa	NC_021447.1	NC_021447.1	
Anodontostoma chacunda		Anodontostoma	KC466691.1	KC466691.1	
Clupanodon thrissa		Clupanodon	NC_018600.1	NC_018600.1	
Sardinops sagax		SardinSag	JF494412.1	HQ592235.1	
Sardinops melanostictus		SardMel	JF952843.1	AB246173.1	
Pellona flavipinnis		PellFlav	KU289001.1	DQ912064.1	DQ912134.1
Ilisha elongata		IlisElon	HM030780.1	FJ870910.1	DQ912160.1

Coilia Mystus	CoiliaMy	KF056322.1	KF056322.1	DQ912162.1
Anchoviella lepidentostole	AnchoLep	JQ365222.1	EU552735.1	JQ012635.1
Chirocentrus dorab	ChirDor	AP006229.1	AP006229.1	DQ912163.1
Etrumeus teres	EtrTer	NC_009583	NC_009583	DQ912143.1
Denticeps clupeoides	Dclupeoides	NC007889.1	NC007889.1	DQ912133.1

* Samples donated by Dr. Joel D. Anderson from Texas Park and Wildlife Department, TX/USA.
° Samples donated by Dr. Andrew Bentley from the University of Kansas, KS/USA.
^ Samples donated by Dr. Luis Gustavo Cardoso from Universidade Federal de Rio Grande (FURG), RS/ Brazil and colected by Dr. Luciano G. Fischer from Universidade Federal do Rio de Janeiro (UFRJ).
# Samples donated by Dr. Luiz R. Malabarba from Universidade Federal do Rio Grande do Sul (UFRGS), RS/ Brazil.

Table 2: Pairwise genetic differences between (*F*st's Distance Method) and within (Theta pi) species. Above diagonal: nuclear pairwise differences between species; diagonal in bold: mitochondrial/nuclear pairwise differences within species; below diagonal: mitochondrial (COI+16S) pairwise differences between species.

	B. aurea	*B. gunteri*	*B. patronus*	*B. pectinata*	*B. smithi*	*B. tyrannus*
B. aurea	**3.01/ 1.48**	0.73	0.85	0.09	0.72	0.94
B. gunteri	0.84	**5/ 3.35**	0.80	0.73	0.07	0.89
B. patronus	0.89	0.87	**5.53/ 5.15**	0.86	0.79	0.79
B. pectinata	0.18	0.84	0.89	**3.23/ 1.37**	0.74	0.94
B. smithi	0.83	0.11	0.87	0.83	**5.58/ 3.64**	0.88
B. tyrannus	0.81	0.79	0.04	0.82	0.79	**12.5/ 1.66**

Table 3: Number of migrants per generation between species. Above diagonal: number of migrants between species inferred by nuclear RAG2 sequences; below diagonal: number of migrants between species inferred by the mitochondrial (COI+16S) sequences.

	B. aurea	*B. gunteri*	*B. patronus*	*B. pectinata*	*B. smithi*	*B. tyrannus*
B. aurea		0.09	0.04	2.44	0.09	0.01
B. gunteri	0,09		0.06	0.08	2.91	0.02
B. patronus	0,05	0.06		0.03	0.06	0.06
B. pectinata	2.2	0.09	0.05		0.08	0.01
B. smithi	0,09	3.82	0.07	0.09		0.03
B. tyrannus	0.11	0.12	9.76	0.1	0.13	

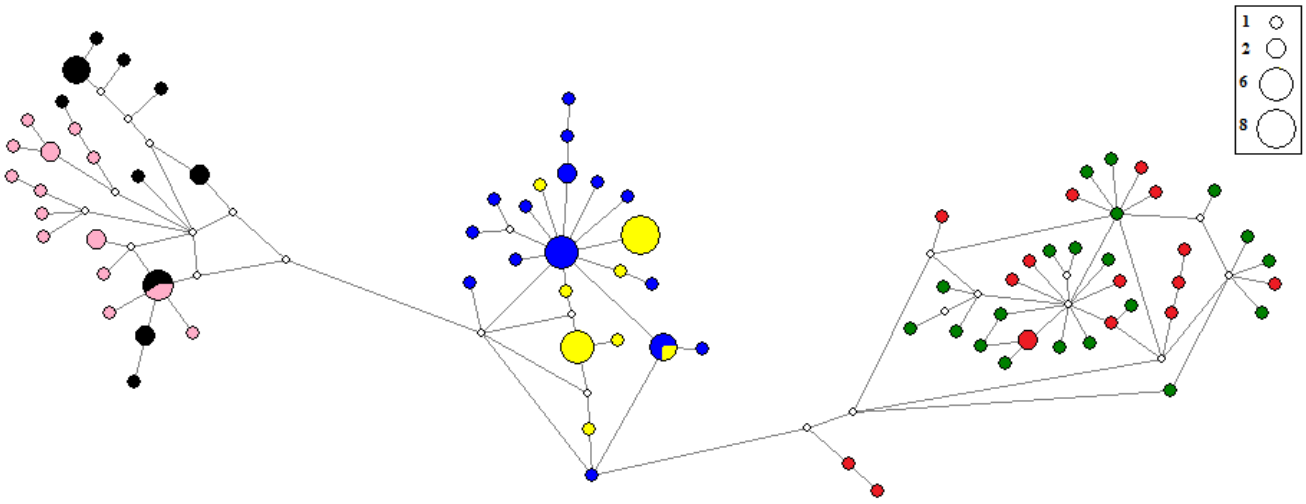

Figure 1: Haplotype network of mitochondrial sequences (COI+16S). Sizes of circles represent the haplotype frequency, upper right black box, related circle sizes with the number of specimens that presents that haplotype. Red=*B. tyrannus*; green=*B. patronus*; yellow=*B. aurea*; blue=*B. pectinata*; pink=*B. gunteri*; black=*B. smithi*. White circles indicate missing haplotypes.

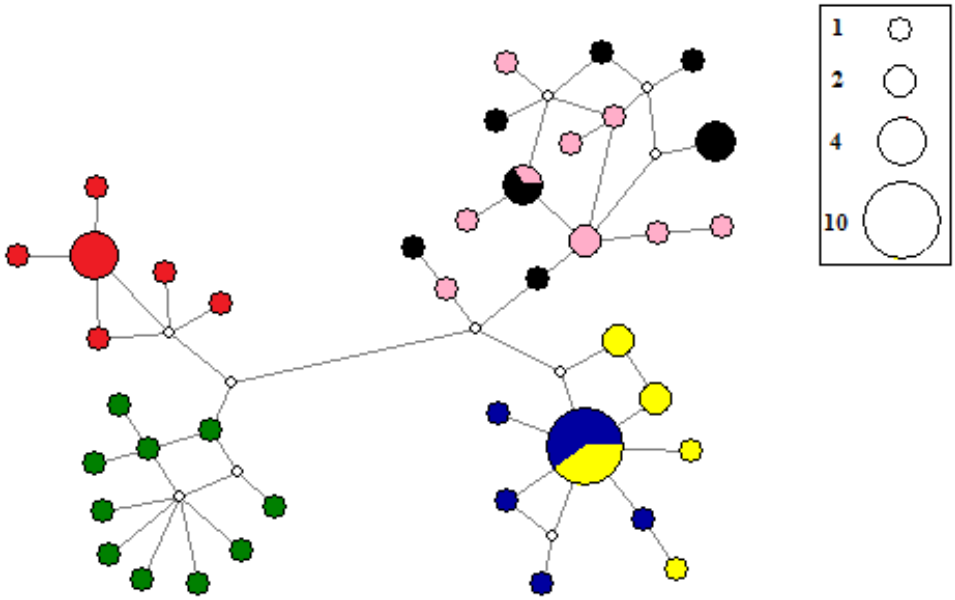

Figure 2: Haplotype network of nuclear sequences. Sizes of circles represent the haplotype frequency, upper right black box, related circle sizes with the number of specimens that presents that haplotype. Red=*B. tyrannus*; green=*B. patronus*; yellow=*B aurea*; blue=*B. pectinata*; pink=*B. gunteri*; black=*B. smithi*. White circles indicate missing haplotypes.

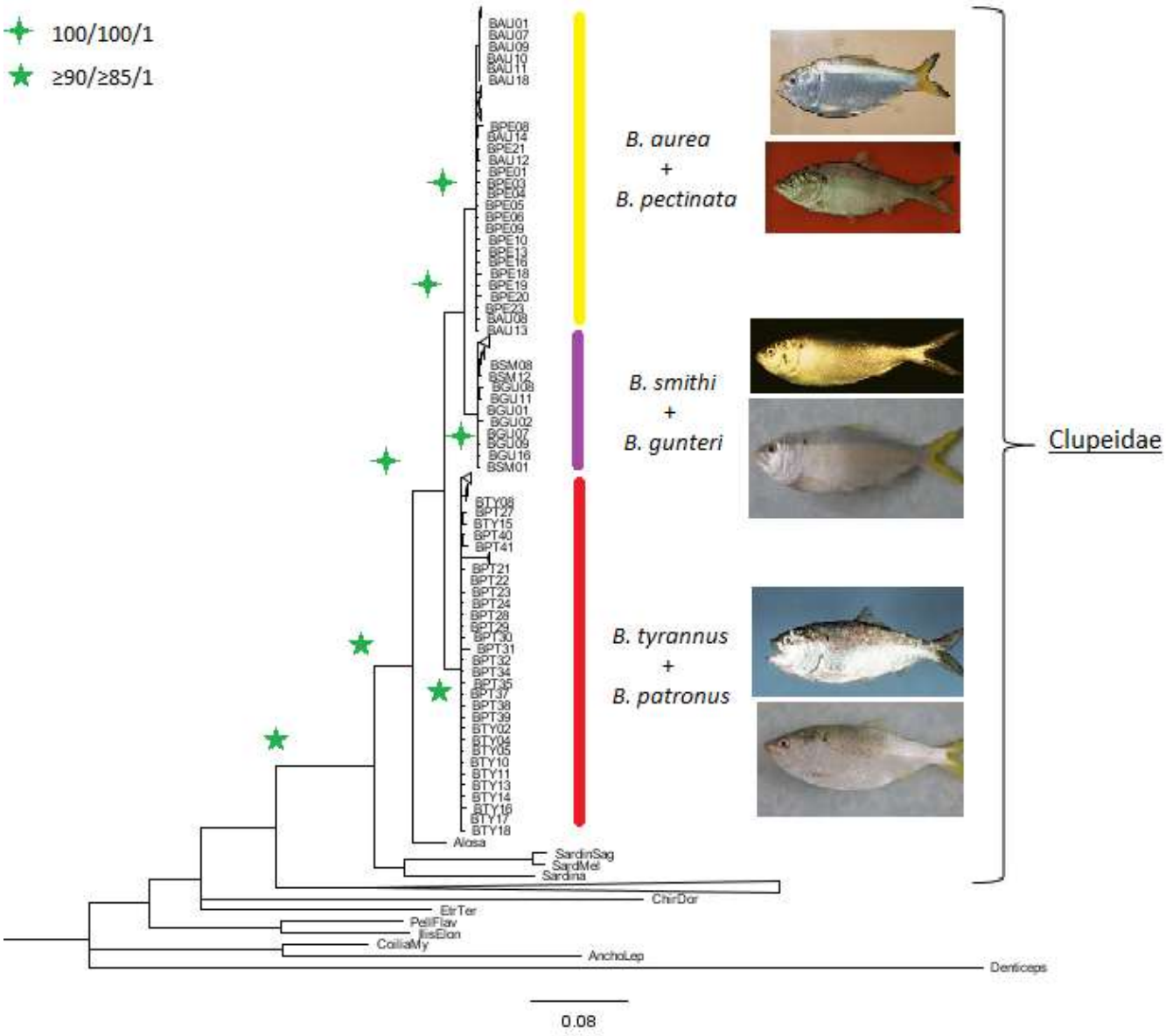

Figure 3: Bayesian tree topology based on the combined dataset of 2116 bp of 16s, COI and RAG2 gene sequences. Numbers on branches represent *bootstrap* support for Maximum Likelihood/Parsimony analyses/Posterior Probabilities from the Bayesian analysis, respectively. The symbol "-" indicates the situation where the particular branch was not recovered by one particular optimality criteria.

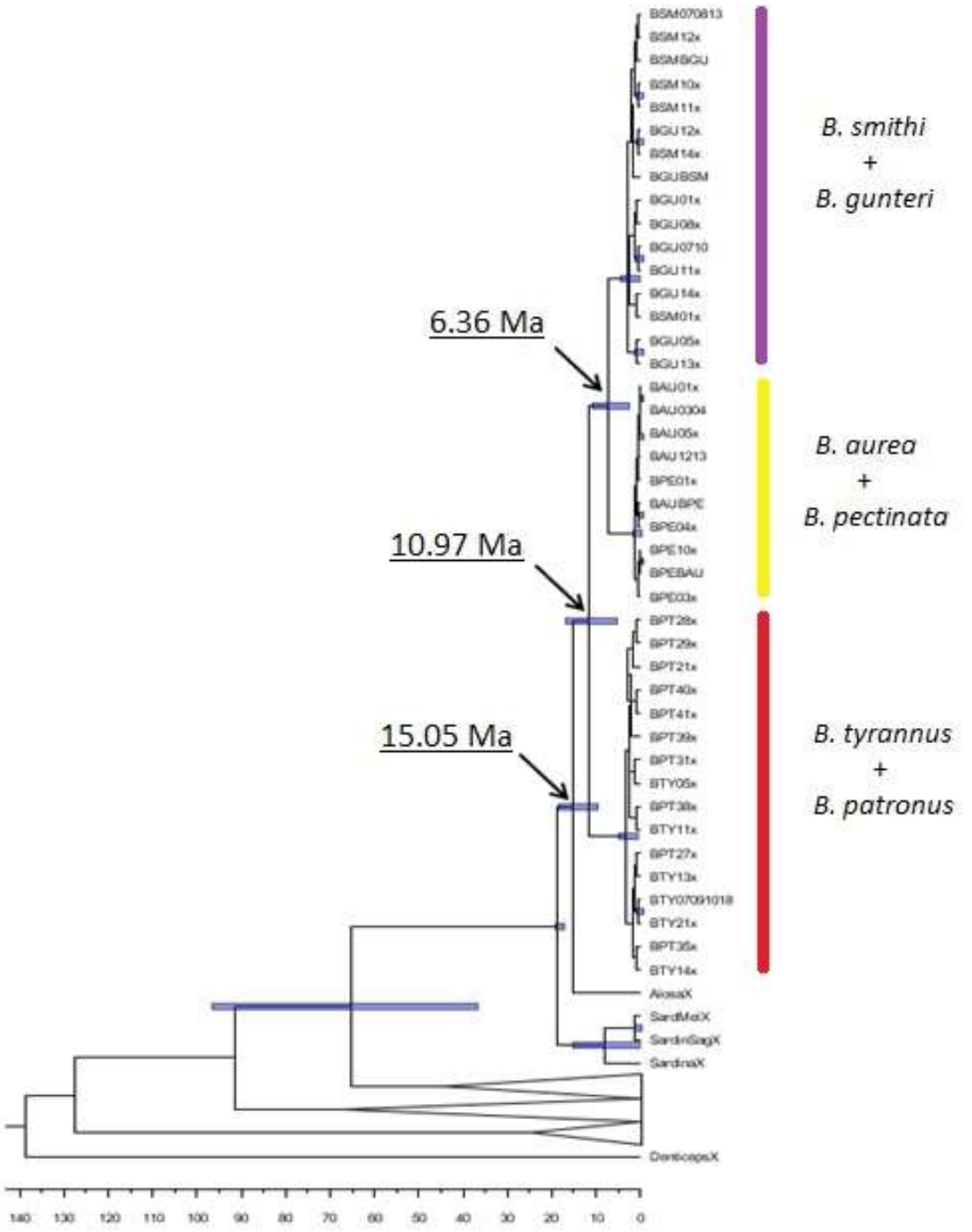

Figure 4: Species tree inferred by a Bayesian multispecies coalescent approach. Node dates were estimated using fossil calibrations (details are discussed in the text). The scale (abscissa) represents millions of years (Mya) before present. Bars at nodes reflect 95% confidence intervals. Collapsed nodes (white triangles) represent outgroup taxa in the analyses.

Capítulo 2

One species of *Brevoortia* (Clupeiformes: Clupeidae) in the western South Atlantic: evidence from morphology and molecular data

Abstract

Brevoortia is a taxonomically problematic genus of the Clupeidae, with six currently recognized species distributed in the western Atlantic Ocean. Two of those species are reported for the western South Atlantic: *B. aurea* (Spix & Agassiz 1829) and *B. pectinata* (Jenyns, 1842). In this study, morphological and molecular data were employed to test the validity of both species. A total of 254 specimens of *Brevoortia* were analyzed for meristic (13 counts) and morphometric (27 measures) data. In the molecular analyses, three molecular markers, being two mitochondrial (COI and 16S) and one nuclear (RAG2), were amplified in 68 specimens. Phylogenetic analyses based on molecular data were performed under the Maximum Parsimony, Maximum Likelihood and Bayesian methods. Collection locality of specimens examined for both morphology and molecular data cover the reported distribution of the genus in the western South Atlantic (southeastern Brazil to Argentina). Overall, meristic and morphometric data indicate that a single species should be recognized in the region, with a latitudinal gradient detected for meristic data. Phylogenetic trees and haplotype networks also failed to recover species-specific clades among sequences examined. Overlaping genetic divergences frequencies within and between geographic localities, in the western South Atlantic, were detected. Positive numbers of migrants were also observed among different localities in the western South Atlantic, indicating the presence of gene flow throughout the region. Therefore, results from both morphology and molecular data support the hypothesis that a single species of *Brevoortia* (*B. aurea*, the senior synonym) should be recognized in the western South Atlantic.

Introduction

The proper recognition of taxonomic entities at the species level and reasonably accurate projections of geographic distributions coupled with assessment of the genetic variability of fish stocks are necessary steps towards the development of more effective sustainable management strategies (Margules & Pressy 2000; Linke et al. 2007). The combined use of morphological and molecular data offers conditions for more precise resolutions of those questions, especially in the case of cryptic species that are not recognized by traditional morphological characters (Thomas et al. 2014). In the last years, the combined use of these two sets of characters has indeed grown in studies related to taxonomy and evolution (e.g., Vasconcellos et al. 2008; Oliveira et al. 2011; Amaral et al. 2013; Thomas et al. 2014).

All species of the genus *Brevoortia* Gill 1861 are exploited in artisanal and industrial fisheries (Franklin, 2007; FAO, 2012; Pinho, 2016), despite some controversy about how many species should be recognized in the genus both in the western North and western South Atlantic. In the western South Atlantic, *B. aurea* (Spix & Agassiz 1829) and *B. pectinata* (Jenyns, 1842) are currently considered as valid (e.g, Figueiredo & Menezes, 1978; Whitehead, 1985; Fricke et al., 2018), but both species are morphologically very similar and presumably have overlapping geographic distributions in southern Brazil, Uruguay and Argentina (Whitehead, 1985). *Brevoortia aurea* have a geographic distribution reported going from the Rio de Janeiro state (Brazil) to the mouth of the Plata river (Argentina), *B. pectinata*, in turn, have a geographic distribution reported from São Paulo state (Brazil) to Bahia Blanca (Argentina) (Whitehead, 1985). Hildebrand (1948) indicated that the two species are distinct in the following characters: size of scales, number of oblique series of scales crossing the middle of the lateral portion of the body, number of longitudinal rows of scales in the caudal peduncle, number of vertical series of scales between the tip of pectoral fin and the pelvic-fin base, and the distance between the end of pectoral fin and the base of the pelvic fin. In his review, Hildebrand (1948) particularly noted the relatively small scales of *B. aurea* when compared with *B. pectinata*. According to him: "…a difference actually greater than shown, by the enumerations (of scales oblique series) given in the description." (Hildebrand, 1948: 27). Summing up Hildebrand's

observations on the differences of the two species in terms of the number of scales, in *B. aurea* size of scales at the middle region of the body at the origin of dorsal fin tend to be about two-thirds of those in the same region of *B. pectinata*; this difference cannot be presumably fully appreciated from the "enumeration" (number) of the oblique series of scales along the middle of the side since in *B. aurea* scales are less imbricated than in *B. pectinata*. Whitehead (1985), in what is the last morphological review of the taxonomy of clupeoids of the world, recognized *B. aurea* and *B. pectinata* as valid, based on some of the diagnostic features indicated by Hildebrand (1948): number of scales in lateral series (48-56 in *B. aurea* vs. 35-46 in *B. pectinata*) and number of scales between the pectoral fin tips and the pelvic fin base (3-7 in *B. aurea* vs 0-3 in *B. pectinata*). However, Whitehead (1985: 214) also considered that "More work (is) needed to separate it (*B. pectinata*) from *B. aurea* and clarify if their ranges really overlap."

Cousseau & Díaz de Astarloa (1993) presented meristic and morphometric data of 484 specimens of *Brevoortia* collected in the region between the Rio Grande do Sul (Brazil) and Bahia Blanca (Argentina), comparing with available information supposedly indicative of the validity of *B. aurea* and *B. pectinata*. The authors concluded that only one species of *Brevoortia* is present in the region analyzed by them and that species should be recognized as *B. aurea*, the senior synonym. Recently, García et al. (2008) reached the same conclusions of Cousseau & Díaz de Astarloa (1993), this time based on molecular data. The authors analyzed the mitochondrial DNA (*cytochrome b*) of 240 specimens collected between Rio Grande do Sul (southern Brazil) and Mar Chiquita (Argentina), and also concluded that their results strongly supported the existence of only one species in the region.

In spite of the relevance of the results of Cousseau & Díaz de Astarloa (1993) and García et al. (2008) to the understanding of the taxonomy of *Brevoortia* in the western South Atlantic, in both studies specimens were obtained only from the south limit of the geographic distribution of the genus. In this study, the validity of *B. aurea* and *B. pectinata* is assessed based on the analyses of both morphological and molecular data of specimens collected along the reported distribution of the genus in the western South Atlantic, which

ranges from northern Rio de Janeiro State (Brazil; 21° 37'S) to Mar del Cobo, Argentina (37° 47'S; Whitehead, 1985; Menezes et al., 2003).

Material and Methods

Specimens

A total of 254 specimens of *Brevoortia* from the western South Atlantic were examined in the study. Specimens were tentatively identified based on current literature (e.g., Hildebrand, 1948; Whitehead, 1985; Cousseau & Díaz de Astarloa, 1993), resulting in the identification of 190 specimens as *Brevoortia* cf. *aurea* and 64 as *Brevoortia* cf. *pectinata*. Specimens were analyzed from three different Brazilian States along the southern coast of Brazil: Rio de Janeiro (189 specimens), São Paulo (15 specimens) and the Rio Grande do Sul (45 specimens). Five additional specimens collected in Mar del Cobo, Argentina, in the vicinity of the southern limit of the geographic distribution of the genus according to literature, were also examined (Table 1).

Amongst the total of 254 specimens examined, 151 were collected for this study in fish markets and landing sites. After processing in the laboratory for tissue sample collection, those specimens were fixed in formalin 10% and subsequently stored in alcohol 70% in the Fish Collection of the Núcleo em Ecologia e Desenvolvimento Socioambiental de Macaé, Universidade Federal do Rio de Janeiro (NPM; Table 1).

Museum specimens and tissue samples were obtained as loans and/or donations from the fish collections of Museu Nacional, Rio de Janeiro (MNRJ), Museu de Zoologia da Universidade de São Paulo (MZUSP), Coleção de Peixes da Universidade Federal do Rio Grande do Sul (UFRGS), Universidade Federal de Rio Grande (FURG), Universidad Nacional de Mar del Plata (UNMDP), Texas Parks and Wildlife Department (TPWD), and University of Kansas (KU).

Morphology and type specimens

A total of 27 morphometric characters, defined according to Hildebrand (1948), Hubbs & Lagler (1964) and Whitehead (1985; Fig. 1-3), were measured preferentially on

the left side of all 254 specimens of *Brevoortia* available for the study, using a digital caliper with 0.01 mm precision (Table 2). Morphometric characters measured in this study are: anal-fin base length (AFBL), measured from the insertion of the first anal-fin ray to the posterior margin of the basis of the last anal-fin ray; axillar scale length (ASL), measured as a straight line between the anterior margin of the axillar scale to its posterior tip; body height (BH), measured as the largest body height, at the origin of dorsal fin; caudal peduncle height (CPH), the largest vertical distance between the dorsal and ventral margins of the caudal peduncle; caudal peduncle length (CPL), measured as a straight line between the posterior margin of the basis of the last anal-fin ray to the posterior margin of the third hypural; dorsal-fin base length (DFBL), measured from the insertion of the first dorsal-fin ray to the posterior margin of the basis of the last dorsal-fin ray; distance between nostrils (DN), measured as the largest distance between left and right nostrils; distance between orbit and upper maxilla (DOM), measured as the shortest distance between the ventral margin of the orbit to the dorsal margin of the upper maxilla; distance between pelvic-fin base and anal fin (DPFAF), measured as a straight line between the insertion of the pelvic-fin to the basis of the first anal-fin ray; distance between pectoral fin and pelvic fin (DPFPF), measured as a straight line between the posterior tip of the pectoral fin to the to the insertion of the pelvic fin; distance between symphysis of the upper maxilla to the anterior margin of nostril (DSN); eye diameter (ED), measured as a horizontal line between anterior and posterior margins of the eye; head height (HH), the largest vertical height of the head at the posterior margin of the operculum; head length (HL), measured as a straight line from the tip of the snout to the posterior margin of operculum; head width (HW), measured as the largest transversal distance of the head at the posterior margin of the opercle; interorbital distance (IOD), measured as the largest transversal distance between left and right orbits; lower jaw length (LJL), measured as the distance between the anterior and posterior margins of the lower jaw; pre-anal distance (PAD), from the tip of the snout to the insertion of the first anal-fin ray; pre-dorsal distance (PDD), measured from the tip of the snout to the dorsal-fin base, at the insertion of first dorsal-fin ray; pectoral-fin length (PFL), measured as a straight line between the insertion and the posterior tip of the longest pectoral-fin ray; post-orbital distance (POD), measured as a horizontal line between the posterior margin of the orbit to the posterior margin of the operculum; pre-pectoral distance

(PPD), measured from the tip of the snout to the insertion of the upper pectoral-fin ray; pre-pelvic distance (PVD), measured from the tip of the snout to the insertion of the first pelvic-fin ray; standard Length (SL), measured from the tip of the snout to the posterior margin of the third hypural; snout length (SNL), measured from the tip of the snout to the anterior margin of the orbit; upper maxilla height (UMH), largest length measured between dorsal and ventral margins of upper maxilla; upper maxilla length (UML), measured as the distance between the anterior and posterior margins of the upper maxilla.

In addition to the morphometric characters listed above, 13 meristic characters were also coded for the same 254 specimens, following Hildebrand (1948), Hubbs & Lagler (1964), Whitehead (1985), Segura & Díaz de Astarloa (2004) (Table 3). Twelve specimens were cleared and stained for visualization of bones and cartilages (Taylor & Van Dyke 1985; Song & Parenti 1995). Osteological observations were complemented by x-ray images of 40 specimens, obtained with a Digital Faxitron X-Ray Machine model LX-60.

Multivariate Discriminant Analyses were employed in the PAST v2.17c software (Hammer et al. 2001) separately for the meristic and morphometric data. Before the analyses were performed, size and distance values (morphometric data) were logarithmized (log10), normalizing the variation of the characters. The Multivariate Discriminant Analyses aimed at exploring the patterns of morphometric discrimination among a priori identified groups along few discriminant functions or canonical variates calculated from the product of intergroup variance-covariance matrix and the inverse intragroup variance-covariance matrix, which represents the maximum intergroup variance-covariance. These functions allow the computation of scores for specimens based on their original measurements, and the representation of theses individual scores along bivariate scatterplots formed the basis for the description of discrimination patterns among groups. The importance of each canonical variate to the discrimination patterns was estimated by their eigenvalues and the contribution of each character to the discrimination along the axes was estimated based on the correlation coefficients among characters and scores. In a preliminary Multivariate Discriminant Analysis, pre-established groups of specimens reflect five larger geographic samples, which also reflects a latitudinal gradient from north to south along the geographic distribution of the genus *Brevoortia* in the western South

Atlantic: North of the Rio de Janeiro State ("North RJ"), Rio de Janeiro city and vicinities ("Rio"), São Paulo State ("SP"), Rio Grande do Sul State ("RS"), and Argentina ("Arg").

A second Multivariate Discriminant Analysis, were all pre-established groups indicated in the preliminary analyses with the exception of "SP" and "Arg" were further divided in a total of eight smaller geographic unities, was also performed, resulting in the following arrangement of groups: "SJBarra/RJ" and "Macaé/RJ", which were included in "North RJ" in the preliminary analysis; "SaqMar" and "RioCity", which were included in "Rio" in the preliminary analysis; "North RS" and "South RS", which were included in "RS" in the preliminary analysis.

Morphometric and meristic data of the syntypes of *Brevoortia aurea* (MHNN 1159) (Fig. 4) and *B. pectinata* (BMNH 1843.2.8.47 and BMNH 1917.7.25.1) (Fig. 5) were obtained through photographs and x-ray images. In order to test where the type specimens of both species are spatially located in the scatterplot of all specimens examined along the two principal canonical axes according to the examined data, three Multivariate Discriminant Analyses were conducted. The first one was performed with the morphometric data alone, including all the 254 specimens plus the syntypes of both species. The second analysis was performed only with meristic data available for 43 x-rayed specimens, including the syntypes of both species, from which the number of vertebrae was also known. The third Multivariate Discriminant Analysis including the syntypes was conducted with all the 254 specimens examined, but in this case, only the three meristic characters that were assessed in all specimens were considered (number of dorsal-fin rays, number of anal-fin rays, and number of abdominal scutes).

Molecular data

Among the 254 specimens of *Brevoortia* examined in the anatomical study, tissue samples of 60 specimens (40 identified as *Brevoortia* cf. *aurea* and 20 identified as *Brevoortia* cf. *pectinata*) were available for the molecular study. In addition, eight tissue samples of specimens previously identified as *B. pectinata* from south Brazil (TEC3428A/B-UFRGS) were also sequenced and included in the analyses (total=68 specimens). Molecular sequence data of one specimen of *B. gunteri* (Hildebrand, 1948) and

one specimen of *B. patronus* (Goode, 1878), both from the North Atlantic, were also obtained in this study and included in the analyses. Sequence data of one specimen of *Alosa pseudoharengus* and *Opisthonema oglinum*, species of the Clupeidae that are apparently related to *Brevoortia* (e.g., Lavoué et al., 2013; Egan et al., 2018), were also included in the analyses as outgroup taxa. The outgroup taxa also included representatives of the families Pristigasteridae (*Pellona flavipinnis*) and Engraulidae (*Anchoviella lepidentostole*), in addition to *Denticeps clupeoides*, the sole living member of the Denticipitoidei in the Clupeiformes (Greenwood, 1958; Grande, 1985; Di Dario and de Pinna, 2006; de Pinna and Di Dario, 2010; Lavoué et al., 2014). All sequence data of the outgroup taxa, with the exception of *B. gunteri* and *B. patronus*, were obtained in the National Center for Biotechnology Information (NCBI- http://www.ncbi.nlm.nih.gov/nuccore; Table 4).

DNA isolation and sequencing

Total DNA was obtained through fins or muscle tissue samples using the protocol of DNA extraction of Sambrook (2001). Primer Chain Reaction (PCR) was performed using the primers described by Ivanova et al. (2007) for the mitochondrial genes (COI and 16S). The nuclear gene (RAG2) was amplified using the primers described by Lovejoy & Collette (2001) and previously used in *B. tyrannus* and *B. patronus* by Li & Orti (2007). PCR products were sequenced using the same primers of the PCR reaction in the international platform for sequencing, Macrogen Inc., and in the Applied Biosystems 3130 Genetic Analyzer (ABI 3130) of the California Academy of Sciences (CAS).

Amplification of 16S sequences was successfully obtained for the 68 specimens included in the molecular analyses (20=“North RJ”, 20=“Rio”, 23=“RS” and 5=“Arg”). For the COI region, sequences of 63 specimens were successfully amplified (15=“North RJ”, 20=“Rio”, 23=“RS” and 5=“Arg”). RAG2 sequences, in turn, were successfully obtained from 49 specimens (5=“North RJ”, 19=“Rio”, 20=“RS” and 5=“Arg”).

Molecular sequences were aligned in DAMBE (Xia & Xie, 2001), which implements the ClustalW algorithm (Thompson et al. 1997). Parameters set in DAMBE are “10” for the gap open penalty and “0.1” for gap extension. After alignment, each sequence was carefully eye checked with sequencing chromatograms for corrections of obvious

misalignments. Phylogenetic analyses, described below, were performed for the molecular markers alone (COI= 651 bp, 16S= 583 bp, and RAG2= 864 bp) and for the three sequences concatenated (AllGenes= 2098 bp).

Phylogenetic analyses

Maximum Parsimony (MP) was employed in PAUP*v. 4.0b10 (Swofford, 2002). Phylogenetic trees were obtained by heuristic search with TBR/Branch Swapping in 100 stepwise random additions of taxa. Only the topologies with the smallest number of steps were retained, and branches with zero lengths were collapsed. Maximum Likelihood analyses (ML) were performed in the RAxML v8.2.X (Stamatakis, 2014) with a mixed/partitioned model that estimated parameters independently for each gene partition. The Bayesian analyses (BY) was performed in MrBayes 3.2.6. (Ronquist et al., 2011) with parameters from TIM2+I+G model, for the concatenated sequences, selected by jModeltest2 software (Darriba et al., 2012) under the Bayesian Information Criterion. The analysis was run with two groups of four simultaneous MCMC (Monte Carlo Markov Chains) for two million generations, sampling trees every 100 generations. The first generated trees were discarded as "burn-in" (25%), the remaining were used to create a majority rule consensus tree. Due to the heavy computational power required for the analyses, ML and BY were performed in the "CIPRES Science Gateway" (https://www.phylo.org/).

Bootstrap analyses (Felsenstein, 1985) were implemented in the topologies obtained in the MP and ML methods with 1000 replicates. Posterior probabilities indicate the supports for nodes in the BY method. In addition to the analyses with the molecular markers concatenated in a supermatrix, a Multi-Species Coalescent analysis (*BEAST) was also performed on BEAST v2.4.3 (Bouckaert et al., 2014). This analysis estimates the species tree that is most probable given the multi-individual, multi-locus sequence data. The analysis requires that the user define a previous identification (species or group) for each sequence examined. Specimens were therefore grouped by their shared haplotype, in an attempt to recover species-specific clusters based on the assumption that similar/shared haplotypes would indicate a single origin or at least a more cohesive population structure, suggestive of a species.

Tracer v1.6 (Rambaut et al., 2013) was employed to measure effective sample size of each parameter and TreeAnnotator (from the BEAST package) was used to summarize the trees obtained in the analyses after discarding 25% of the first trees sampled in each run. Posterior probabilities indicate the support for nodes in the tree. In all phylogenetic analyses, trees were rooted at the branch between *Denticeps clupeoides*, the sole living member of the Denticipitoidei, and the clade formed by the remaining taxa.

Genetic structure and gene flow estimates

The relationship between and within the two putative species of *Brevoortia* (*B. aurea* and *B. pectinata*) was inferred using haplotype networks, developed through the Median-joining method (Bandelt et al., 1999) in Network 5.0 (www.fluxus-engineering.com/sharenet.htm). Arlequin 3.5.1.2 (Excoffier & Lischer 2010) was used to check parameters of genetic diversity (*p*-distance) and number of migrants per generation (Nm), an indicator of gene flow and hybridization between species. Analyzes aimed at assessing genetic structure and gene flow estimates were performed with mitochondrial sequence data separately from nuclear DNA sequences.

Species delimitation

Recently, Stern et al. (2017) proposed the combination of three different approaches for the recognition of possible species in *Sardinella*, another member of the Clupeidae, using molecular data. The first of those approaches would be a "tree-based approach", using a matrix of concatenated sequences (Bayesian Poisson Tree Processes). The other approaches were not based on trees, and were conducted only for the COI dataset by Stern et al. (2017): *p*-distance comparison, and the Automatic Barcoding Gap Discovery method (ABGD) (Flot 2015).

The Bayesian Poisson Tree Process model (bPTP) provides an objective approach for delimitating putative species boundaries that are consistent with the phylogenetic species concept. The models indicate species based on a phylogenetic topology and do not require an ultrametric input tree or models of speciation by directly using the number of substitutions to reach results (Zhang et al. 2013). The bPTP web server (http://www.species.h-its.org) was employed to conduct the analyses with the concatenated

ML topology as the input tree. Default settings were used in the analyses, except for the definition of the outgroup, which was indicated as *Denticeps*.

The first "non-tree based" approach consists in a pairwise-comparison distance matrix. In this analysis, the specimens were grouped among themselves by the collection locality. Genetic divergence within and between groups were compared in two simple histograms. The second "non-tree based" approach employed the Automatic Barcoding Gap Discovery method (ABGD). This analysis was performed twice, with and without outgroups, at the ABGD web server (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). Defaults settings were used, except for the "gap width", for which three different values were indicated (X= 0.5, 1.0, 1.5) to assess the consistency of the inferred groups under varying gap-width values. The ABGD method uses the genetic distance-based matrix to detect a "barcode gap" separating putative species based on non-overlapping values of intra (represented by "P") and interspecific genetic distances, independent of any tree-topology (Hebert et al. 2003; Puillandre et al. 2012).

Results

Morphometric and meristic data

When five groups of specimens of *Brevoortia* from the western South Atlantic are organized by their collecting localities ("North RJ"; "Rio"; "SP"; "RS"; "Arg"), the first two axes in the Multivariate Discriminant Analysis explain 94.25% of the total variance observed in the morphometric data (axis 1: 67.93%; axis 2: 26.32%; Fig. 4A). Overall, specimens of different groups did not occupy exclusive regions of the multivariate morphospace generated by the two axes, and there is no absolute distinction among groups based on the locality of collection. Specimens from "SP", "RS" and "Arg", in particular, are all superimposed in the same section of the bivariate plot. Specimens from "North RJ" and "Rio", in turn, tend to be more segregated on opposite extremities of Axis 1, although just a few specimens shared between those two sections. Specimens from "Rio", in particular, are segregated from "SP", "RS" and "Arg", but tend to form a continuum in the

morphospace when “North RJ” is considered as intermediate between those two assemblages. Larger values of “caudal peduncle length” and “anal-fin base length” are associated with the divergence of most specimens from “North RJ” relative to other samples (Fig. 6A). “Dorsal-fin base length” and “body height”, in turn, have larger values associated with the distribution of most specimens from “SP”, “RS” and “Arg” in the morphospace, whereas larger values in the “distance from orbit to upper maxilla”, “snout length”, “axillar scale length” and “distance between symphysis of the upper maxilla to anterior nostril” are more responsible for the distribution of most specimens from “Rio” (Fig. 6A).

For the meristic data in the Multivariate Discriminant Analysis, when the same five groups of localities are recognized, the first two axes explain 89.4% of the total variance observed in the data (axis 1: 65.99%; axis 2: 23.41%; Fig. 6B). The results observed are similar to those indicated by the morphometric data, *i.e.*, there is not a clear segregation of groups according to the collection locality. Larger values in the “number of transverse scale rows along the body” and “horizontal scale series around caudal peduncle” influenced the distribution of most specimens from “North RJ” in the morphospace, whereas larger values of “scales below pre-dorsal series” and “pre-dorsal scales” are associated with the distribution of specimens from “Rio” (Fig. 6B).

In the second round of the Multivariate Discriminant Analyses, specimens were grouped into eight assemblages based on collection locality: “SJBarra/RJ”, “Macaé/RJ”, “RioCity”, “SaqMar”, “SP”, “North RS”, “South RS”, “Arg” (Fig. 7). In the analysis based on morphometric data, specimens from “SJBarra/RJ”, “Macaé/RJ”, “RioCity” and “SaqMar” are distributed along axis 1, from left to right, without forming distinct groups (Fig. 7A). Specimens from the remaining regions (“SP”, “North RS”, “South RS”, and “Arg”) form themselves a cluster that is superposed with “SJBarra/RJ” and “Macaé/RJ” on the upper section of the morphospace. Larger values of “caudal peduncle length” and “anal-fin base length” are associated with the distribution of specimens from “Macaé/RJ” in the morphospace (Fig. 7A). Larger values of “dorsal-fin base length”, in turn, are associated with the position of specimens from “SJBarra/RJ”, whereas larger values of “body height” are associated with the distribution of specimens from “SP”, “North RS”, “South RS” and

"Arg" in the morphospace. Larger values of "snout length" and "distance from orbit to upper maxilla" are more relevant for the distribution of specimens from "RioCity" and "SaqMar".

Meristic data, when those same eight locality-based groups are defined, also failed to indicate distinct assemblages in the Multivariate Discriminant Analysis, even though most specimens from "Macaé/RJ" tended to be located on the right side of the scatterplot, mostly due larger values of "horizontal scale series around caudal peduncle" and "transverse scale rows along the body" (Fig. 7B).

Vertebral counts of 40 specimens (25 from "Rio" and 15 from "RS"), based on x-rays, were checked. No differences were observed in the number of pre-caudal vertebrae [max=28(14); min=26(14); median=27] and caudal vertebrae [max=19(5); min=17(15); median=18)] among specimens of different regions (Table 3). Counts of gill rakers in the lower branch of the first branchial arch of six clear and stained specimens (four from "Rio" and two from "RS") are also similar ("Rio"=169, 173, 178, 180; "RS"=170, 183).

Syntypes

Morphometric and meristic data of the single syntype of *B. aurea* and of the two syntypes of *B. pectinata* are not associated with any particular, segregated, group in the morphospace defined by the first two axes in the three different Multivariate Discriminant Analyses that include those specimens (Fig. 8A-C).

Molecular data

Phylogenetic analyses

Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian (BY) analyses were first conducted for the molecular markers separated. The topologies obtained were overall congruent for the same marker in all methodologies employed. Analyses with the concatenated genes and with a partitioned matrix data are also overall congruent among themselves, regardless of the optimality criteria employed (MP, ML and BY; Fig. 9).

A clade with high statistical support, containing all 68 western South Atlantic specimens of *Brevoortia* included in the molecular analyses, was recovered. Inside this clade, no species-specific clades can be defined. A monophyletic assemblage including all 20 specimens collected in "North RJ" was recovered in the ML and BY approaches (Fig. 9; LI01-LI20). However, both the accumulated mutation between the "North RJ" group and other specimens of western South Atlantic *Brevoortia*, expressed by the size of the branches, and the low statistical support of the clade indicates that genetic differences between that assemblage and remaining specimens are low (Fig. 9).

Species-specific clades were not recovered as well in the Multi-Species Coalescent analysis (Fig. 10), with the different haplotype groups not indicating the existence of any taxonomic putative species or geographically isolated populations. Basically, this analysis indicates the existence of one large clade that includes all the western South Atlantic specimens of *Brevoortia* divided into three groups with an admixture of haplotypes from different collection sites. Interestingly, haplotypes of specimens of the "North RJ", which form a monophyletic assemblage in previous phylogenetic analyses, do not form a monophyletic group in the Multi-Species Coalescent analysis (Fig. 10; LI).

Haplotype networks and genetic diversity

Analyses of haplotype networks and genetic diversity were conducted with the mitochondrial markers separated from the nuclear sequence data, due to the expected differences in mutational rates of these two classes of molecular markers. A total of 29 haplotypes were identified in the mitochondrial dataset (Fig. 11). One haplotype is shared between specimens of "North RJ" and "Rio", a second distinct haplotype is shared between "Rio" and "RS", and another haplotype is shared between specimens from "RS" and "Arg". Overall haplotype diversity for the mitochondrial data is high (h= 0.426) and nucleotide diversity (π) is equal to 3.401 (Table 5). The genetic divergence, which is measured as pairwise differences between distinct collection sites ranges from 2.05 ("North RJ" *vs*. "RS") to 5.41 ("Rio" *vs*. "Arg"). The average number of pairwise differences within geographic regions varies from 0.40 ("North RJ") to 7.06 ("Arg").

Summing up, the haplotype network of the mitochondrial sequences fails to indicate species-specific groups within or among distinct geographical regions (Fig. 11A). Those results are more in agreement with the hypothesis that a single species is present in the dataset. The positive number of migrants per generation (Nm) observed among distinct regions, with the exception of "North RJ" *vs*. "Arg" (Table 6), which are located at the opposite limits of the latitudinal gradient sampled, also agrees with this hypothesis.

In the nuclear sequences of the RAG2 region, 20 haplotypes were detected. One is shared between specimens from "North RJ" and "Rio", another is shared between "North RJ" and "RS", and a third one is shared among specimens from "Rio", "RS" and "Arg". Overall haplotype diversity is high (h= 0.408) and nucleotide diversity (π) is equal to 2.843. The genetic divergence, measured as pairwise differences, between collection sites ranges from 2.06 ("Rio" *vs*. "RS") to 3.92 ("North RJ" *vs*. "Arg") (Table 5). The average number of pairwise differences within geographical regions, in turn, ranges from 1.35 ("RS") to 4.60 ("North RJ"; Table 5).

As in the case of the mitochondrial genes, haplotype networks for RAG2 sequences failed to indicate clusters of species-specific haplotypes, with haplotypes shared among distinct geographical regions (Fig. 11B). Those results are also in agreement with the hypothesis that a single species is present in the data set. The positive number of migrants per generation (Nm) observed also agrees with this hypothesis, with migrants present among all geographic regions (Table 6).

Species-delimitation analyses

Analyses of the bPTP model identified eight putative species when all sequence examined are included in the analyses are evaluated, including those of the outgroup taxa. Most notably, all samples of western South Atlantic specimens of *Brevoortia* are grouped in a single lineage, with a significant support value of 0.888. In the second species-delimitation approach, the frequencies of genetic divergence estimated within *vs.* between geographic regions in the western South Atlantic are similar, indicating the existence of a single species of *Brevoortia* in the region (Fig. 12). In the third species-delimitation approach, which is the analyses of the ABGD model, similar results were obtained

regardless of the relative gap widths assumed (X= 0.5, 1.0, 1.5) or the presence of outgroup taxa. The main partition identified by the model divided the dataset into eight groups, which are identical in content as those revealed by the bPTP model, *i.e*, seven outgroups "species" plus one cluster of sequences of *Brevoortia* specimens from the western South Atlantic, when intraspecific divergence (P) is equal to 0.0046. Higher values of "P" (0.0077, for example) results in six groups, one with all specimens of *Brevoortia* presents in the analyses (western South Atlantic specimens+*B. gunteri* and *B. patronus* from North Atlantic), this means that, there is no intra-inter specific relationship that recognize more than one group in the western South Atlantic *Brevoortia* analyzed.

Discussion

Morphometric and meristic data

The results obtained in the present study overall indicate that a single species of *Brevoortia* is present in the western South Atlantic. Among the morphological features examined, segregation of specimens into distinct assemblages is suggested only by the morphometric data, as in the case of the ordination of specimens from "North RJ"+"Rio" *vs*. "SP"+"RS"+Arg" along Axis 2 in Fig. 6A. Interestingly, this particular ordination of specimens might reflect a latitudinal gradient of morphological variation of some of the characters examined in the study. One feature in particular is the number of transverse scale rows along the body, which shows a clear latitudinal gradual variation with differences between the two extremes of distribution (Fig. 13). We suspect that this latitudinal variation was perhaps responsible for the conclusion that two species of *Brevoortia* inhabit the western South Atlantic. Even though the type locality of *B. aurea* might be wrong (Bahia, Brazil; see discussion below), it is presumed that the species were recognized based on specimens collected at the north and south limits of the geographic distribution of the genus in the region. In summary, when only specimens at the more extreme north/south range of distribution are considered, without morphologically intermediate forms, it is indeed reasonable to conclude that they represent distinct species according to some morphological data. A similar situation was recently reported by Menezes et al. (2010) for lebranche

mullets, genus *Mugil*, in the western South Atlantic. Previous studies reported the presence of up to three species in the region (*M. liza*, *M. platanus*, *M. cephalus*). However, morphological analyses including representative specimens collected along the whole distributional range of the genus in the region revealed the presence of a single species. The seemingly morphological differences attributed to the previously recognized species actually are related to a pattern of gradual variation through the latitudinal range.

Additional circumstantial evidence suggesting the existence of a single species of *Brevoortia* in the western South Atlantic with a marked latitudinal variation in morphology was also provided by Cassia & Rosa (1993), based on the study of larvae. Morphological variation in close geographical regions was also previously reported for the western South Atlantic populations of *Brevoortia* by Díaz de Astarloa & Cousseau (1993). Their data revealed the existence of minor differences in meristic and morphometric data between specimens collected in the northern *vs* southern Argentina. According to the authors, those differences might be related to different levels of salinity in each region.

North to south morphocline variation was also reported for another clupeiform, the Pacific Sardine *Sardinops sagax*, by Hedgecock et al. (1989). The authors presented data relating size of Pacific Sardines with a specific age and compared different collections sites along the north-south distribution of the specimens, reinforcing the presence of a geographical morphocline variation. This morphocline variation was previously interpreted as indicative of the existence of genetically distinct populations, but the results presented by Hedgecock et al. (1989) refuted this hypotheses, since low levels of genetic variation among different collections sites were actually observed. Safford & Booke (1992), in the analyses of putative stocks of the Northwest Atlantic herring, *Clupea harengus*, also reported low levels of genetic divergence associated with significant morphological differentiation in the species throughout its range. They suggested that the different morphs recognized within the species resulted from the development in different environmental conditions. Kinsey et al. (1994), in turn, employed morphological and molecular analyses to explore the population structure of the Spanish sardine (*Sardinella aurita*) in the Florida peninsula, finding no signs of genetic structuration among collection sites despite some segregation in morphological traits, which were attributed to “embayment collection sites”

against “oceanic collection sites”. More recently, based on a shallow genealogy across a global scale, Stern et al. (2017) presented convincing evidence that a single cosmopolitan pelagic species of *Sardinella* (*S. aurita*), with two parapatric subspecies, should be recognized in what was previously hypothesized as a subgenus containing five presumably morphologically distinguished species.

Giving the apparent influence of particular ecological or physiographic conditions in the development of some species of marine Clupeiformes and in western South Atlantic populations of *Brevoortia*, as indicated by Hedgecock et al. (1989), Safford & Booke (1992), Díaz de Astarloa & Cousseau (1993), and Kinsey et al. (1994), an additional Multivariate Discriminant Analyses was conducted with the morphometric data and specimens of *Brevoortia* grouped according to their collecting habitat: “coastal lagoon”, “estuary” or “ marine coastal” (Fig. 14). Interestingly, a gradual ordination of specimens was observed along axis 1, with those collected in estuaries located in an intermediate position in the morphospace between “coastal lagoons” and “marine coastal”. Those results indicate that perhaps a complex correlation between latitudinal variation and ecological/habitat conditions play a significant role in the morphological development of menhadens in the western South Atlantic. In addition, patterns of latitudinal migration or the degree of fidelity to particular “growing sites” are largely unknown in the western South Atlantic populations of *Brevoortia*. Results uncovered herein, however, indicate some degree of fidelity and perhaps a reduced capacity of latitudinal migration, as also indicated by the recovery of a monophyletic lineage (albeit with an extremely shallow genetic divergence from other lineages) containing all specimens collected in a single coastal lagoon in Northern Rio de Janeiro State (LI01-LI20; Fig. 9). Those results are potentially significant for the fisheries management of stocks of “savelhas” in the western South Atlantic.

Molecular data

North Atlantic species of *Brevoortia* and related genus (*Alosa* and *Sardinops*) also have a complex taxonomic situation at the species level, reminiscent and perhaps elucidative of the results obtained herein for the western South Atlantic populations of menhadens. Anderson (2007) detected the existence of two main lineages of *Brevoortia* in

the North Atlantic based on molecular markers (nuclear and mitochondrial). Those lineages were referred to by him as the "large-scales" and the "small-scales" groups of menhadens (*B. tyrannus*+*B. patronus* and *B. smithi*+*B. gunteri*, respectively), following Hildebrand (1948) who defined the same groups based on morphology. No species-specific clusters were identified by Anderson (2007) within those lineages. Anderson et al. (2007) later attributed similar results to an incomplete genetic divergence between *B. gunteri* and *B. smithi*. In another study based on molecular data, Lynch et al. (2010) presented evidence that the genetic divergence found between specimens identified through morphology and collection locality as *B. tyrannus* and *B. patronus* is more characteristic of conspecific populations rather than that of truly separated, valid, species. In another study using morphological and molecular data, Anderson & McDonald (2007) presented evidence that *B. patronus* and *B. gunteri*, which represent the "large-scales" and the "small-scales" groups of menhadens and are sympatric in some regions of the Gulf of Mexico, can be separated on the bases of meristic characters and the existence of a high value of *Fst*, which is an indication of reduced gene flow. The results of the present study regarding the western South Atlantic species of *Brevoortia*, however, indicate a positive and significative number of migrants among the different geographic localities (an index related to *Fst*). The sharing of haplotypes among distinct regions and the absence of a clear segregation among groups of specimens also support the existence of a single species in the region.

Similar taxonomic complex situations have also been detected in the supposedly related genus *Alosa* by Faria et al. (2006), who identified shared haplotypes between *A. aestivalis* and *A. psedoharengus* and credited this result to an incomplete lineage sorting between those species. Bowen et al. (2008), in turn, suggested that results obtained do not support the hypothesis of an incomplete lineage sorting, since high values of genetic divergence is observed between *A. psedoharengus* and *A. aestivalis* when compared with intraspecific genetic divergence of each species, again recognizing *A. aestivalis* and *A. psedoharengus* as valid. In the present study, shared haplotypes were also observed among specimens of *Brevoortia* from different geographic localities, and genetic divergence between and within groups were not significantly different from each other (Fig. 12), supporting the hypothesis that a single species of the genus is present in the western South Atlantic. In another case involving *Alosa*, Chapman et al. (1994) considered *Alosa*

alabamae and *A. sapidissima* as synonyms based on a lack of congruence between mitochondrial and nuclear markers, a situation that indicates an incomplete lineage sorting according to the authors. Bowen et al. (2008), however, again concluded that *Alosa alabamae* and *A. sapidissima* are valid, based on the perception that the molecular differences observed between them are a reflex of recent segregation rather than of incomplete lineage sorting. Indeed, all specimens of *A. alabamae* included in the study of Bowen et al. (2008) are grouped in a monophyletic assemblage, nested within a polytomy involving several clades containing specimens identified as *A. sapidissima*. This situation is different from the one recovered herein for the species of *Brevoortia* of the western South Atlantic, where no clear species-specific clades with substantial support in terms of genetic divergence were recovered based on the molecular data examined.

Species delimitation

Carl Linnaeus, a botanist of the early 18 century, revolutionized the issue of species definition and delimitation with the establishment of a universal binominal system for naming and classification of all organisms, but this question can be traced back at least to ancient Greek texts (e.g., Wilkins, 2009). In the Clupeidae, which typically have a complex taxonomic scenario at the species level, the situation is similar. Thomas et al. (2014), using mitochondrial/nuclear sequences and morphological data, recently recognized a cryptic species in *Sardinella gibbosa*, the gold stripe sardine, from the Indo-West Pacific. Results of molecular data showed two clearly distinct clades in all haplotype networks and phylogenetic trees recovered, one broadly distributed and containing specimens collected throughout all the sampling area (Southeast Asia), and a second with specimens collected solely in the Cagayan Province, Philippines. Interestingly, their data indicated that this second clade of *Sardinella gibbosa* is more related to other species of *Sardinella* (*S. fimbriata* and *S. hualiensis*) than to specimens clearly recognized as *Sardinella gibbosa* sensu stricto, which were actually related to another species of *Sardinella*, *S. lemuru*. This phylogenetic scheme in association with the high values of divergence between the two clades of *S. gibbosa* and the haplotype network, which also showed clear isolation between the two clades of *S. gibbosa*, clearly indicated the existence of two sympatric species within the complex. This hypothesis was further corroborated by morphology, since a Principal

Component Analysis indicated two clearly distinct polygons representing the two "species" that should be recognized under *S. gibbosa*.

The situation revealed by Thomas et al. (2014) in the case of *S. gibbosa* is one of the few recent examples in the taxonomy of the Clupeidae where practically all examined data clearly converge to the resolution of a taxonomically complex scenario. In the case examined herein, molecular data and, perhaps to a lesser degree, morphological data, converge to the conclusion that a single species of *Brevoortia* should be recognized in the western South Atlantic. No species-specific clades were recovered in the phylogenetic trees generated by the molecular data. Haplotypes are also shared among different collection sites, and genetic divergence within and between groups had no significant differences. Multivariate Discriminant Analyses of morphological data also failed to reveal clearly distinct groups, with the possible exception of the apparent latitudinal morphocline suggested by the analysis of the morphometric data in figure 6A, as discussed above.

Results obtained herein for the western South Atlantic *Brevoortia* are actually more similar to the ones provided by Stern et al. (2017) in the case of *Sardinella* (subgenus *Sardinella*). In their study, the bPTP species-delimitation method merged all the samples containing the putative species of the subgenus *Sardinella* to a single lineage, with a significant support value of 0.99. The ABGD model, in turn, provided a main partition of the data into three groups, one including all specimens of the putative species (*S. aurita*), a second one with two exceptional haplotypes from the Pacific, and a third group containing all the remaining specimens (intraspecific divergence=0.0016). Genetic pairwise comparisons between morphospecies did not reveal a clear segregation, with projecting overlapping *p*-distances and without a delimiting gap between them. The haplotype network presents a positive number of migrants among distant geographical sites, despite being divided among the different places sampled.

In the present study, as in the case presented by Stern et al. (2017), the bPTP species-delimitation method merges all the western South Atlantic specimens of *Brevoortia* included in the analysis into a single lineage, with a significant support value of 0.88. The ABGD model provides a main partition that delimitated the dataset into eight groups including the seven outgroup taxa, *i.e.*, all the western South Atlantic specimens of

Brevoortia were included in a single group (intraspecific divergence=0.0046). The genetic pairwise comparison also agrees with the hypothesis of a single species, with no genetic differences found between and within geographical collection sites (Fig. 12). Positive numbers of migrants were also observed among all localities, indicating a considerable level of gene flow. Different from the situation revealed by Stern et al. (2017), who still recognized two subspecies in *Sardinella aurita* (sensu lato), no geographic clusterings were observed in the haplotype networks or in the phylogenetic trees, and no morphospecies could be clearly delimitated in the morphological analyses of the examined specimens of *Brevoortia* from the western South Atlantic.

Taxonomic resolution

The results obtained herein support the hypothesis that *Brevoortia aurea* (Spix & Agassiz, 1829) and *B. pectinata* (Jenys, 1842) should not be recognized as distinct species. This conclusion is in accordance with the results obtained by Cousseau & Diaz de Astarloa (1993) and García et al. (2008) that indicated the existence of a single species of the genus in the region between Uruguay and Argentina. Together, results obtained in those different studies and the ones presented herein constitute a strong set of evidence, based on a significant number of specimens collected throughout the known range of distribution of the genus in the western South Atlantic and from different data sources (molecules and morphology), for the existence of a single species of *Brevoortia* in the region. Therefore, it is proposed that *Brevoortia aurea* (Spix & Agassiz, 1829), the senior synonym, should be recognized as the single valid species of *Brevoortia* in the western South Atlantic.

Kottelat (1988: 84) noted that two specimens of *Clupanodon aureus* Spix & Agassiz (1829), upon which the species was described, were apparently originally deposited in the Museé d'Histoire Naturelle de Neuchâtel, Switzerland (MHNN), according to Agassiz's annotations available in the "Archives de Louis Agassiz; Fonds de l'Institut de Géologie de l'Université de Neuchâtel, Suisse". In the description of *Clupanodon aureus*, Spix & Agassiz (1829) indicated that two specimens were collected in the expedition of Spix & Martius (1817-1820), and that those specimens were sent to the Munich Museum, Germany: "In Museo Monacensi specimina duo egregia in spiritu vini servantur" (Spix & Agassiz, 1829: 53). However, according to Kottelat (1988), only one of those specimens

can still be located in the MHNN collection (MHNN 1159; Fig. 4). It is possible that the second specimen referred to by Spix and Agassiz (1829) was actually left in the Munich Museum upon Agassiz's transfer to Neuchâtel in 1832. If that is the case, then that second specimen is permanently lost, as all specimens deposited in the Munich Museum worked by Spix and Agassiz were destroyed during the Second World War (Whitehead & Myers, 1971). Kottelat (1988) proposed that the single specimen deposited in Neuchâtel (MHNN 1159) should be recognized as a syntype, apparently given the likely assumption that two specimens were used in the original description of the species. It is however possible that the description of *Clupanodon aureus* was mostly based on that single surviving specimen (MHNN 1159), given Agassiz's notation in Neuchâtel of just the TL of that specimen, without a further reference to the second specimen mentioned by Spix & Agassiz (1829). Regardless of the relevance of this second syntype specimen of *Clupanodon aureus* to the description of the species, it is highly likely that only one specimen of the original series collected during Spix & Martius' expedition is still available.

It is therefore proposed that MHNN 1159 should be recognized as the lectotype of *Brevoortia aurea* (Spix & Agassiz, 1829). We also believe that the recognition of the single specimen deposited in the lot MHNN 1159 as the proper lectotype of *Clupanodon aureus* Spix & Agassiz, 1829 will help to enhance the stability of the nomenclature of an otherwise taxonomically complex species, in accordance with Article 74 of the ICZN (1999). Meristic and morphometric data of the lectotype of *Clupanodon aureus* (MHNN 1159) is presented in Table 7.

The notation made by Spix & Agassiz (1829:53) about *Clupanodon aureus*, "Habitat Bahiae et alibi in ora Brasiliae orientali", has also led some past authors (e.g., Hildebrand, 1948; Figueiredo & Menezes, 1978; Cousseau & Diaz de Astarloa, 1993) to conclude that the type-locality of the species is actually the state of Bahia, northeastern Brazil (8°32'S-18°18'S). However, this region is broadly outside the known geographic range of distribution of the species based on all other western South Atlantic specimens of *Brevoortia* deposited in fish collections, including those collected during this study (northern Rio de Janeiro, Brazil, south to Mar de Cobo, Argentina). Indeed, since at least the 1960's and in most of the recent reviews or catalogs of the genus in the western South

Atlantic (e.g., Berry, 1964; Whitehead, 1985; Menezes, 2003; Fricke et al., 2018), the type locality of *Clupanodon aureus* is simply stated as "Brazil". Papavero (1971) noted that some boxes containing specimens collected by Spix and Martius' expedition in Brazil (1817-1820) were dispatched to Europe (Hamburg) from the city of Salvador (Bahia state), and that might be the original source of confusion regarding the type locality of the species. Based on the information available so far, the precise type locality of *Clupanodon aureus* is therefore uncertain; it should indeed be referred to simply as "Brazil".

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Specimens examined (names according to original identification):

Clupanodon aureus: MHNN 1159 (syntype), 1, 171 mm SL, Brazil, Bahia, 1829.

Alosa pectinata: BMNH 1843.2.8.47 (syntype), 1, 150 mm SL, Argentina, Bahia Blanca, 1842. BMNH 1917.7.25.1 (syntype), 1, 230 mm SL, Argentina, Bahia Blanca, 1842.

Brevoortia aurea: BRAZIL: MNRJ 12101, 23, 90-136 mm SL, Rio de Janeiro/RJ, Rodrigo de Freitas lagoon, 12/03/1990. MNRJ 13349, 3, 26-33 mm SL, Maricá/RJ, 1986. MNRJ 17464, 2, 127-145 mm SL, Maricá/RJ, 22/08/1986. MNRJ 36978, 2, 113-124 mm SL, Maricá/RJ, 22/08/1986. MNRJ 39383, 1, 148 mm SL, São Gonçalo/RJ, 27/08/2009. MNRJ 39388, 1, 145 mm SL, São Gonçalo/RJ, 31/08/2009. MNRJ 39856, 2, 162-171 mm SL, Rio de Janeiro/RJ, Guanabara bay, 14/02/2012. MNRJ 42092, 2, 119-148 mm SL, Saquarema/RJ, Saquarema lagoon, 09/1975. MZUSP 1310, 9, 125-156 mm SL, São João da Barra/RJ, Paraíba river, 12/1911. MZUSP 3325, 1, 209 mm SL, Rio de Janeiro/RJ, Copacabana beach, 1942. MZUSP 11728, 3, 123-160 mm SL, Cananéia/SP, 15/08/1961. MZUSP 11729, 4, 100-105 mm SL, Cananéia/SP, 23/02/1949. MZUSP 11730, 2, 77-88 mm SL, Cananéia/SP, 10/1977. MZUSP 16393, 2, 147-148 mm SL, Cananéia/SP, 19/06/1952. NPM 1794, 2, 219-238 mm SL, Casimiro de Abreu/RJ, São João river, 28/10/2012. NPM 3039, 51, 173-244 mm SL, Rio de Janeiro/RJ, 08/05/2015. NPM Uncatalogued, 80, 113-143 mm SL, Macaé/RJ, Imboassica lagoon, 2016.

Brevoortia pectinata: BRAZIL: MNRJ 25502, 7, 23-42 mm SL, Rio de Janeiro/RJ, Marapendi lagoon, 21/12/2002. MNRJ 42123, 3, 75-130 mm SL, Saquarema/RJ, Saquarema lagoon, 08/1980. MZUSP 1337, 2, 49-67 mm SL, State of Rio Grande do Sul, 1890. MZUSP 11726, 1, 155 mm SL, Cananéia/SP, 14/07/1961. MZUSP 11727, 3, 94-118 mm SL, Cananéia/SP, 1961. MZUSP 11734, 3, 116-126 mm SL, Imbé/RS, Imbé-Tramandaí bridge, 06/04/1974. MZUSP 11735, 2, 75-96 mm SL, Rio Grande/RS, Cassino beach, 07/1965. MZUSP 11736, 1, 35 mm SL, southern Brazil, 02/1974. MZUSP 14157, 5, 186-192 mm SL, Tramandaí/RS, Tramandaí lagoon, 19/04/1977. MZUSP 14158-14163, 6, 133-186 mm SL, Tramandaí/RS, Tramandaí lagoon, 10/05/1977-18/05/1978. MZUSP 14164, 1, 106 mm SL, Tramandaí/RS, Armazém lagoon, 25/10/1977. MZUSP 18320, 2, 14-16 mm SL, Rio Grande/RS, Cassino beach, 21/01/1972. MZUSP 18451, 8, 58-70 mm SL, Tramandaí/RS, near the fishing platform, 1974. NPM 2795, 15, 210-244 mm SL, Rio Grande/RS, 18/07/2015. ARGENTINA: NPM 5601, 5, 164-282 mm SL, Mar del Cobo/Buenos Aires province, 18/11/2016.

Tables and Figures

Table 1: List of specimens examined for morphology, grouped by voucher specimens deposited in Fish Collections and the five main geographic regions considered in the Multivariate Discriminant Analyses.

	North RJ n=91	Rio n=98	SP n=15	RS n=45	Arg n=5	Total n=254
MNRJ (10 vouchers)		n=7 (25502-Rio de Janeiro/RJ) n=2 (36978-Maricá/RJ) n=3 (13349-Maricá/RJ) n=1 (39383-São Gonçalo/RJ) n=3 (42123-Saqurema/RJ) n=2 (42092-Saquarema/RJ) n=2 (17464-Maricá/RJ) n=2 (39856-Guanabara bay/RJ) n=1 (39388-São Gonçalo) n=23 (12101-Rodrigo de Freitas lagoon/RJ)				
MZUSP (17 vouchers)	n=9 (1310-São João da Barra/RJ)	n=1 (3325-Rio de Janeiro/RJ)	n=3 (11727-Cananéia/SP n=1 (11726-Cananéia/SP) n=2 (11730-Cananéia/SP) n=4 (11729-Cananéia/SP) n=3 (11728-Canaéia/SP) n=2 (16393-Cananéia/SP)	n=2 (1337-Rio Grande do Sul) n=2 (11735-Rio Grande/RS) n=6 (14158 to 14163-Tramandaí/RS) n=1 (11736-South of Brazil) n=2 (18320-Rio Grande/RS) n=8 (18451-Tramandaí/RS) n=3 (11734-Imbé/RS) n=1 (14164-Tramandaí/RS) n=5 (14157-Tramandaí/RS)		
NPM (5 vouchers)	n=2 (1794-Casimiro de Abreu/RJ) n=80 (NPM- Macaé/RJ)	n=51 (3039- Rio de Janeiro/RJ)		n=15 (2795-Rio Grande/RS)	n=5 (NPM- Mar de Cobo/Argentina)	

Table 2: Comparative morphometric data of specimens of *Brevoortia*, grouped by geographic regions. Numbers indicate minimum and maximum measurements (mm); medians are indicated in parentheses (mm).

	North RJ n=91	Rio n=98	SP n=15	RS n=45	Arg n=5	Total n=254
Standard Length	113-238 (127)	23-244 (177)	77,6-160 (108)	35-244 (145)	164-282 (237)	23-282 (129)
Body Height	38,8-88,6 (46,2)	6,7-86,9 (60,95)	33,7-64 (46,6)	14,3-94,7 (58,9)	70-112,4 (93)	6,7-112,4 (47,2)
Head Length	32,5-73 (36,4)	7-76,9 (58,9)	25,6-51,8 (33,6)	12,3-75,4 (46,8)	51,2-82,8 (75,1)	7-82,8 (38,1)
Head Height	15,2-65,2 (35)	5,7-70,6 (52)	23,4-46,8 (30,7)	12,1-70,2 (42,4)	49,5-76,1 (68,9)	5,7-76,1 (36,2)
Snout length	7,8-19,9 (9,2)	1,5-28,8 (16,95)	6,4-14,6 (9,4)	2,8-22,3 (12,3)	14-23,8 (21,5)	1,5-23,8 (9,7)
Distance between Symphysis of the upper maxilla to nostrils	3-10 (4,6)	0,7-11,4 (7,7)	3,4-6,7 (4,4)	1-12,4 (6)	6-11,8 (9,4)	0,7-12,4 (5)
Eye Diameter	7,1-11,3 (8,3)	2-12,3 (9,7)	7-10,4 (8,8)	3,1-11,9 (9,8)	9,7-11,4 (10,8)	2-11,9 (8,7)
Post-Orbital Distance	17,1-43 (19,7)	3,5-61,2 (32,7)	12,8-29,9 (17,8)	6-45,7 (25,6)	27,9-50 (43,7)	3,5-61,2 (20,7)
Distance Between Orbit and Upper Maxilla	1,3-8 (3)	0,3-9,9 (5,7)	2,2-4,6 (2,9)	0,7-9,5 (3,3)	2,8-9,2 (8,6)	0,3-9,9 (3,2)
Upper Maxilla Length	13,4-30,7 (15,3)	2,5-33,3 (24,6)	10,4-22,1 (14,1)	5-34,4 (19,8)	22,4-37,3 (35)	2,5-37,3 (16)
Upper Maxilla Height	3,2- 8,8 (4,8)	0,9-10 (7,15)	3,7-7,2 (4,5)	1,8-10,8 (6,3)	6,7-11,7 (9,4)	0,9-11,7 (5,1)
Lower Jaw Length	17,2-37,8 (19,3)	3,3-41,6 (30,8)	13,3-27,3 (17,8)	5,6-44,6 (25)	28,6-45 (42,3)	3,3-45 (20)
Pre-Dorsal Distance	52,3-120,3 (60)	10,3-131,3 (91,5)	40,5-83,2 (55,6)	17,5-130,2 (76,6)	83,6-142,3 (120,7)	10,3-142,3 (61,8)
Pre-Pectoral Distance	31,1-70,3 (35,7)	7,2-74,7 (56,4)	22,6-52,9 (33,6)	12,9-73,1 (46,6)	49,4-79 (73,3)	7,2-79 (37)
Pre-Pelvic Distance	37,3-128 (62,9)	12,1-127,9 (97,05)	42-88,3 (58,1)	21,7-133,8 (81,9)	88,7-149 (129,5)	12,1-149 (65,9)
Pre-Anal Distance	80-180,8 (91,2)	16,4-178,2 (133,45)	58,1-121,3 (82,1)	28,1-187,4 (111,7)	127,4-213,6 (184,1)	16,4-213,6 (94)
Anal Fin Base Length	14,7-41,9 (25,6)	3,7-43 (30,7)	14,4-32,4 (22,1)	6-41,4 (28,5)	30-46,7 (39,5)	3,7-46,7 (26,1)
Dorsal Fin Base Length	22,5-45 (26,3)	4,4-46,1 (32,3)	16,1-36,4 (25,1)	7,4-49 (31,4)	34,7-55 (48,4)	4,4-55 (27)
Pectoral Fin Length	14,6-38,5 (19,9)	3,1-44,1 (30,7)	13,2-29 (19,1)	6,3-48,8 (26,1)	31,2-52,3 (44,1)	3,1-52,3 (20,4)
Distance between Pectoral Fin and Pelvic Fin	3,8-21,9 (9,7)	2-23 (10)	5,9-14,4 (8,7)	2,9-22,5 (9,7)	5,9-25,8 (10)	2-25,8 (9,7)
Distance Between Pelvic-Fin base and Anal Fin	10,8-63 (31,5)	4,3-65,1 (41,3)	19,1-42,2 (28,2)	8,6-68,7 (38,5)	44,3-74,2 (63,2)	4,3-74,2 (32,5)
Caudal Peduncle Length	11,7-26,3 (16,3)	1,6-27,3 (17,9)	7,7-18,4 (12,7)	2,5-37,5 (15,4)	18,3-53,5 (27,8)	1,6-53,5 (16,4)
Caudal Peduncle Height	11,3-24,4 (14,1)	2,2-24,4 (17,7)	9,3-19,7 (12,9)	4,2-28 (17)	20,4-29,7 (25,9)	2,2-29,7 (14,3)
Axillar Scale Length	5-21,6 (9,1)	1,5-25,5 (15,2)	6,4-19,4 (10,8)	2,8-26,6 (16)	15,8-31,9 (24,4)	1,5-31,9 (10,4)
Head Width	13-33,2 (15,1)	2,3-32,4 (22,2)	10,2-23,1 (14,9)	4,3-35,3 (19,4)	22,9-39,4 (30,5)	2,3-39,4 (15,6)
Distance between Nostrils	2,3-9,1 (4,3)	0,6-10,8 (7,3)	2,8-6,1 (4,6)	1-10,6 (6)	5,4-9,2 (8,1)	0,6-10,8 (4,6)
Interorbital Distance	8-18 (9,2)	1,6-22 (14,7)	6,9-14,7 (9,2)	2,1-22 (11,7)	14-25 (19,5)	1,6-25 (9,7)

Table 3: Comparative meristic data of specimens of *Brevoortia*, grouped by geographic regions. Numbers indicate minimum and maximum values; medians are indicated between parentheses.

	North RJ n=91	Rio n=98	SP n=15	RS n=45	Arg n=5	Total n=254
Dorsal rays	17-20 (18)	15-20 (18)	12-19 (19)	15-21 (18)	17-18 (18)	12-21 (18)
Pectoral rays	11-17 (15)	13-18 (15)	13-17 (15)	12-17 (15)	15-17 (16)	11-18 (15)
Anal rays	17-24 (22)	16-24 (21)	16-24 (19)	16-24 (20)	17-21 (20)	16-24 (21)
Pelvic scutes	16-19 (17)	14-20 (18)	15-19 (17)	15-19 (17)	17-18 (18)	14-20 (17)
Post-pelvic scutes	11-15 (12)	10-14 (12)	11-14 (13)	11-14 (13)	12-13 (13)	10-15 (13)
Transverse scale rows along the body	52-64 (58)	42-68 (56)	52-64 (55)	43-64 (51)	44-49 (46)	42-68 (56)
Pre-dorsal scales	31-47 (38)	28-55 (44)	31-47 (38)	30-47 (40)	38-45 (41)	28-55 (40)
Scales below pre-dorsal series	7-16 (9)	9-17 (12)	7-15 (13)	7-15 (11)	9-11 (10)	7-17 (11)
Horizontal scale series around caudal peduncle	13-19 (16)	12-18 (14)	13-18 (15)	11-18 (13)	12-14 (14)	11-19 (15)
Horizontal scale series on caudal peduncle	6-9 (7)	5-9 (6)	6-9 (7)	5-9 (6)	5-6 (6)	5-9 (7)
Operculum ridges	5-14 (8)	1-19 (10)	5-14 (9)	1-17 (9)	8-12 (11)	1-19 (8)
Pre-caudal vertebrae		26-27 (26)		27-28 (28)		26-28 (27)
Caudal vertebrae (including pleurostile)		18-19 (18)		17		17-19 (18)

Table 4: Specimens examined in the molecular study with respective localities, dates of collection, and Codes used in this study and assessed in GenBank.

	Collections sites/Year	Code in analyses	COI Genbank Code	16s Genbank Code	RAG2 Genbank Code
Brevoortia cf. *aurea*	Rio de Janeiro- RJ-Brazil/ 2015 Macaé-RJ-Brazil/ 2017	BAU (01-20) LI (01-20)			
Brevoortia cf. *pectinata*[#^+]	^Rio Grande- RS-Brazil/ 2015	BPE (01-15)			
	[#]Tramandaí-RS-Brazil/ 2013	BPE (16-23)			
	[+]Mar de Cobo-Argentina/ 2017	AR (1-5)			
*Brevoortia gunteri**	*Sabine lake-Texas- USA/ 2004	BGU 01			
Brevoortia patronus°	°Atlantic Ocean-Texas-USA/?	BPT 21			
Alosa pseudoharengus		Alosa	NC_009576.1	NC_009576.1	DQ912149.1
Opisthonema oglinum		Opisthonema	GU702358.1	EU552783.1	DQ912144.1
Anchoviella lepidentostole		AnchoLep	JQ365222.1	EU552735.1	JQ012635.1
Pellona flavipinnis		PellFlav	KU289001.1	DQ912064.1	DQ912134.1
Denticeps clupeoides		Denticeps	NC_007889.1	NC_007889.1	DQ912133.1

* Tissue samples donated by Dr. Joel D. Anderson, Texas Parks and Wildlife Department, USA.
° Tissue samples donated by Dr. Andrew Bentley, University of Kansas, KS, USA (KU:IT5103).
[#] Tissue samples donated by Dr. Luiz R. Malabarba, Universidade Federal do Rio Grande do Sul (UFRGS), RS, Brazil (TEC3428A/B).
^ Specimens donated by Dr. Luiz Gustavo Cardoso, Universidade Federal de Rio Grande (FURG), RS, Brazil and colected by Dr. Luciano G. Fischer from Universidade Federal do Rio de Janeiro (UFRJ).
[+] Specimens donated by Dr. Juan M. Diaz de Astarloa, Universidad Nacional de Mar del Plata, Argentina.

Table 5: Pairwise genetic differences between (*F*st's Distance Method) and within (Theta pi) groups of western South Atlantic specimens of *Brevoortia* defined by collection sites, in addition to *B. gunteri* and *B. patronus* (North Atlantic). Above diagonal: nuclear pairwise differences between groups/species; diagonal, bold: mitochondrial/nuclear pairwise differences within groups/species; below diagonal: mitochondrial (COI+16S) pairwise differences between groups/species.

	North RJ	Rio	RS	Arg	*B. gunteri*	*B. patronus*
North RJ	**0.4/ 4.6**	3.61	2.94	3.92	8.0	22.0
Rio	2.09	**2.42/ 2.61**	2.06	3.04	7.1	21.1
RS	2.05	3.2	**3.17/ 1.35**	2.22	6.5	20.5
Arg	4.52	5.41	5.35	**7.6/ 2.8**	7.2	21.2
B. gunteri	23.1	23.05	23.6	25.4	-	16.0
B. patronus	39.0	39.05	39.43	41.4	40.0	-

Table 6: Number of migrants per generation between western South Atlantic specimens of *Brevoortia* grouped by collection sites. Above diagonal: number of migrants between groups inferred by nuclear RAG2 sequences; below diagonal: number of migrants between groups inferred by mitochondrial (COI+16S) sequences.

	North RJ	Rio	RS	Arg
North RJ	-	4.86	2.2	4.2
Rio	1.03	-	5.86	1.91
RS	3.65	3.52	-	1.56
Arg	0.76	2.07	5.89	-

Table 7: Meristic and morphometric data of the lectotype of *Clupanodon aureus* (MHNN 1159)*:

Total Length	203 mm
Standard Length	171 mm
Furcal Length	175 mm
Body Height	58 mm
Head Length	53 mm
Horizontal Eye Diameter	11 mm
Pre-Dorsal Distance	77 mm
Pre-Pectoral Distance	50 mm
Pre-Pelvic Distance	84 mm
Pre-Anal Distance	126 mm
Pectoral Fin Length	25 mm
Caudal Peduncle Length	25 mm
Caudal Peduncle Height	19 mm
Dorsal Fin Rays	19
Anal Fin Rays	19
Pectoral Fin Rays	15
Ventral Scutes	31
Vertebrae Count	45

*Data provided by Dr. Juan M. Diaz de Astarloa, Universidad Nacional de Mar del Plata, Argentina.

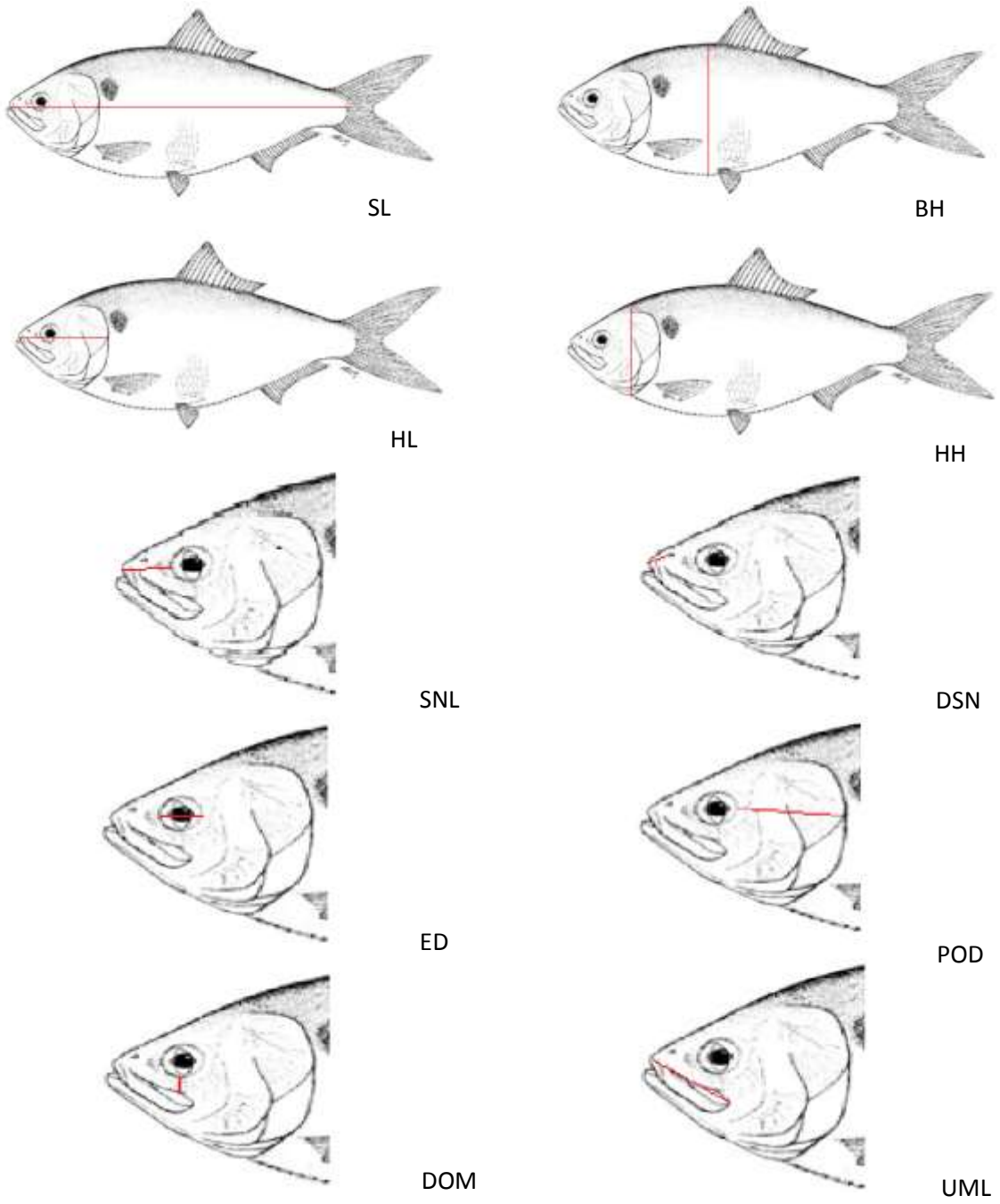


Figure 1: Morphometric measurements included in this study, part 1 (in red): Standard Length (SL), Body Height (BH), Head Length (HL), Head Height (HH), Snout length (SNL), Distance between symphysis of the upper maxilla to nostrils (DSN), Eye Diameter (ED), Post-Orbital Distance (POD), Distance Between Orbit and Upper Maxilla (DOM), Upper Maxilla Length (UML).

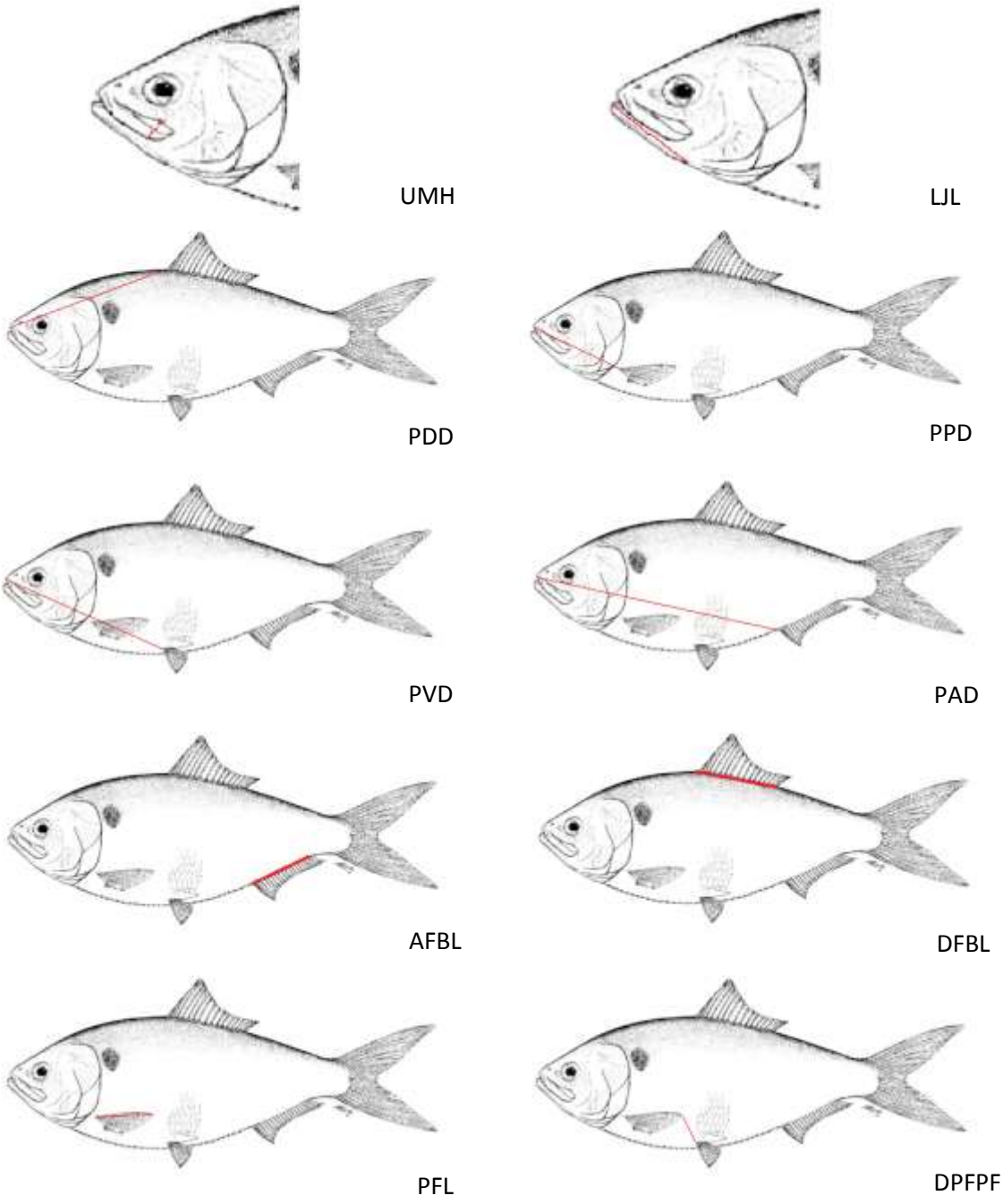


Figure 2: Morphometric measurements included in this study, part 2 (in red): Upper Maxilla Height (UMH), Lower Jaw Length (LJL), Pre-Dorsal Distance (PDD), Pre-Pectoral Distance (PPD), Pre-Pelvic Distance (PVD), Pre-Anal Distance (PAD), Anal-fin Base Length (AFBL), Dorsal-fin Base Length (DFBL), Pectoral-fin Length (PFL), Distance between Pectoral Fin and Pelvic Fin (DPFPF).

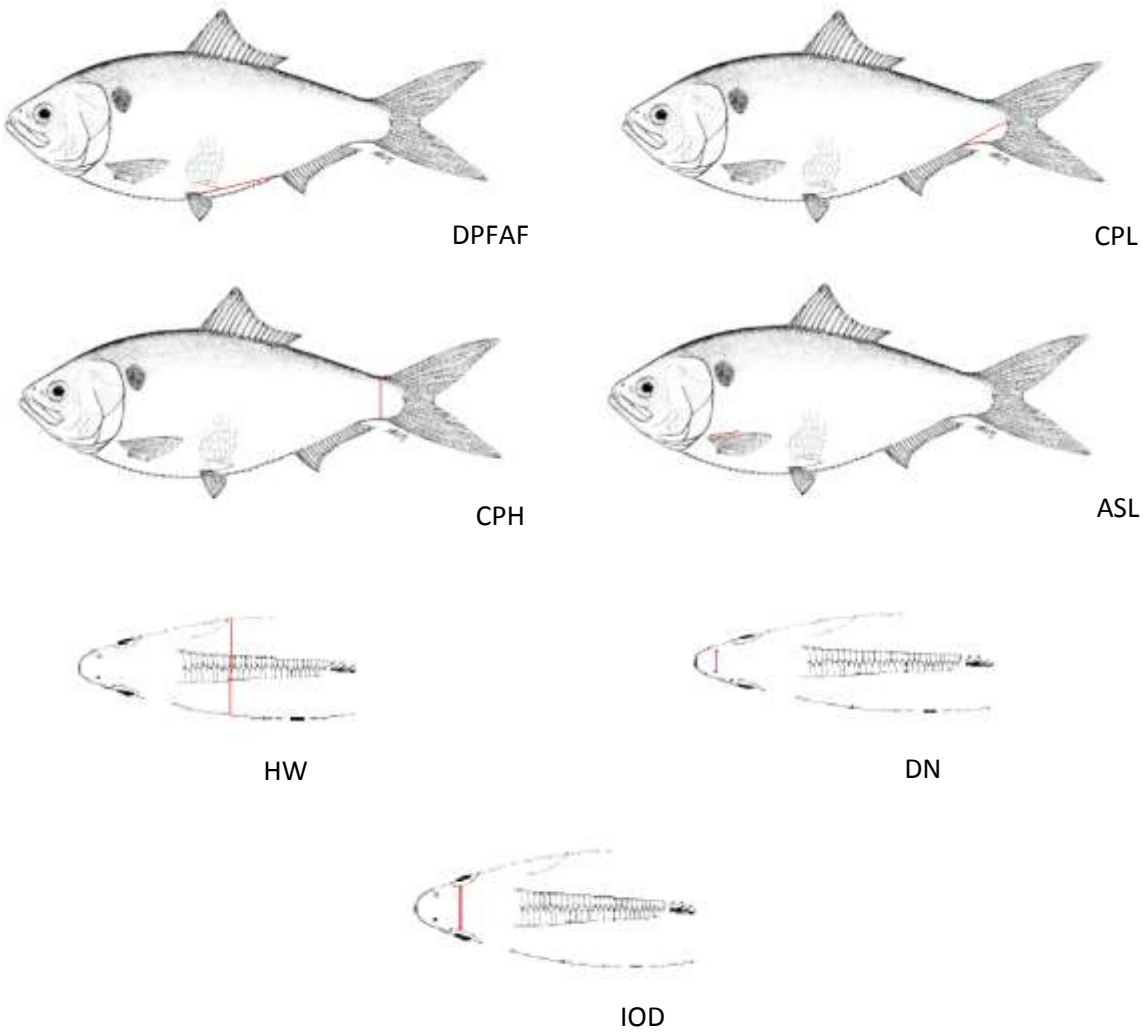


Figure 3: Morphometric measurements included in this study, part 3 (in red): Distance Between Pelvic-fin base and Anal Fin (DPFAF), Caudal Peduncle Length (CPL), Caudal Peduncle Height (CPH), Axillar Scale Length (ASL), Head Width (HW), Distance between Nostrils (DN), Interorbital Distance (IOD).

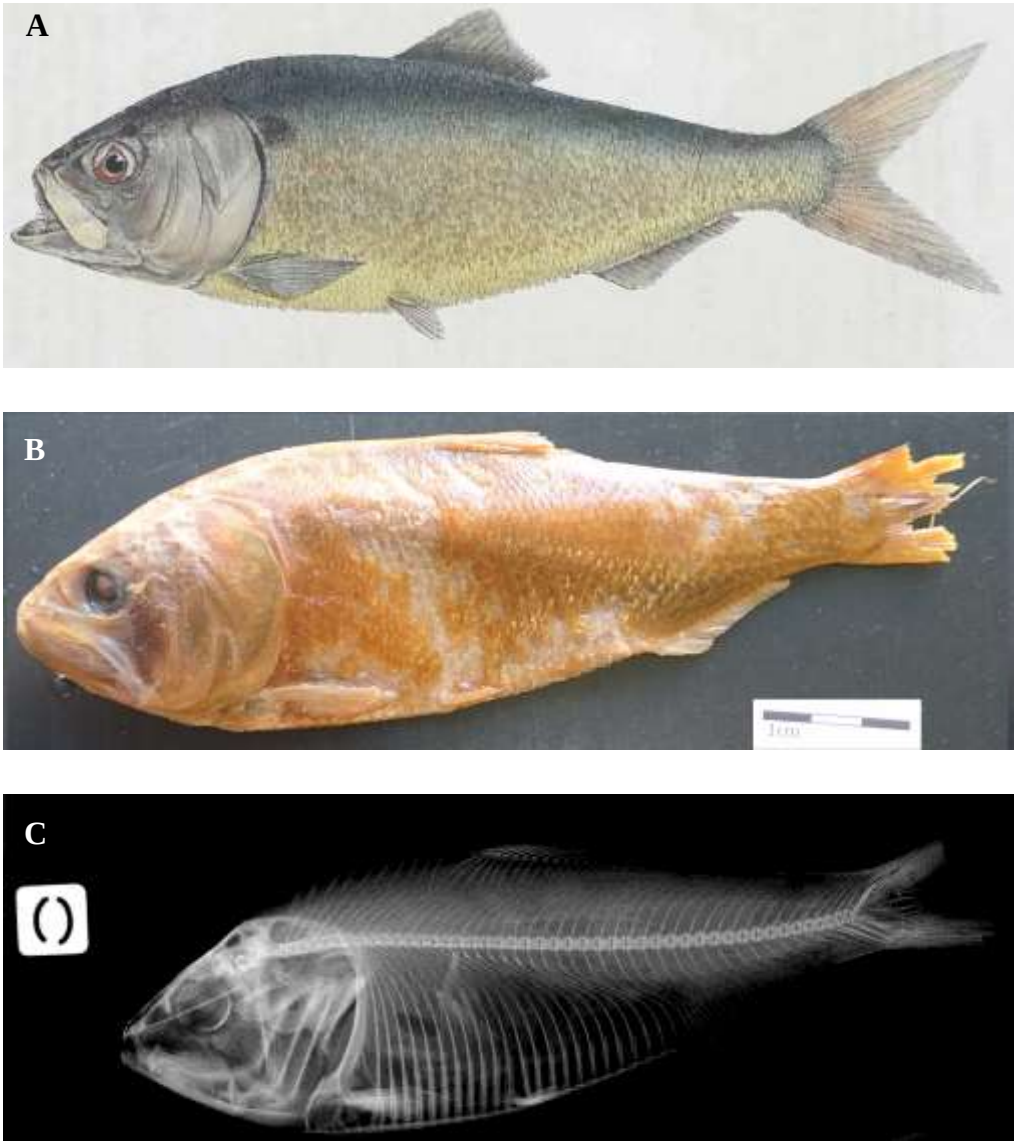


Figure 4: *Clupanodon aureus*; **A**, Original drawing in Spix & Agassiz (1829); **B**, photograph of syntype MHNN 1159, provided by Jessica Litman, Musée d'Historie Naturelle Neuchâtel, Swiss; **C**, x-ray image of the syntype (MHNN 1159), provided by Dr. Juan M. Diaz de Astarloa, Universidad Nacional de Mar del Plata, Argentina).

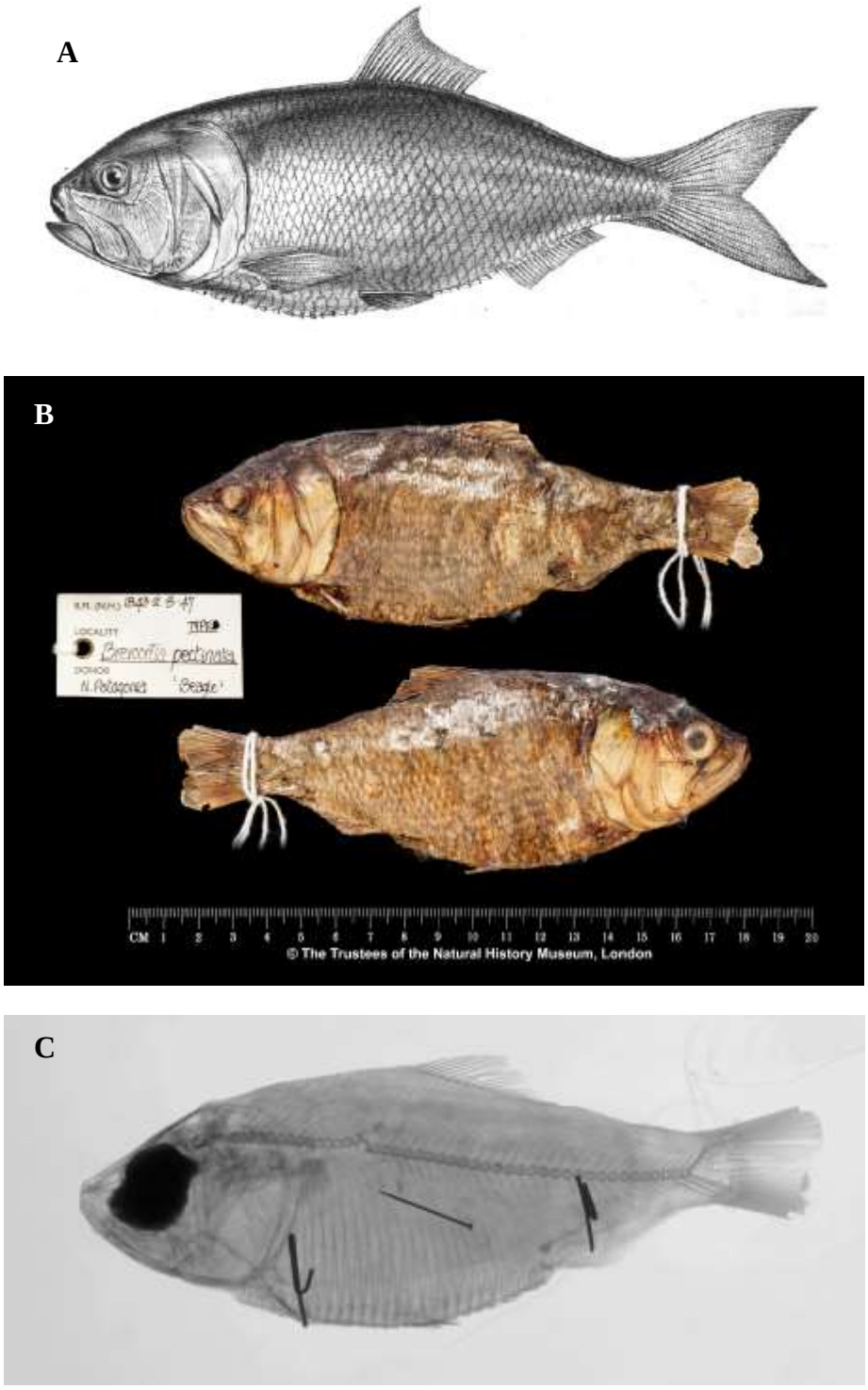


Figure 5: *Alosa pectinata*; **A**, Original drawing in Jenyns (1842); **B**, photograph and **C**, X-ray image of one syntype specimen (BMNH 1843); **D**, photograph and **E**, x-ray image of another syntype (BMNH 1917) (**B**-**E** provided by Dr. Juan M. Diaz de Astarloa, Universidad Nacional de Mar del Plata, Argentina).

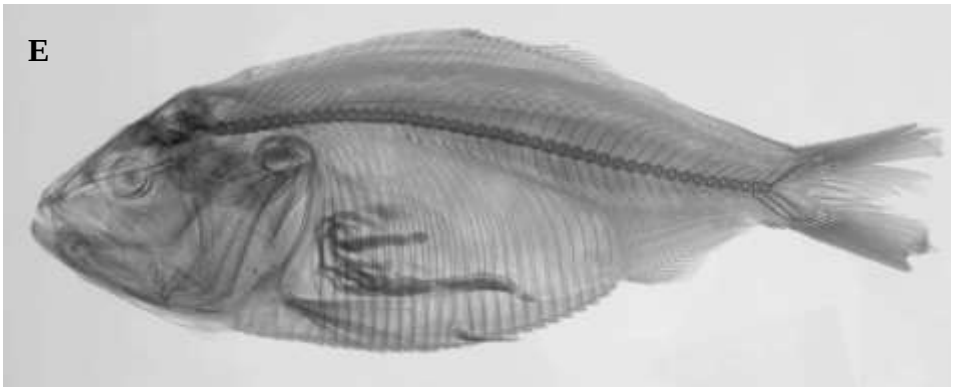


Figure 5: *Alosa pectinata*; **A**, Original drawing in Jenyns (1842); **B**, photograph and **C**, X-ray image of one syntype specimen (BMNH 1843); **D**, photograph and **E**, x-ray image of another syntype (BMNH 1917) (**B**-**E** provided by Dr. Juan M. Diaz de Astarloa, Universidad Nacional de Mar del Plata, Argentina).

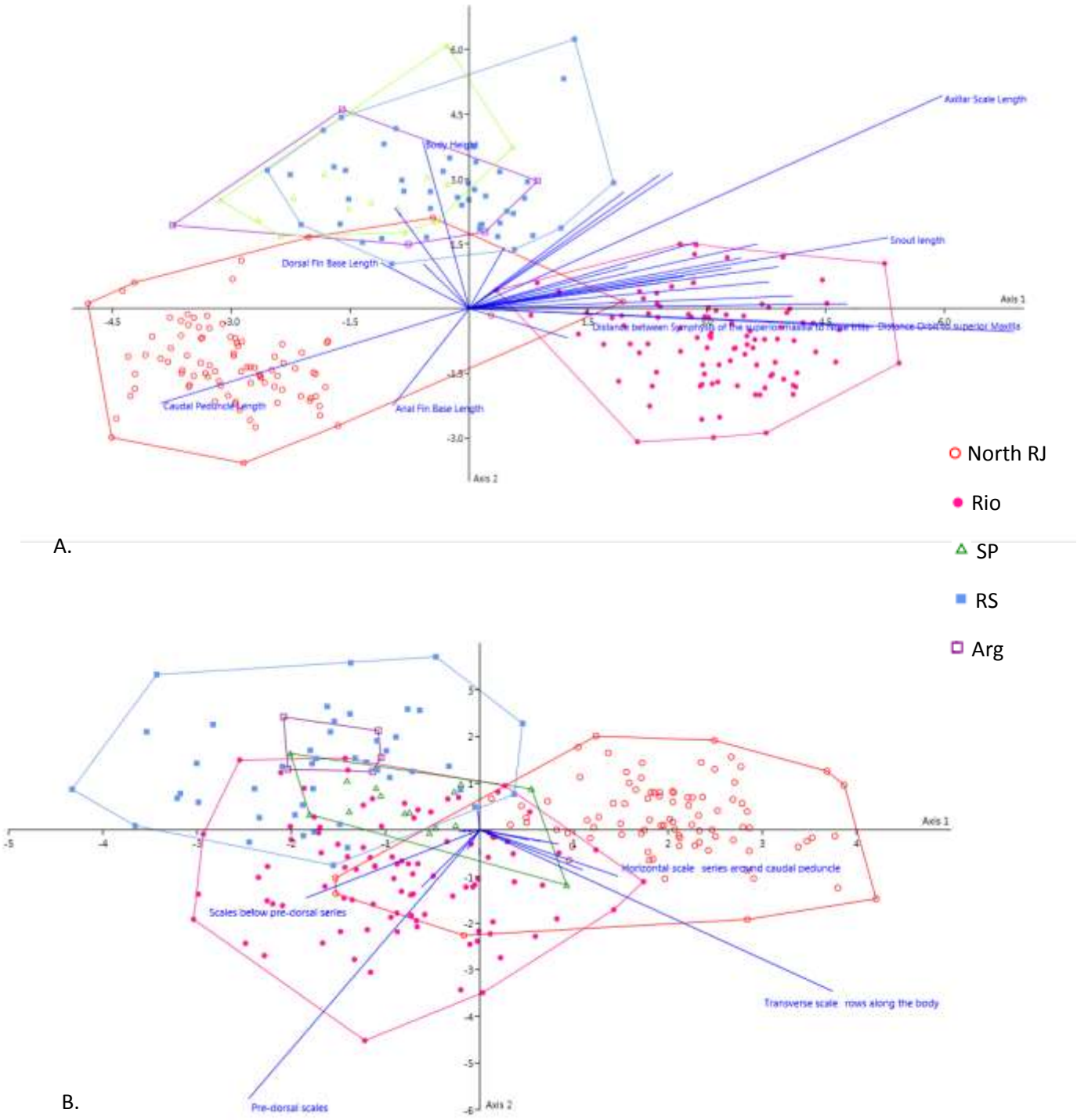


Figure 6: Multivariate Discriminant Analysis with data divided into five groups (A. Morphometric data, B. Meristic data). Colors represent collection sites: Open red circle=North of the Rio de Janeiro State (North RJ); closed pink circle=Rio de Janeiro city and vicinities (Rio); open green triangle=São Paulo state (SP); closed blue square=Rio Grande do Sul state (RS); open purple square=Argentina (Arg).

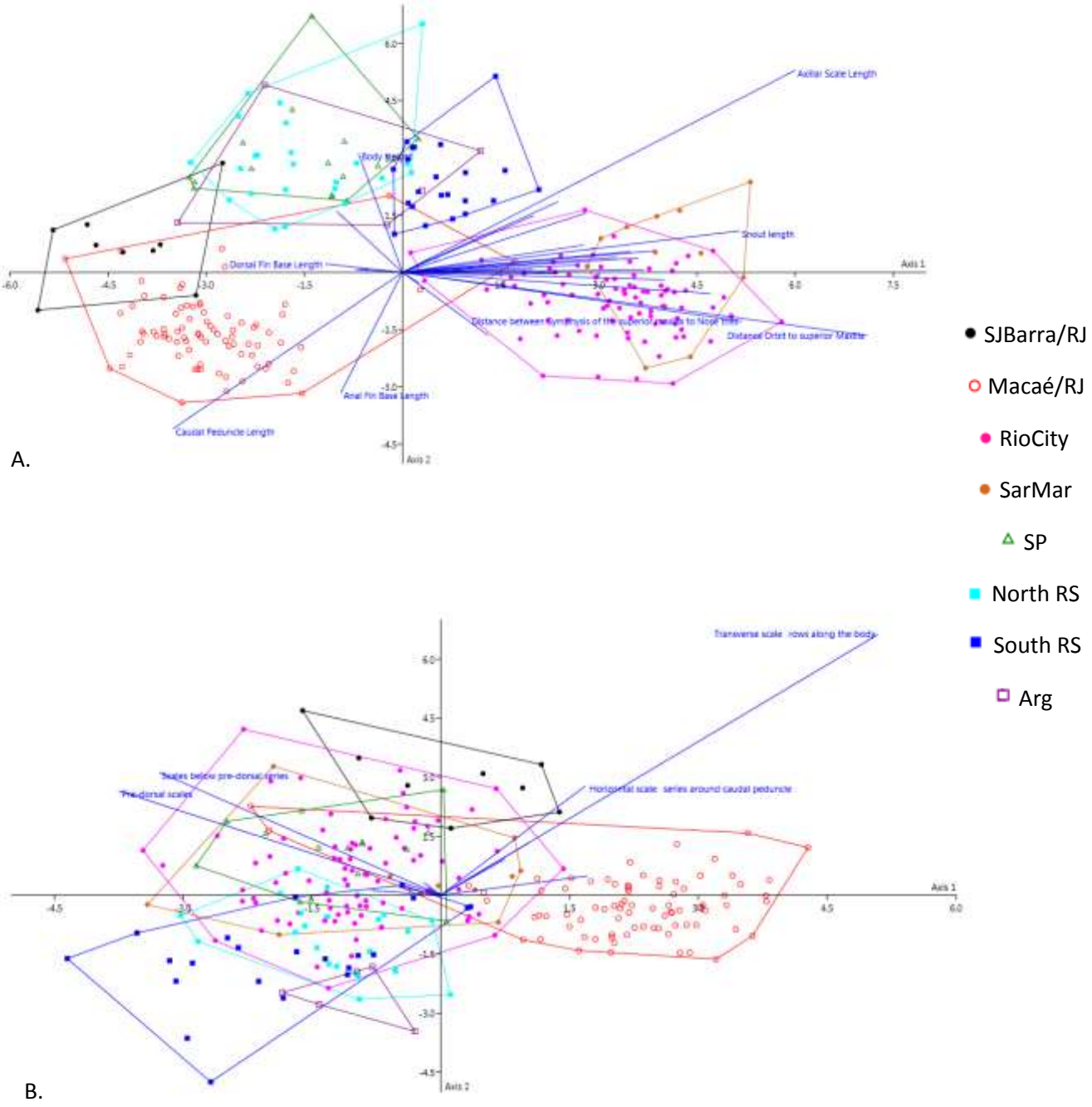
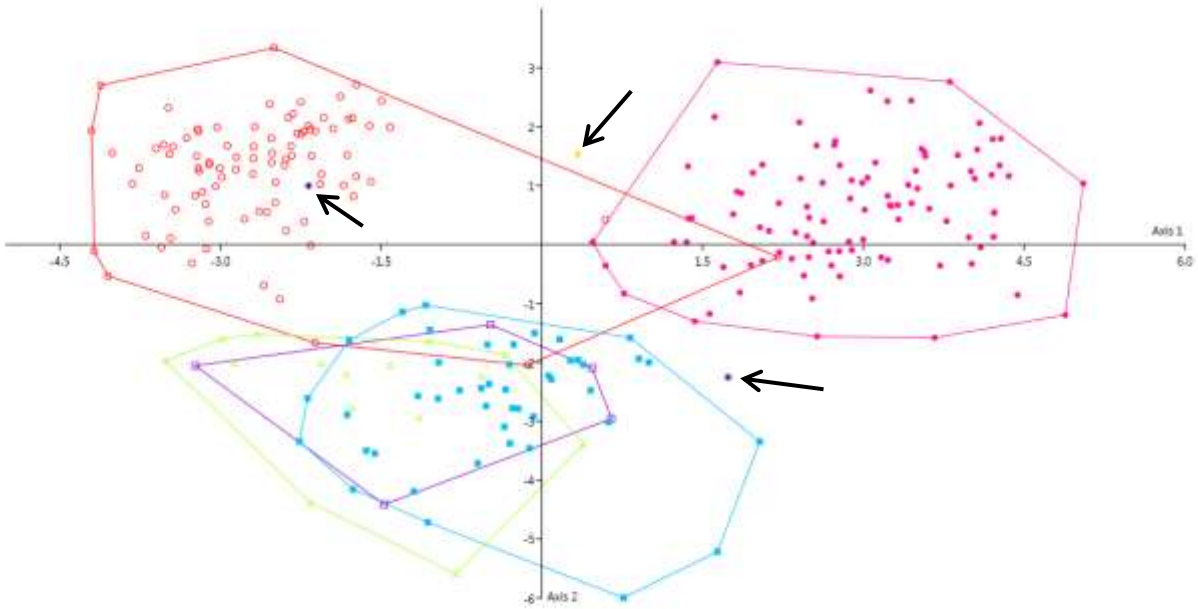
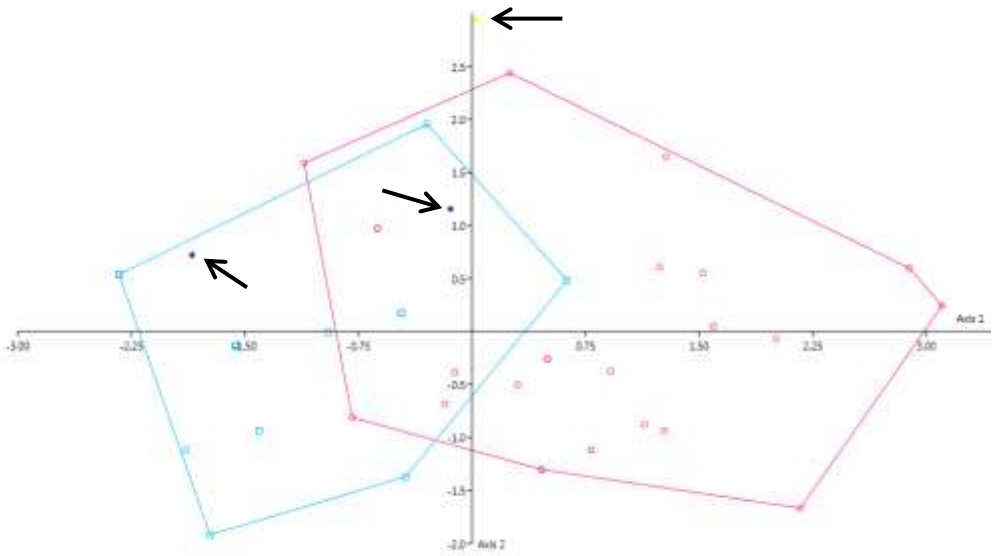


Figure 7: Multivariate Discriminant Analysis with data divided into eight groups of specimens (A. Morphometric data, B. Meristic data). Colors represent collection sites: Closed black circle=São João da Barra /RJ (SJBarra/RJ); open red circle=Macaé /RJ (Macaé/RJ); closed brown circle=Saquarema and Maricá /RJ (SaqMar); closed pink circle=Rio de Janeiro (RioCity); open green triangle=São Paulo state (SP); closed light blue square=North Rio Grande do Sul (North RS); closed dark blue square=South Rio Grande do Sul (South RS); open purple square=Argentina (Arg).

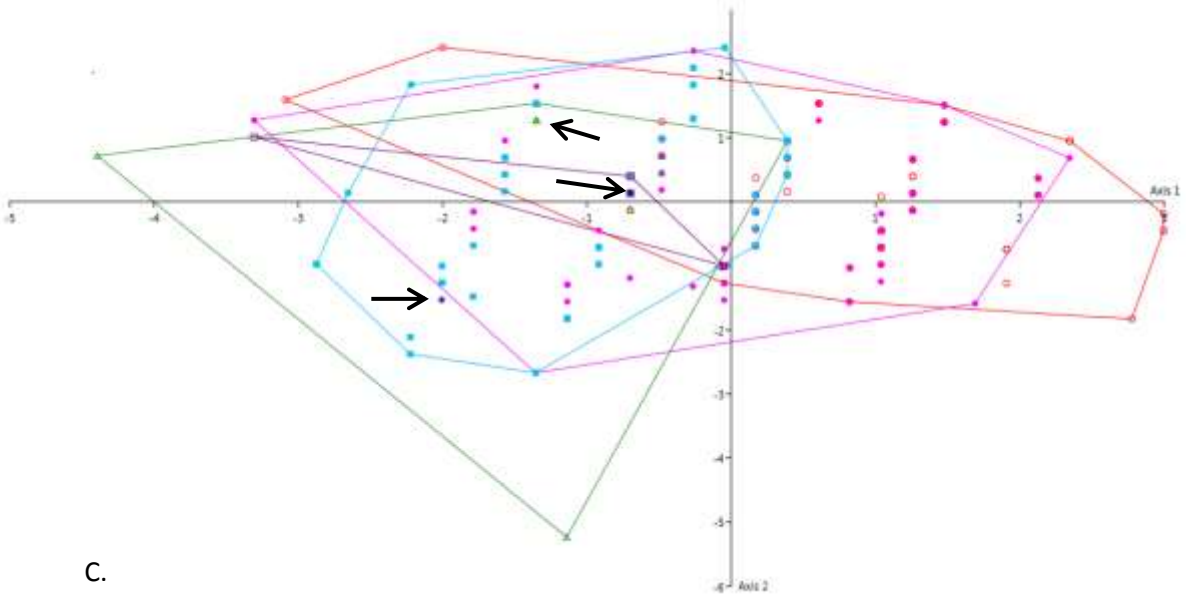


A.

✳ *B .aurea* syntype/ ○ North RJ/ ● Rio/ △ SP/ ■ RS/ □ Arg/ ✱ *B. pectinata* syntypes



B.



C.

Figure 8: Multivariate Discriminant Analysis with the syntypes of *Brevoortia aurea* and *B. pectinata* (A. Morphometric data; B. Meristic data, including vertebrae count (40 specimens+3 syntypes); C. Meristic data without vertebrae count but with all specimens examined (254 specimens+3 syntypes). Colors represent collections sites: Open red circle=North Rio de Janeiro State (North RJ); closed pink circle=Rio de Janeiro and vicinities (Rio); open green triangle=São Paulo (SP); closed light blue square=Rio Grande do Sul (RS); open purple square=Argentina (Arg). Syntypes indicated by black arrows: *Brevoortia aurea*=Closed yellow asterisc; *B. pectinata*=Closed dark blue asterisc.

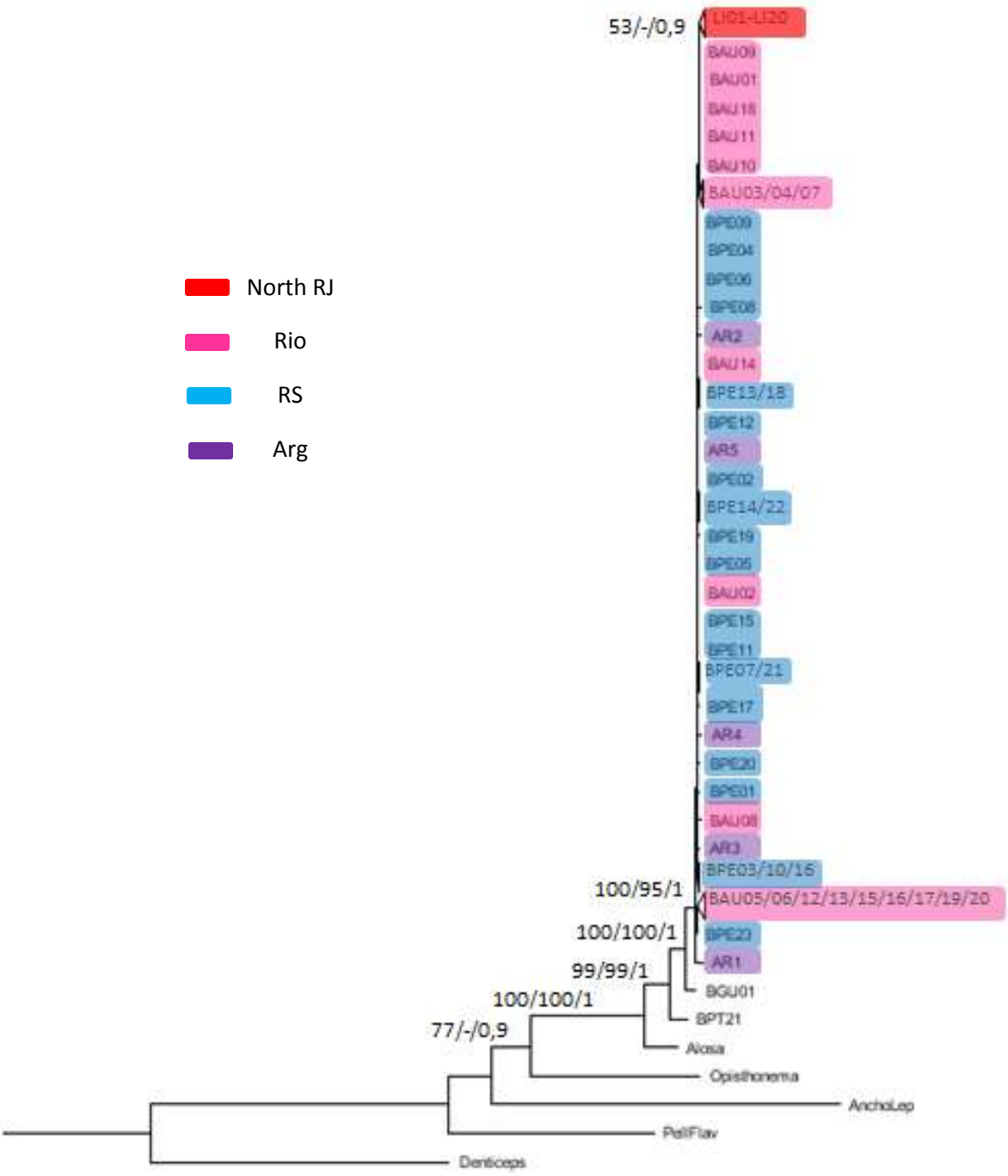


Figure 9: Altered Maximum Likelihood phylogenetic tree of the combined gene dataset (16s, COI, RAG2). Colors represent collections sites: Red=North Rio de Janeiro (North RJ); pink=Rio de Janeiro city and vicinities (Rio); blue=Rio Grande do Sul (RS); purple=Argentina (Arg). Numbers above branches are *bootstrap* support for ML, MP and Posterior Probabilities for BY, respectively ("-" indicates that the optimality criteria didn't recover the branch).

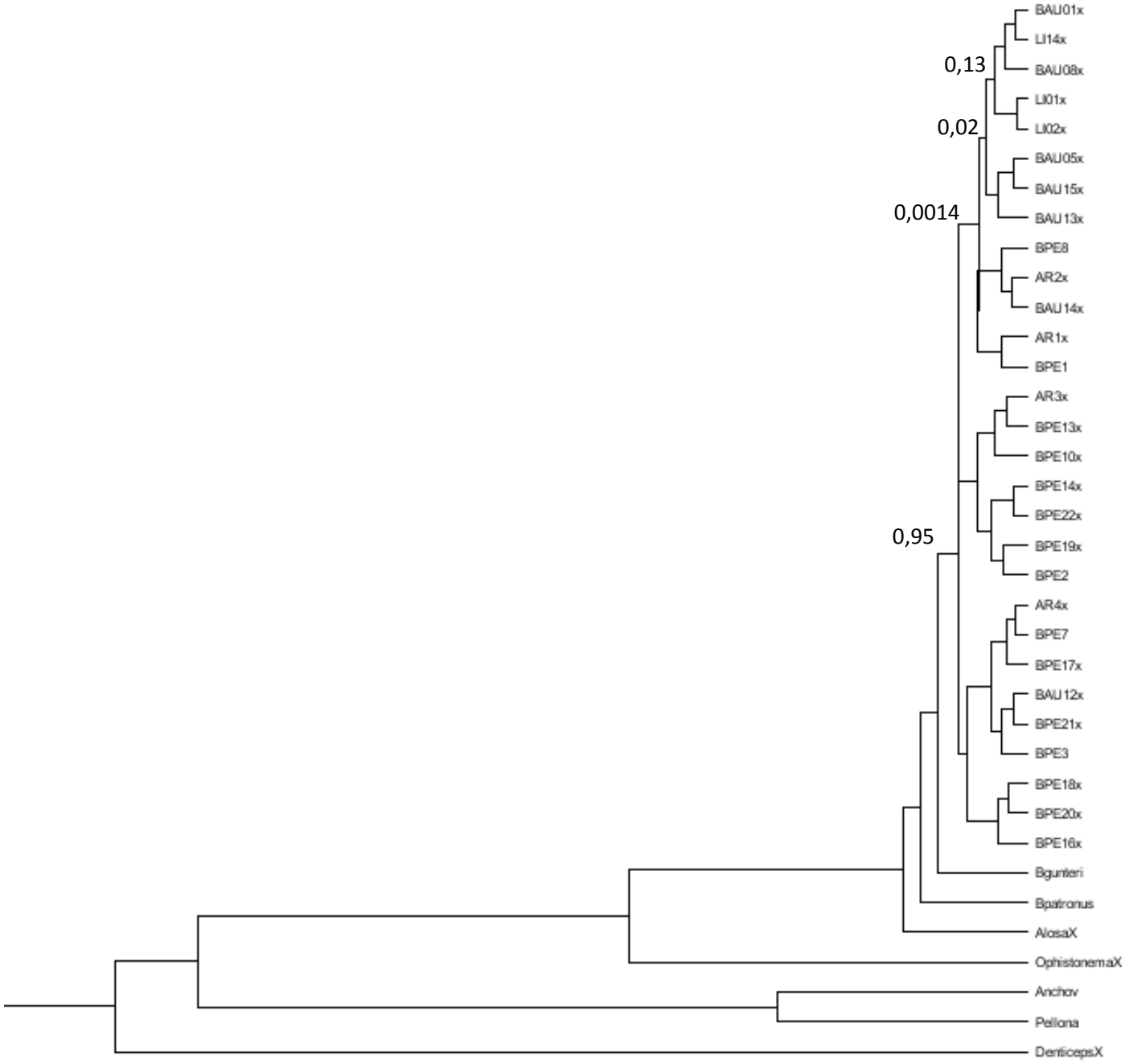


Figure 10: Multispecies Coalescent tree for the combined gene dataset (16s, COI, RAG2). Specimens were grouped by their respective haplotypes a priori, following the results obtained in the haplotype network. Numbers above branches are Posterior Probabilities.

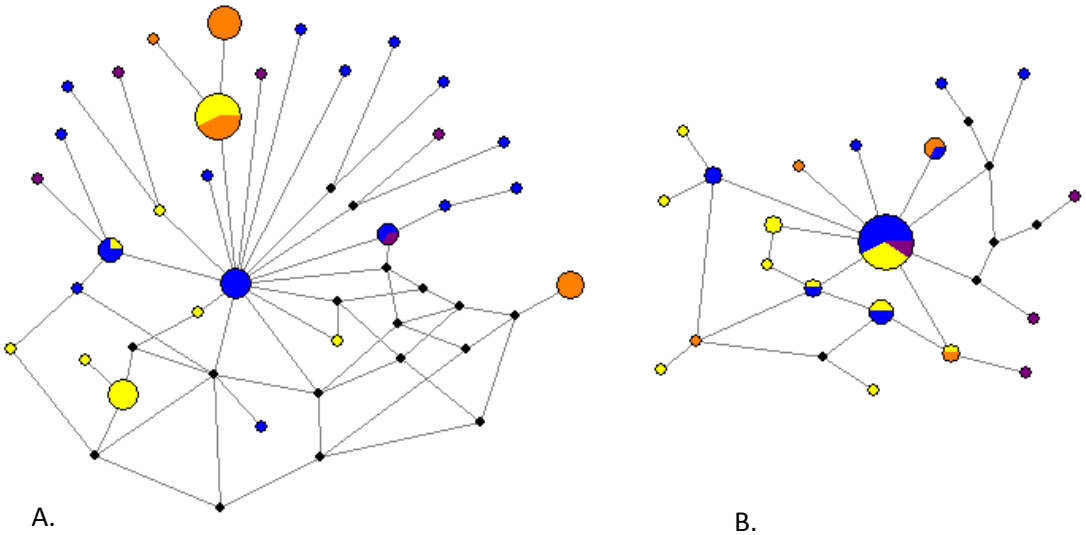
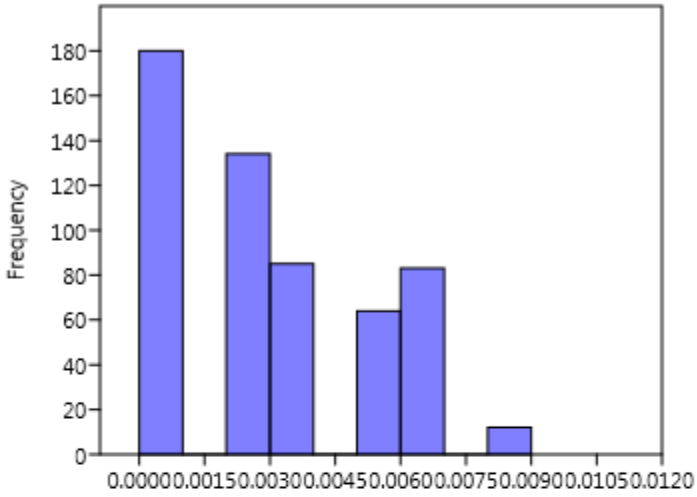


Figure 11: Haplotype networks (A. mitochondrial data; B. nuclear sequences). Size of circle represent haplotype frequency. Orange=North RJ; yellow=Rio; blue=RS; purple=Arg. Black circles represent missing haplotypes.

A.



B.

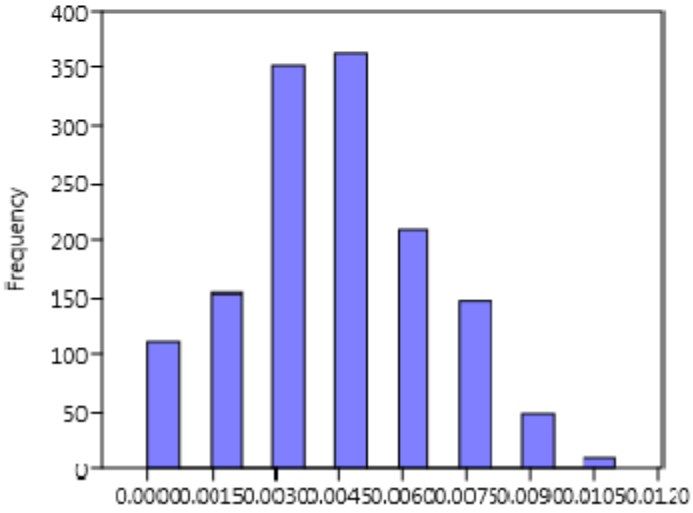


Figure 12: Genetic divergence comparisons: A, within geographic regions; B, between geographic regions.

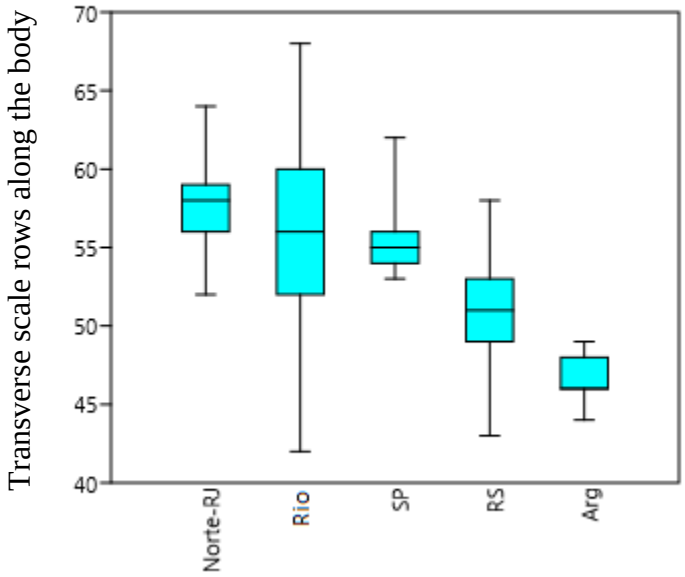


Figure 13: Number of transverse scale rows along the body of western South Atlantic specimens of *Brevoortia* when five collection sites are considered in a latitudinal gradient.

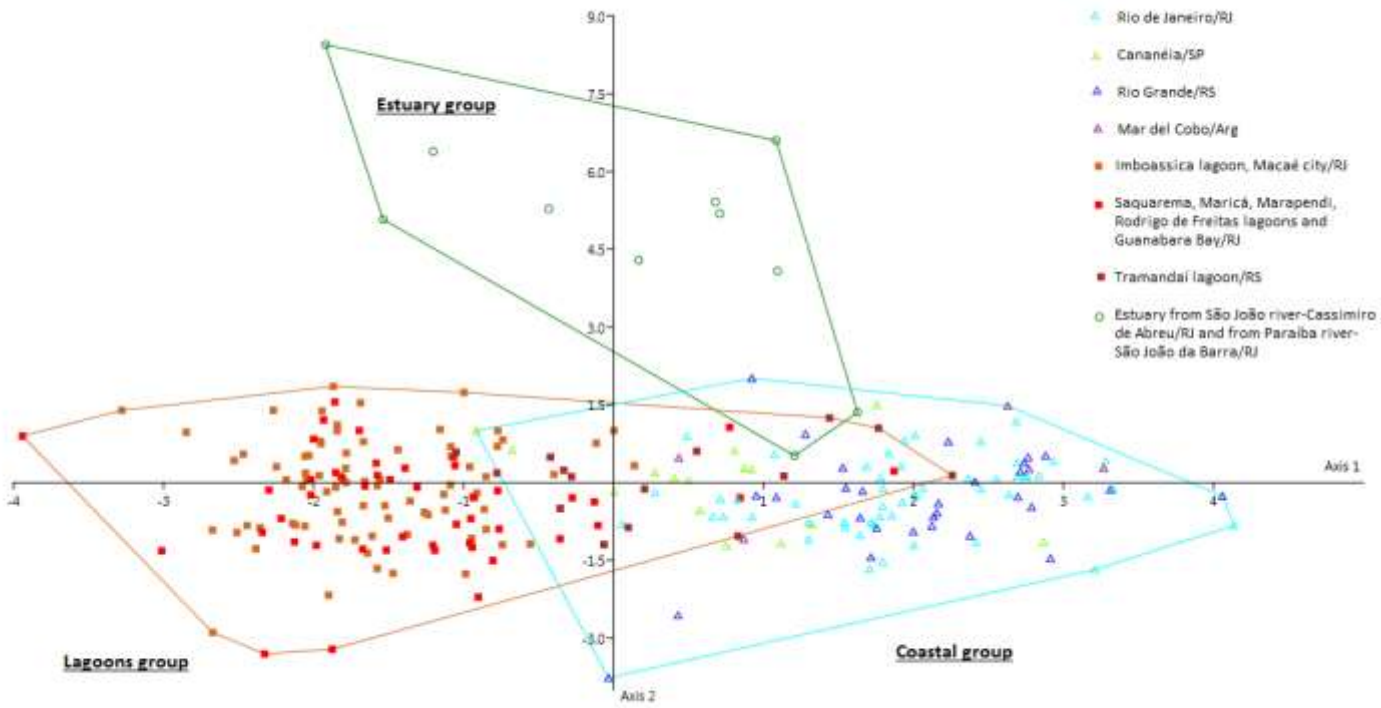


Figure 14: Scatter plot from the Multivariate Discriminant Analysis (morphometric) with data divided into three groups, represented by the polygons (coastal lagoons=red; estuary=green; marine coastal=light blue). Colors of geometric symbols represent collection sites of specimens: Light blue triangle=Rio de Janeiro/RJ; light green triangle=Cananéia/SP; dark blue triangle=Rio Grande/RS; purple triangle=Mar del Cobo/Argentina; light brown square=Imboassica lagoon, Macaé/RJ; orange square=Saquarema, Maricá, Marapendi, Rodrigo de Freitas lagoons and Guanabara bay/RJ; dark brown square=Tramandaí lagoon/RS; dark green circle=estuaries of the São João and Paraíba rivers, Northern RJ.

Conclusões gerais

O presente estudo abordou questões relacionadas à diversidade (genética e morfológica) do gênero *Brevoortia* no Atlântico Sul ocidental, sendo também o primeiro estudo a lidar com as relações filogenéticas de todas as espécies consideradas válidas, até então, para o gênero. Mesmo não sendo obrigatório no formato de tese adotado, consideramos pertinente que seja brevemente apresentado um breve sumário das conclusões gerais da tese, em língua portuguesa, complementar ao resumo geral apresentado no início desta tese.

Em termos de relações filogenéticas (Capítulo 1), foram encontrados três grandes clados que abrigam "duplas de espécies". Uma maior proximidade filogenética foi proposta entre o clado que contém *B. gunteri+B. smithi*, do Atlântico Norte, com o clado que inclui as espécies descritas para o Atlântico Sul ocidental (*B. aurea+B. pectinata*). Um terceiro clado, mais basal, inclui *B. tyrannus* e *B. patronus*, também do Atlântico Norte.

As análises também geraram informações para que fosse desenvolvida uma hipótese relacionada à origem, evolução e dispersão do gênero no Atlântico Norte. Nela, o ancestral comum de todas as espécies de *Brevoortia* teria se separado do ancestral compartilhado com *Alosa* (gênero mais próximo filogeneticamente de *Brevoortia* de acordo com as análises moleculares) por volta de 15 milhões de anos, no início do Mioceno. Nesse período, as temperaturas das águas ao redor do mundo estavam mais baixas, criando condições para que os espécimes do gênero colonizassem regiões mais ao sul, por exemplo, o Golfo do México e o Atlântico Sul ocidental.

O primeiro evento de cladogênese no gênero, que separou o grupo que contém *B. tyrannus* e *B. patronus* do clado formado pelas outras espécies do gênero, ocorreu por volta de 11 milhões de anos. O ancestral comum dos grupos remanescentes, dadas às condições mais frias das águas tropicais durante o Serravaliano (13,82-11,63ma), teria se dispersado ao Atlântico Sul ocidental. Presume-se que um aumento das temperaturas dos oceanos, durante o Mioceno médio, por volta de 7,5 milhões de anos atrás, gerou uma barreira que impediu ou limitou drasticamente o fluxo gênico entre as populações no norte e sul do Atlântico Ocidental, a partir da contração da população ancestral deste clado em direção às

latitudes mais extremas, por fim originando os grupos que contém *B. smithi+B.gunteri* em um ramo e *B. aurea+B. pectinata* em outro, por volta de seis milhões de anos atrás.

Os resultados das análises sistemáticas do gênero reforçaram questões recorrentes na literatura científica acerca do numero de espécies presente em *Brevoortia*, principalmente no Atlântico Sul ocidental, onde estudos anteriores indicavam a possibilidade da existência de apenas uma espécie, contra as duas que são atualmente consideradas como válidas (*B. aurea* e *B. pectinata*). Com o intuito de trazer novas informações que pudessem elucidar essa questão, foi feita uma amostragem em toda a distribuição conhecida do gênero no Atlântico Sul ocidental (Capítulo 2), sendo utilizados caracteres morfológicos (anatomia externa e contagem de vértebras) e moleculares (mitocondrial: 16S e COI; nuclear: RAG2) para acessar a diversidade do gênero na região.

Os resultados obtidos corroboram a hipótese de que apenas uma espécie de *Brevoortia* é encontrada no Atlântico Sul ocidental, e que a mesma deve ser reconhecida como *B. aurea* (Spix & Agassiz, 1829), o sinônimo sênior. Arvores filogenéticas e redes de haplótipos não recuperaram nenhum agrupamento espécie-específico, também sendo observado um baixo valor de divergência genética entre os locais amostrados, quando comparados com suas divergências internas, além da presença de um numero considerável de migrantes (um indicador de fluxo gênico) entre as localidades. Diferentes abordagens foram testadas para verificar a existência de grupos de espécimes de *Brevoortia* estruturalmente coesos no Atlântico Sul ocidental, mas todas as abordagens apontaram para apenas uma unidade taxonômica na região.

Um padrão de variação morfológica latitudinal foi proposto, principalmente em relação ao numero de fileiras de escamas transversais ao longo do corpo. Esse caráter foi tradicionalmente reconhecido como sendo diagnóstico entre as duas espécies (*B. aurea* e *B. pectinata*). É proposto, portanto, que a ausência de estudos de revisão abrangentes, compreendendo toda a distribuição geográfica reconhecida do gênero no Atlântico Sul ocidental, tenha em parte sido responsável pela falha de autores prévios em reconhecer a sinonímia das duas espécies, devido à ausência de formas intermediárias entre os dois extremos latitudinais de onde os exemplares tipos de ambas aparentemente provêm.

Também foi observada uma separação incipiente entre os espécimes dos grupos Norte RJ e Rio (com grupos remanescentes em posição intermediária aos dois), principalmente nas análises utilizando caracteres morfométricos. Essa separação, nas Análises Multivariadas Discriminantes, encontra-se no eixo oposto onde é observada a diferenciação entre os grupos de regiões geograficamente opostas (norte X sul), e é explicada provavelmente por condições ambientais distintas encontradas nos diferentes pontos amostrais. Resultados de outra Análise Multivariada, onde exemplares foram previamente agrupados a partir do ambiente onde foram coletados (lagoas costeiras; estuários; região marinha costeira), forneceram subsídios adicionais de que fatores ambientais são responsáveis por algum grau de diferenciação morfológica entre os exemplares de *Brevoortia* do Atlântico Sul ocidental.

Análises morfológicas também foram conduzidas com os síntipos conhecidos de *B. aurea* (1 exemplar) e *B. pectinata* (2 exemplares). Os resultados das Análises Multivariadas realizadas, tanto com os caracteres merísticos quanto com os morfométricos, indicam que os síntipos posicionam-se em uma zona de sobreposição indistinta aos demais espécimes de ambos os gêneros, provenientes de diferentes regiões amostradas. Após um levantamento histórico, é proposta a alteração do status de síntipo para o de lectotípo para o espécime que se encontra no Museu de História Natural de Neuchantel (MHNN 1159), o único remanescente no lote utilizado para a descrição de *Clupanodon aureus* Spix e Agassiz (1829), o nome original atribuído à espécie.