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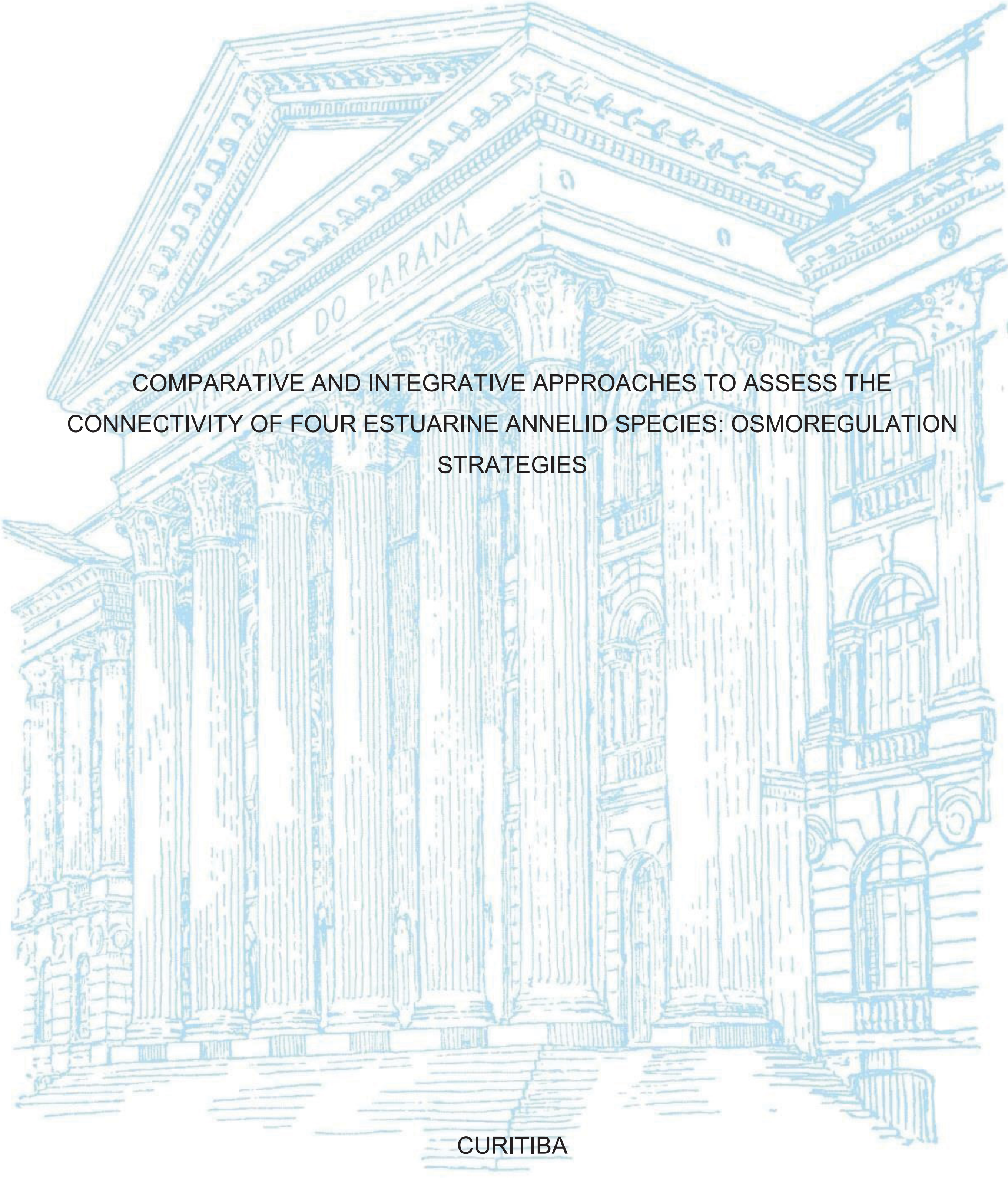

COMPARATIVE AND INTEGRATIVE APPROACHES TO ASSESS THE CONNECTIVITY OF FOUR ESTUARINE ANNELID SPECIES: OSMOREGULATION STRATEGIES

CURITIBA

2021

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Tese apresentada ao curso de Pós-Graduação em Zoologia, Setor de Ciencias Biologicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Zoologia.

Orientador: Prof. Dr. Paulo Da Cunha Lana

Co-orientador: Prof. Dr. Maikon Di Domenico

CURITIBA

2021

Universidade Federal do Paraná
Sistema de Bibliotecas
(Giana Mara Seniski Silva – CRB/9 1406)

Mucciolo, Serena
Comparative and integrative approaches to assess the connectivity of four estuarine annelid species : osmoregulation strategies. / Serena Mucciolo. – Curitiba, 2021.
173 p.: il.

Orientador: Paulo Da Cunha Lana.
Coorientador: Maikon Di Domenico

Tese (doutorado) - Universidade Federal do Paraná, Setor de Ciências Biológicas. Programa de Pós-Graduação em Zoologia.

1. Anelídeo. 2. Ecologia dos estuários. 3. Animais - População. 3. Osmorregulação. I. Título. II. Lana, Paulo da Cunha, 1956. III. Domenico, Maikon Di, 1980-. IV. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pós-Graduação em Zoologia.

CDD (22. ed.) 597.959

MINISTÉRIO DA EDUCAÇÃO
SETOR DE CIÊNCIAS BIOLÓGICAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO ZOOLOGIA -
40001016008P4

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ZOOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **SERENA MUCCIOLO** intitulada: **Comparative and integrative approaches to assess the connectivity of four estuarine annelid species: osmoregulation strategies**, sob orientação do Prof. Dr. PAULO DA CUNHA LANA, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 26 de Fevereiro de 2021.

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01/03/2021 14:45:52.0
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27/02/2021 16:01:39.0
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Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

ACKNOWLEDGEMENTS

This four-year journey has come to an end and I would love to thank everybody who contributed to making this experience unforgettable.

Firstly, I wish to thank my supervisor Prof. Paulo Lana and my co-supervisor Prof. Maikon Di Domenico, who both accepted me and my enthusiasm in their lab and life when I was still on the other side of the world. Thanks for all the conversations, the lessons and to teach me the importance of being persuasive. Thank you for all the great time together outside the lab, the dinners and the trips. Thank you for helping me grow, not only professionally.

I thank Prof. Carolina Freire and Prof. Viviane Prodocimo, for fully support my ideas and let me study the physiology of these fascinating animals. Thank you for all the time spent together and your kindness and friendship. I loved the time spent in your lab!

I would love to thank Prof. Giuseppe Calamita, who first introduced me in the beauty of the physiology when I was only a bachelor student, and then, fully supported my ideas and received me in his lab when I came back as a PhD student in love with annelids. Thank you for sharing with me your experience, your passion and your time.

I thank Prof. Carmela Gissi, for all the kindness and for receiving me in her lab. Thank you for all the time shared with me, for the conversations, the nights and the holidays spent together and to let me discover the “cool side” of the molecular biology.

Thanks to Prof. Francesco Mastrototaro, who contributed to the development of part of the thesis, helping me in the search of the polychaetes when I was in Bari.

To Prof. Maria Mastrodonato, for allowing me to study polychaetes from a different “point of view” and to sharing with me her knowledge on histology. Thank you for the friendship and for all the time spent together looking at those cute sliced worms.

Thank you, Paulo, Maikon, Carolina, Viviane, Giuseppe, Carmela, Francesco and Maria, you all made me realize the “thesis of my dreams”, celebrating with me the successes and supporting me when I needed during this journey. Thank you for your friendship!

I would love to thank Prof. Emanuel Maltempi, who readily accepted me in his amazing lab and who always helped me when I needed.

To Valter Baura, for being the best technician a laboratory can have! Thank you for sharing with me your experience, for all the help and the happy moments spent together.

To Maria Angelica Haddad, for letting me feel at home since the beginning and for always be there when I needed it. Thank you for hosting me and for all the conversations, the pizzas, the laughs and the nights together.

To Prof. Walter Boeger who inspired the initial idea of this project, and Prof. Rodolfo Angulo who helped me understanding the geomorphology and the paleogeographic evolution of these beautiful estuaries. My gratitude to both of you for your support.

I would love to thank Prof. Michal Grabowski and Prof. Tomasz Mamos from the University of Lodz to accepting me in their labs, allowing me to start a new exciting adventure!

Thanks to the members of my Comitê de Acompanhamento, Prof. Carolina Freire, Prof. Paulo Paiva, Prof. Maikon Di Domenico, Prof. Marcelo Kitahara, for accompanying me in these years and be always ready to give me advice when I needed.

Thanks to the members of my thesis assessment committee, Prof. Marcos Barbeitos, Prof. Paulo Paiva, Prof. Marta Souza, Prof. Paulo Pagliosa for the delighting discussion and comments about my thesis.

To all the drivers and the sailors of the CEM, for all their patience and who always helped me in the fieldworks. Thank you all for your friendship and to help me with the Portuguese since the beginning.

To Israel from the Centro de Tecnologias Avançadas em Fluorescência, for all his patience, his kindness and for being ready to help me when I needed.

To the amazing secretaries from the PGZOO, for the infinite patience and for always helping me with a smile on their face.

I thank my mother, my father, my sister and Flea for the unconditional love and to be always there for me. Thank you for celebrating with me each small success I reached. I haven’t enough words to express my gratitude! Thank you for “spreading the word” of how cool and fascinating polychaetes are!

I thank Andrea, for being a constant in my life and showing me what unconditional love is. Thanks to Gorgonzola, for his sweet, infinite and “healing” purring.

I thank Estela, the best friend with whom share life and work. Thank you for all the laughs, the support and for letting me discover the “real” Brazil and all its beauties.

Thanks to Ricardo, for all the “swims in the mud” looking for polychaetes, for always having my back and for sharing happy moments all over the world.

To Luana, for all the love and always having my back.

To Thaise and Marco, for their love and support since the beginning.

To Paulo’s family for all the smiles, the laughs and for letting me feel like part of the family since I landed in Brazil.

To Selene, who chose me as her co-supervisor. Thank you for let me share with you my passion for worms and all the help during the craziness of the experiments.

To Mariana, the first smiley person in the lab who talked with me when I arrived. Thank you for all the support and the great/crazy time we had in Costa Rica. Thanks to all the friends and colleagues of the benthos lab! Thank you, André Cattani, for being always ready to help, for all the coffee and the lovely time in the lab.

I would love to thank my life friend Rosa, for always being there for me since I can remember!

Thank you, Marika, for all the friendship and support during my stay in Bari. Thank you for sharing with me your experience and your time both in the lab and outside. I loved to meet you!

Thanks to Frenk, Frine and Martina, for their love and support. Thanks to Luca and Veronica to hosting me during my stay in Bari, allowing me to do part of the thesis and sharing with me their time. Thank you for everything!

I thank the Brazilian polychaetologists, for let me feel part of this amazing scientific family since the beginning, and all the great people I met during my congresses. You all made these moments unforgettable!

Last but not least, I would love to thank CAPES and Brazil to fund my project and allow me to live this amazing experience.

Thank you all for being part of this journey!

RESUMO

As distribuições populacionais atuais e a diversidade genética dos organismos estuarinos são o resultado de uma combinação de fatores históricos e contemporâneos, que influenciam a dispersão e o fluxo gênico entre populações. Devido às rápidas mudanças climáticas que influenciaram direta e indiretamente o nível do mar e a linha de costa em todo o globo, o Pleistoceno afetou profundamente a distribuição e a diversidade genética de vários táxons. Os estuários são ambientes altamente dinâmicos, caracterizados por grandes flutuações de salinidade. Os organismos estuarinos desenvolveram adaptações fisiológicas e ecológicas para lidar com tais variações estuarinas diárias ou sazonais. Este trabalho visa investigar como fatores contemporâneos e históricos modelaram e modelam os atuais padrões de distribuição e a composição gênica de quatro espécies-alvo de anelídeos em três estuários subtropicais do sul do Brasil, explorando vários níveis de organização biológica por meio de uma abordagem bastante integrativa. As espécies-alvo foram escolhidas em função da sua abundância e maior ou menor tolerância às variações de salinidade ao longo de baixios entre-marés dos estuários. Testamos duas espécies com nichos de salinidade mais amplos, os nereidídeos *a priori* chamados de *Alitta succinea* e *Laeonereis culveri*, e espécies com nichos de salinidade mais restritas, o neftídeo *Nephtys fluviatilis* e o melinídeo *Isolda pulchella*. A tese foi dividida em quatro capítulos, que avaliaram a importância de forçantes históricas e contemporâneas na distribuição e composição genica das quatro espécies, em diversos níveis de organização biológica:

- O primeiro capítulo investigou a tolerância à variação da salinidade e a espessura do tegumento das quatro espécies alvo. Ambos os nereidídeos apresentaram estabilidade de peso corporal e tegumento espesso, compatível com sua eurihalinidade. O neftideo foi nitidamente menos eurihalino, mas tolerou baixas salinidades e água doce, enquanto o melinídeo apresentou um comportamento estenohalino marinho e não tolerou diluição da água do mar. Ambas as espécies apresentaram um tegumento fino.
- O segundo capítulo revelou a expressão e localização do cotransportador NKCC por imunofluorescência nos corpos das quatro espécies de anelídeo. Sua expressão não foi homogênea, refletindo claramente o estilo de vida dos anelídeos. Os anelídeos de vida livre (ambos os nereidídeos eurihalinos e o neftídeo estenohalino), exibiram um sinal para NKCC espalhado e detectado ao longo de seus corpos, em contraste com o melinídeo sedentário, no qual o sinal foi restrito às regiões anteriores do corpo em contato direto com a coluna d’água.
- O terceiro capítulo investigou a presença das aquaporinas em anelídeos por meio de uma abordagem in sílico, através de uma busca nos bancos de dados públicos, e a confirmou experimentalmente em *A. succinea*. A filogenia de MIPs dos anelídeos foi reconstruída, potenciais ortólogos das aquaporinas dos vertebrados AQP1-like, AQP8 e as AQP11-like foram recuperados, e a presença de três parálogos de *A. succinea* foram confirmados por RT-PCR.
- O quarto capítulo investigou os padrões de diferenciação genética de populações das quatro espécies-alvo em três estuários subtropicais do sul do Brasil. Alto fluxo gênico foi recuperado para os nereidídeos entre as baías, resultado congruente com os dados do primeiro capítulo e esperado em função de sua eurihalinidade. Marcada segregação foi recuperada para as populações de neftídeo da Baía da Babitonga, como reflexo de seu baixo grau de eurihalinidade e de sua

distribuição limitada aos setores oligohalinos mais confinados. Panmixia entre os estuários foi recuperada para o melinídeo, refletindo seu comportamento “marinho” e sua ocorrência restrita aos setores euhalinos. As análises moleculares indicaram a existência de uma nova espécie de *Alitta*, ainda por ser formalmente descrita. Validaram ainda trabalhos recentes que haviam mostrado que populações antes referidas ao nome específico *Laeonereis culveri*, pertencem de fato a *Laeonereis acuta* e *Laeonereis pandoensis*, ocorrendo em simpatria ou alopatria.

Palavras-chave: Anelídeos. Osmorregulação. NKCC. Aquaporinas. Conectividade entre populações.

ABSTRACT

Present-day distribution and genetic diversity of estuarine animals are the result of a combination of historical and contemporary factors that have influenced both their dispersal and gene flow. For instance, Pleistocene glaciations influenced sea-level and the coastal lines worldwide, strongly affecting the distribution patterns of coastal and estuarine species belonging to different taxa. Estuaries are highly dynamic environments, characterised by regular or irregular salinity fluctuations, and estuarine organisms share a variety of physiological and ecological adaptations to face such daily or seasonal variations. This work aimed to investigate how contemporary and historical factors have modelled and are still modelling the current distribution of four estuarine annelid species from different sectors of three subtropical estuaries of southern Brazil, by exploring different biological organization levels through an integrated approach. The target species were chosen considering their distribution and the variability in the salinity regimes of their ranges of occurrence. We tested two species with broader salinity niches, the nereidids treated *a priori* as *Alitta succinea* and *Laeonereis culveri*, and two species with narrower salinity niches, or more restricted spatial ranges in the bay, the nephtyid *Nephtys fluviatilis* and the melinnid *Isolda pulchella*. The thesis was articulated into four chapters, assessing contemporary or historical drivers which potentially led to genetic structure, or lack of it, at diverse organization levels:

- Chapter one investigated the tolerance to salinity variation and the integument thickness of the four annelid species. Both nereidids displayed body weight stability and a thick integument, compatible with their high euryhalinity. The nephtyid was distinctly less euryhaline but tolerated low salinities and fresh water, while the melinnid presented a marine stenohaline behaviour and did not tolerate seawater dilution. Both species have a thin integument.
- Chapter two revealed the expression and localization of the NKCC cotransporter through immunofluorescence in the bodies of the four annelids. NKCC expression was not homogeneous, and clearly reflected the lifestyle of each species. The free-living/burrowers (both euryhaline nereidids and the stenohaline nephtyid) displayed a widespread signal for NKCC along their bodies, in contrast to the stenohaline sedentary melinnid, in which the signal was restricted to the body regions exposed to the surrounding environment.
- Chapter three investigated the presence of aquaporins in annelids through an *in silico* approach against online databases and experimentally confirmed for the first time their expression in *A. succinea*. The phylogeny of annelid MIPs was reconstructed, putative orthologs of the vertebrate AQP1-like, AQP8 and the AQP11-like subfamilies were recovered, and three paralogs of A. succinea were confirmed by RT-PCR.
- Chapter four investigated patterns of genetic differentiation in populations of the four selected annelid species along three subtropical estuaries from southern Brazil. High genetic flow among the bays was reported for the nereidids, congruent with their high euryhalinity. Segregation was recovered for the nephtyid populations from Babitonga Bay, reflecting their low degree of euryhalinity and their limited distribution to the more confined, oligohaline sectors. Panmixia among the estuaries was recovered for the melinnid, reflecting its “marine” behaviour and its restricted occurrence in the euhaline sectors. As a result of our molecular analyses, we found a putative new species related to the complex *A. succinea*, and validated the recent

findings from other authors that the species name *L. culveri*, as yet used for local populations of *Laeonereis*, corresponds in fact to two distinct species, *Laeonereis acuta* and *Laeonereis pandoensis*.

Key-words: Annelids. Osmoregulation. NKCC. Aquaporins. Populations connectivity.

SUMÁRIO

GENERAL INTRODUCTION

Estuaries are semi-enclosed systems directly connected to the sea and characterized by marked fluctuations in hydrographic conditions due to regular or stochastic events such as pluviosity, river, tides, waves and storms (Potter et al. 2015). One of their main characteristics is the salinity gradient in response to the mixture of seawater and freshwater. The regular or abrupt salinity changes, caused by tidal fluctuations and spatial or temporal variation in freshwater input, act as drivers and regulate the abundance and distribution of animals and plants along the estuarine gradient. Small- or large-scale estuarine dynamics may affect faunal dispersion and connectivity in different ways, e.g. by creating seasonal barriers, or conversely by promoting the linkage of far apart estuarine habitats (Oglesby 1965b; Whitfield et al. 2012; Chang and Marshall 2016).

The diversity of genuinely estuarine species is often lower than that of marine or freshwater species (Whitfield et al. 2012). Present-day population distributions and their current genetic diversity result from a combination of historical and contemporary factors that may influence both species dispersal and their gene flow (Bilton et al. 2002). For instance, due to fast climate changes that directly and indirectly influenced sea-level and related parameters worldwide, the Pleistocene strongly affected the distribution, and the current genetic diversity, of species belonging to different taxa (Hewitt 2000; Baker and Fritz 2015). There is a general consensus that the estuarine and coastal marine faunas were the most impacted by such changes, since regressive coastlines limited estuarine connectivity and caused the drying up of isolated, temporary lagoons (Hewitt, 2000, 2004). These events potentially led to both the promotion of bottleneck events and population differentiation (Olson et al. 2009; Tschá et al. 2016).

As relevant as historical drivers, contemporary processes also affect the distribution and potential segregation of estuarine animals. To cope with abrupt salinity variations, estuarine animals display a number of osmoregulatory adaptations, from physical avoidance to the regulation of cell volume and ionic concentrations of intra-extracellular fluids (Oglesby 1968; Tait et al. 1981; Preston 2009). In response to osmotic stress, animals may act either as osmoregulators or as osmoconformers.

Osmoregulators are able to maintain the osmotic concentration of the body fluids stable, independently from the external medium, by performing both an isosmotic intracellular osmoregulation (IIR) and an anisosmotic extracellular osmoregulation (AER). Osmoconformers adjust their osmotic concentration to the environment through the IIR. Mechanisms involved include the adjustment of the concentration of osmotic active solutes and the change of the membrane-bound transporters (Evans 2009). One significant difference between tight osmoregulators and osmoconformers is the investment of metabolic energy in transport mechanisms, present in interface epithelia of the former, but absent (or much less abundant) in the latter. Euryhaline conformers, which tolerate a broad range of salinities, supposedly spend more energy in IIR (Kirschner 1991), while stenohaline osmoconformers – which tolerate a narrow range of salinities - are thus restricted to more stable water salinities (Lignot and Charmantier 2015; Rivera-Ingraham and Lignot 2017; Thonig et al. 2019).

Most estuarine invertebrates are osmoconformers (Evans 2009), and, adaptations to osmotic stress have been recognized at different levels. Considering the tissues, a less permeable cuticle is common in these organisms (Oglesby 1965a; Preston 2009). At the cellular level, different membrane proteins work together in order to regulate the cell volume. For instance, membrane proteins such as the Na^+-K^+-$2Cl^-$ (NKCC) cotransporter and the aquaporins, are entitled to transport ions and water through the plasma membranes. The NKCC cotransports sodium, potassium and chloride ions across the cell membrane, normally following the electrochemical gradient for sodium, regulated by intracellular chloride concentration, and cell volume (Russell 2000). As a transporter that relies on electrochemical gradients, it may operate reversibly regulating the cell volume increase (i.e. regulatory volume increase, RVI) or decrease (i.e. regulatory volume decrease, RVD), depending on the anisosmotic or isosmotic conditions, respectively (Russell 2000). Aquaporins facilitate the movement of the water through the lipid bilayer membranes (Madsen et al. 2015). These water channels belong to the membrane intrinsic proteins group (MIPs) and, besides the water transport, are involved in the transport of small solutes, such as mainly glycerol but also ammonia, urea, metalloids and carbon dioxide (Laloux et al. 2018). For these reasons, both membrane proteins (i.e. NKCC and MIPs) play pivotal roles in response to either hyper- or hypoosmotic stress (e.g. Russell 2000; Castellano et al. 2016; Igual Gil et al. 2017).

Annelids seem to be particularly good models to test hypotheses related to the relative importance of historical and contemporary drivers in modulating the current genetic structure of estuarine fauna. They numerically dominate estuarine environments, being represented by infaunal or epibenthic and sessile or errant species (Venturini et al. 2011; Dafforn et al. 2013). Their usual high population densities give them a leading functional role in most benthic communities influencing and creating complex mosaics of micro- and macro- environments relevant for the control of ecosystem functioning (Pischedda et al. 2008; Wrede et al. 2018). Moreover, both their abundance and their suitability to laboratory experiments, allowed them to be often used as representative organisms to assess the ecological status of benthic communities, to carry out ecotoxicological studies and reveal insights into the developmental and evolution biology (e.g. Dean 2008; Seaver 2016; Barros et al. 2018). Despite their abundance and their broad use for scientific purposes, the physiological adaptations to salinity fluctuations in some annelid families are completely disregarded, or else results are contradictory, underlining the need for further investigations.

The present work aimed to investigate how the distribution of contemporary species along three subtropical estuaries, and their genetic characteristics, are shaped by contemporary and historical drivers, such as tolerance to salinity changes, larval dispersal ability and Pleistocene glaciations. Four annelid species were chosen as target species, taking into account both their occurrence along the estuary and their expected salinity tolerance. Species were a) *Alitta succinea* (Leuckart, 1847) - usually related to hard and human-made substrates - and *Laeonereis culveri* (Webster, 1879) - that occurs in soft bottoms - both nereidids with broader salinity niches, abundant in meso/polyhaline sectors (5 – 30 psu) of the estuaries; b) the nephtyid *Nephtys fluviatilis* Monro, 1937, from the oligohaline sectors (0 – 5 psu) of the estuary, and the melinnid *Isolda pulchella* Müller, 1858, usually related to euhaline sectors (30 – 40 psu) of the bays, both with narrower salinity niches.

The thesis was articulated in four chapters, divided in two sections, which aims were:

1. Analyses of contemporary drivers of spatial segregation:

- I chapter: Experimentally investigate the tolerance to salinity variation of the four annelid species, adding data to the current knowledge on the osmotic behaviour of the nereidids, and assessing for the first time the osmotic responses of the nephtyid and the melinnid. The complexity and thickness of the cuticle were also analysed to investigate a possible morphological link to the physiological response.
- II chapter: Reveal the expression and localization of the NKCC cotransporter through immunofluorescence in the bodies of the four annelid species.
- III chapter: Investigate the presence of aquaporins in annelids through an *in silico* approach against online databases and experimentally confirm it in *A. succinea*.

2. Analysis of historical and contemporary drivers of spatial segregation and genetic structure:

- IV chapter: Investigate patterns of genetic differentiation in populations of the four selected annelid species along three subtropical estuaries from southern Brazil.

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CHAPTER I

VARIABILITY IN THE DEGREE OF EURYHALINITY IN NEOTROPICAL ESTUARINE ANNELIDS

Manuscript formatted following the guidelines of the journal: Journal of Experimental Marine Biology and Ecology
ISSN: 0022-0981
Impact Factor: 2.247
Qualis: A2

Variability in the degree of euryhalinity in neotropical estuarine annelids

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Abstract

Estuaries are highly dynamic systems characterized by large daily fluctuations in salinity and high spatial complexity. Accordingly, estuarine animals, especially small animals with limited locomotory capacities, are expected to display variability in their ecological and morphophysiological strategies to address such challenges. The physiological response to salinity challenges was evaluated in four species of neotropical annelids along a subtropical estuarine gradient. Mortality and changes in body weight, treated as proxies for water fluxes, were assessed. Species were chosen considering their distribution and the variability in the salinity regimes of their ranges of occurrence. We tested two species with broader salinity niches, the nereidids *Alitta succinea* and *Laeonereis culveri*, and species with narrower salinity niches, or more restricted spatial ranges in the bay, the nephtyid *Nephtys fluviatilis* and the melinnid *Isolda pulchella.* Worms were exposed to salinities of 0, 5, 15, 25, and 35 psu for 1, 6, 12, and 24 h. Their degree of physiological euryhalinity was assessed by weighing animals before and after exposure to these salinities and assessing their mortality rates (absence of body movements or vascular circulation). Histological characterization of the integument of each species was carried out to investigate a possible morphological link to the physiological response. Both nereidids displayed body weight stability and a thick integument, compatible with their high euryhalinity. The weight in *A. succinea* returned to the initial levels after the first shock for salinities ≥ 5 psu. Body weight did not change in *L. culveri* at salinities ≥ 15 psu. Mortalities were reported only at 0 psu after 12 and 24 h. *Nephtys fluviatilis* showed less variation in weight throughout the whole range, despite showing mortality after 24 h at 0 psu, at its reference salinity (3 psu) and at 35 psu. Thin integument was recovered for this species. Finally, the low capacity for body weight control of *I. pulchella* and its thin integument were compatible with its stenohalinity and occupation of polyhaline marine areas. Weight variation was significant at each salinity, except 35 psu, and mortality occurred at 0 and 35 psu. Our study revealed physiological responses to salinity variation (within 24 h) entirely compatible with the ranges of distribution of the target species. Autecological traits, morphophysiological adaptations and evolutionary history of the species probably all play complementary roles in the distribution of the four annelid species along the estuarine gradient.

Key-words: Annelids. Estuary. Osmoregulation. Laboratory experiment. Histology. Integument

1. Introduction

Estuaries are critical transition zones linking land, freshwater and sea, characterized by marked fluctuations in hydrographic conditions due to regular or stochastic events such as seasonality, tides, and storms (Levin et al., 2001). The regular and abrupt salinity changes (i.e. tidal fluctuations and large precipitation events), act as drivers and limit the abundance and distribution of taxa, affecting their dispersion and connectivity in different ways (Chang and Marshall, 2016; Oglesby, 1965b). To cope with abrupt salinity variation, annelids inhabiting these environments show a variety of osmoregulatory adaptations, such as physical avoidance and variable degrees of regulation of their extracellular fluids and cell volume (Oglesby, 1968; Preston 2009; Tait et al., 1981). For instance, osmoregulatory-driven behaviour is known for vagile, soft-bottom annelids, which may perform diel vertical migrations in the substrate, burrowing deeper during low tides, thus exploring microhabitats and microclimates (Oglesby, 1969; Preston, 2009). Alternatively, sessile annelids such as *Galeolaria caespitosa* Lamarck, 1818 (Serpulidae) may close their own tube with the operculum to isolate themselves from the surrounding water (Tait et al., 1981). In addition to the development of autecological strategies, the epithelium is most likely involved in the osmoregulation of annelids (e.g. Oglesby 1965a, 1965b).

Annelids, as most marine invertebrates, are generally considered osmoconformers, even if some taxa may display an osmotic gradient between their coelomic fluid and the external water at low salinities, in a hyperregulatory pattern (Preston, 2009; Thonig et al., 2019). In general, the tighter the capacity to perform salt transport through interface epithelia, also called anisosmotic extracellular osmoregulation (AER), the less the aquatic animal needs to perform complementary cellular regulation, called isosmotic intracellular regulation (IIR), which means restoring cell volume after extracellular concentration changes (Florkin, 1962). The less tight the osmoregulatory capacity (AER) is, the more conformer the animal is. In order to tolerate salinity fluctuations, that is, to be euryhaline, conformers must be competent in IIR, in addition to avoidance strategies (Evans, 2009; Foster et al., 2010; Freire et al., 2008; Willmer et al., 2005). One significant difference between tight osmoregulators and osmoconformers is the investment of metabolic energy in transport mechanisms, present in interface epithelia of the former but absent (or much less abundant) in the latter. Euryhaline conformers putatively spend more energy in IIR (Kirschner, 1991). Stenohaline osmoconformer animals are thus restricted to stable water salinities (Lignot and Charmantier, 2015; Rivera-Ingraham and Lignot, 2017; Thonig et al., 2019).

Depending on their capability to tolerate salinity variation, annelids may gain or lose water passively through a more or less permeable integument. Oglesby (1965b) carried out salinity experiments with the nereidid *Hediste limnicola* (Johnson, 1903), originally referred as *Nereis limnicola,* usually confined to oligohaline environments, and highlighted the presence of a less permeable body surface and the capability of producing hypoosmotic urine to eliminate excess water.

Osmotic stresses may affect annelids in many ways and at different life stages; in fact, larvae exposed to hypoosmotic stress are unable to beat the cilia to swim, and adults are unable to move, both because of their swollen conditions (Lyster, 1965; Oglesby, 1968, 1969), thus displaying stenohalinity and lack of IIR capacity in regulatory volume decrease. Reduced growth rates and impaired reproduction may also occur, potentially related both to swollen conditions and to energy allocation to IIR (Neuhoff, 1979; Thonig et al., 2019). Indeed, Kuhl and Oglesby (1979) reported dramatic responses to lower salinities of the nereidid *Alitta succinea* (Leuckart, 1847), referred in the original paper as *Nereis succinea*, expressed by lower fertilization success, developmental delays and a general asynchrony between male and female sexual maturity.

Responses to salinity variation may differ among the annelid families because of the level of environmental adaptation (Oglesby 1969; Whitfield et al. 2012). Oglesby (1965b) and Freel et al. (1973) retrieved varying results even working with different populations of the same polychaete species (i.e. *A. succinea* as *Nereis succinea* and *Neanthes succinea*, respectively). *Alitta succinea* showed hyperregulation when exposed to low salinities in the former study, but it behaved as an osmoconformer in the latter. Notwithstanding the diversity of the more than 10,000 accepted species of the “polychaetes” (Read and Fauchald, 2020), and annelids in general, either the literature is still scarce, with many families completely disregarded, or contradictory results underline the need for further investigations.

We experimentally investigated the tolerance to salinity variation of four annelids in a subtropical Brazilian estuary, adding data to the current knowledge on the osmotic behaviour of the nereidids *A. succinea* and *Laeonereis culveri* (Webster, 1879), and assessing for the first time the osmotic responses of the nephtyid *Nephtys fluviatilis* Monro, 1937 and the melinnid *Isolda pulchella* Müller, 1858. According to their estuarine distribution patterns - and presumed realized salinity niches - we expected varying responses to osmotic stress - or physiological degree of euryhalinity - for each species. The two nereidids, displaying a broader distribution, were expected to better tolerate salinity variations, while the nephtyid and the melinnid would hypothetically display a lower degree of euryhalinity, compatible with their more limited range of occurrence, or narrower salinity niches, respectively, in the oligohaline and euhaline sectors of the bay. Responses to osmotic stress were estimated by considering both the variation in weight and mortality rates at varying salinities. In addition, we hypothesized that the complexity and thickness of the cuticle could be correlated with their salinity tolerance.

2. Materials and methods

2.1 Sampling and laboratory acclimation

Sampling was carried out in the Paranaguá Estuarine Complex (PEC), Brazil (Table 1). This estuarine complex is considered a mixed estuary, covering an area of ~612 km². PEC displays a seasonal pattern of variation in salinity and temperature and a gradient of increasing salinity from the inner to the outer sectors (Marone et al., 2005). Target species were chosen taking into account both their occurrence along the estuary and their expected salinity tolerance. Species were a) *Alitta succinea* - usually related to hard and human-made substrates - and *Laeonereis culveri* - that occurs in soft bottoms - both nereidids with broader salinity niches, abundant in meso/polyhaline sectors (5 – 30 psu) of the estuaries; b) the nephtyid *Nephtys fluviatilis*, from the oligohaline sectors (0 – 5 psu) of the estuary, and the melinnid *Isolda pulchella*, usually related to euhaline sectors (30 – 40 psu) of the bays, both with narrower salinity niches. Animals were sampled using a shovel or scraping the fishermen's buoys, depending on the species (Table 1).

In the laboratory, the animals were acclimated for 48 h in plastic containers of 1 l of water and 3-4 cm of sediment, both collected from each respective sampling site. Worms were kept under stable temperature (~20°C), constant aeration, natural photoperiod and were fed with common aquarium fish flakes. To prevent cannibalism, each container held a maximum of 50 specimens, and larger animals were separated from smaller animals.

2.2 Experimental design

The response of each species to osmotic shocks (i.e. physiological degree of euryhalinity) was assessed by their variation in weight and mortality. The experimental design was orthogonal and included two factors: salinity, fixed with five levels plus the reference salinity at each sampling area – used as control treatment, in a total of six levels, and time, fixed with four levels of observation times. The salinities chosen were 0, 5, 15, 25, and 35 psu. Measurements of weight and assessment of mortality were carried out 1, 6, 12, and 24 h after the onset of osmotic shock. To avoid potential bias related to the lower pH of the distilled water, different salinities were achieved by diluting the pre frozen and filtered seawater (35 psu) with local commercial bottled mineral water. Salinities and observation times were chosen to simulate the daily and seasonal salinity fluctuations in the estuary.

Five adults of each species were removed from the sediment and/or tubes and were used for each of the 24 treatments (6 salinities x 4 times), yielding a total of 120 individuals per species. Specimens were not sexed for the experiments. Moreover, regenerating individuals were used, but only if sufficiently advanced in caudal development (i.e. damage occurred prior to sampling), while specimens in reproductive stages were discarded. Individuals were weighed by removing them from their medium and quickly blotting them dry on filter paper, both before exposure to osmotic stress and at the end of each fixed time. Specimens were considered dead in the absence of body movements or vascular circulation, observed under a binocular stereoscope.

2.3 Data processing

To reduce intraspecific variation in size/weight for each species, relative weight variation calculated as ($\frac{w_{tx} - w_{t0}}{w_{t0}}$ x 100) was used for the statistical analyses, with (w_{t0}) being the initial weight at the start of the experiments and (w_{tx}) the weight at fixed times (x).

The Shapiro-Wilk (Shapiro and Wilk, 1965) and Levene tests (Levene, 1960) were carried out to test the normality and homogeneity of variances, respectively. After testing for homoscedasticity, when present, outliers were removed. Generalized linear models (GLMs) (Nelder and Wedderburn, 1972) were performed to assess the best model (i.e. which variable) for the variation of the weights and the mortality (with Gaussian distribution for the first and binomial for the latter). The corrected Akaike Information Criterion (AICc) (Hurvich and Tsai 1989; Sugiura, 1978) was used for model selection, and finally, the model was visually validated (Zuur and Ieno, 2016). In addition, one-way ANOVA (Fisher, 1925) was performed for each level of the best model found (each salinity, time or the interaction between them) to test whether the variation in raw weights from t_0 was significant. Tukey's HSD (honestly significant difference; Tukey, 1953) was performed as *post hoc* analysis, and the results were considered significant when $p \leq 0.05$. All analyses and graphics were carried out using R (R Core Team, 2013), RStudio (RStudio Team, 2015) and the packages *GAD* v. 1.1.1 (Sandrini-Neto and Camargo, 2011), *ggplot2* (Wickham, 2016), *car* v. 2.1-6 (Fox and Weisberg, 2017), and *MuMIn* v. 1.42.1 (Barton, 2018).

2.4 Histological analysis of the epithelium

To evaluate the structure and thickness of the cuticle and the epidermis of each annelid, 7 additional individuals of each species were fixed in 5% paraformaldehyde diluted in filtered water from each sampling site for 24 h after 48 h of acclimation. Animals were then washed and stored in 1% phosphate-buffered saline

(PBS) at 4°C. The protocol described in Mentino et al. (2014) was then followed. Specimens were embedded in Technovit 8100 monomer (EMS, Hatfield, PA), sectioned in semithin slices 2 μm thick, and stained with metachromatic toluidine blue to assess the general morphology of tissues. Colours range from blue/violet (basophilic cellular structures such as nuclei) to red/purple (acidophilic cellular structures such as cytoplasm and mucins), depending on the charge of the stained tissues (Sridharan and Shankar, 2012). The thickness of the cuticle was standardised with the average width of the species (i.e. central portion), measuring specimens previously anesthetized with menthol.

Table 1. Geographical coordinates of sampling points with local salinity at the time of sampling and type of substrate.

Species	Coordinates (DMS)	Salinity (psu)	Substrate
Alitta succinea	S25°26'7.5" W48°42'18.18"	20	Plastic fishermens' hard bottom, buoy
Laeonereis culveri	S25°30'35.52" W48°28'4.32"	28	Sand
Nephtys fluviatilis	S25°14'46.5" W48°24'57.72"	1	Mud
Isolda pulchella	S25°33'25.2" W48°18'37.4"	35	Sand

3. Results

3.1 Tolerance to osmotic stress

3.1.1 Species with broader salinity niches

The best model selected for *Alitta succinea* and *Laeonereis culveri* included the interaction between salinity and time (Table S1).

Alitta succinea gained weight at very low salinities (i.e. 0 and 5 psu) and lost it at salinities higher than the reference salinity of 21 psu 1 h after the shock. Significant differences from the initial weight were recovered at 0, 5, 25 and 35 psu (Fig. 1 – S1.1) after 1 h of exposure. The weight, after a first change, returned to initial levels (i.e. no significant difference from t_0 at all salinities, except for 0 psu, in which it increased further over time (Fig. S1.1). The GLM performed on mortality rates selected both time and salinity (without their interaction). Mortality occurred only after 12 h at 0 psu (Fig. S2).

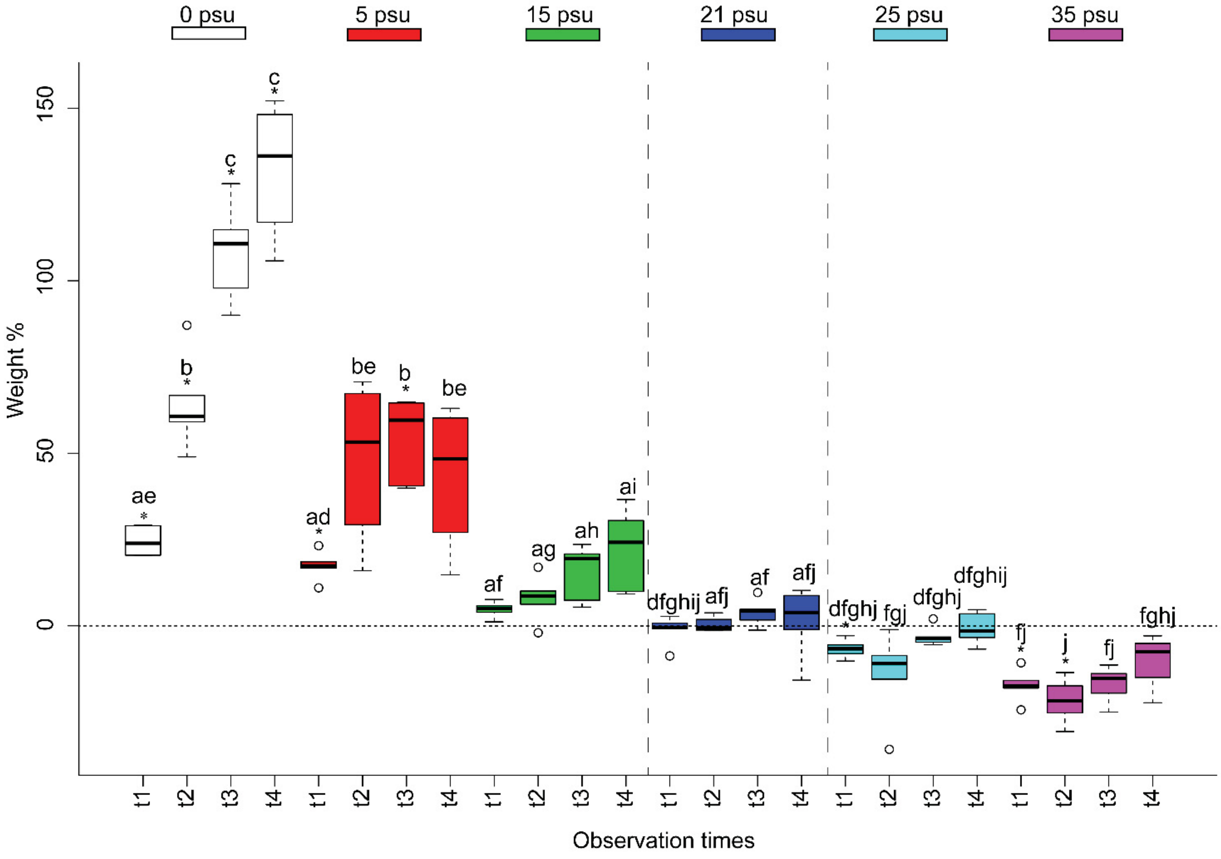


Fig. 1. Percentage of variation in body weights in the interaction between salinity and time for the annelid *Alitta succinea*. Reference salinity delimited by dashes. Horizontal dashed lines indicate w_{t0}. Colours referring to different salinities: white – 0 psu, red – 5 psu, green – 15 psu, blue – 21 psu, light blue – 25 psu, purple – 35 psu. T1 = 1 h, t2 = 6 h, t3 = 12 h, t4 = 24 h. Asterisks showing treatment with weights significantly different from time zero. Letters refer to the results of the pairwise Tukey HSD test.

The weight of *Laeonereis culveri* did not vary significantly over 24 h at the reference salinity (i.e. 31 psu), 25 and 35 psu (Fig. 2). The weight variation was not significant among the salinities after 1 h of exposure, except comparing the two lowest (i.e. 0 and 5 psu) with the two highest (31 and 35 psu). At 0 and 5 psu, weights increased throughout the experiment, while at 15 psu, after an initial increase, weight decreased after 12 h. Significant differences from the initial weights were obtained after each salinity exposure, except for some treatments at 25, the reference (31 psu) and 35 psu (Figs. 1-S1.2). According to the AICc, mortality was independently influenced by time and salinity. Deaths occurred after 12 h at 0 psu but were significant only after 24 h (Fig. S2).

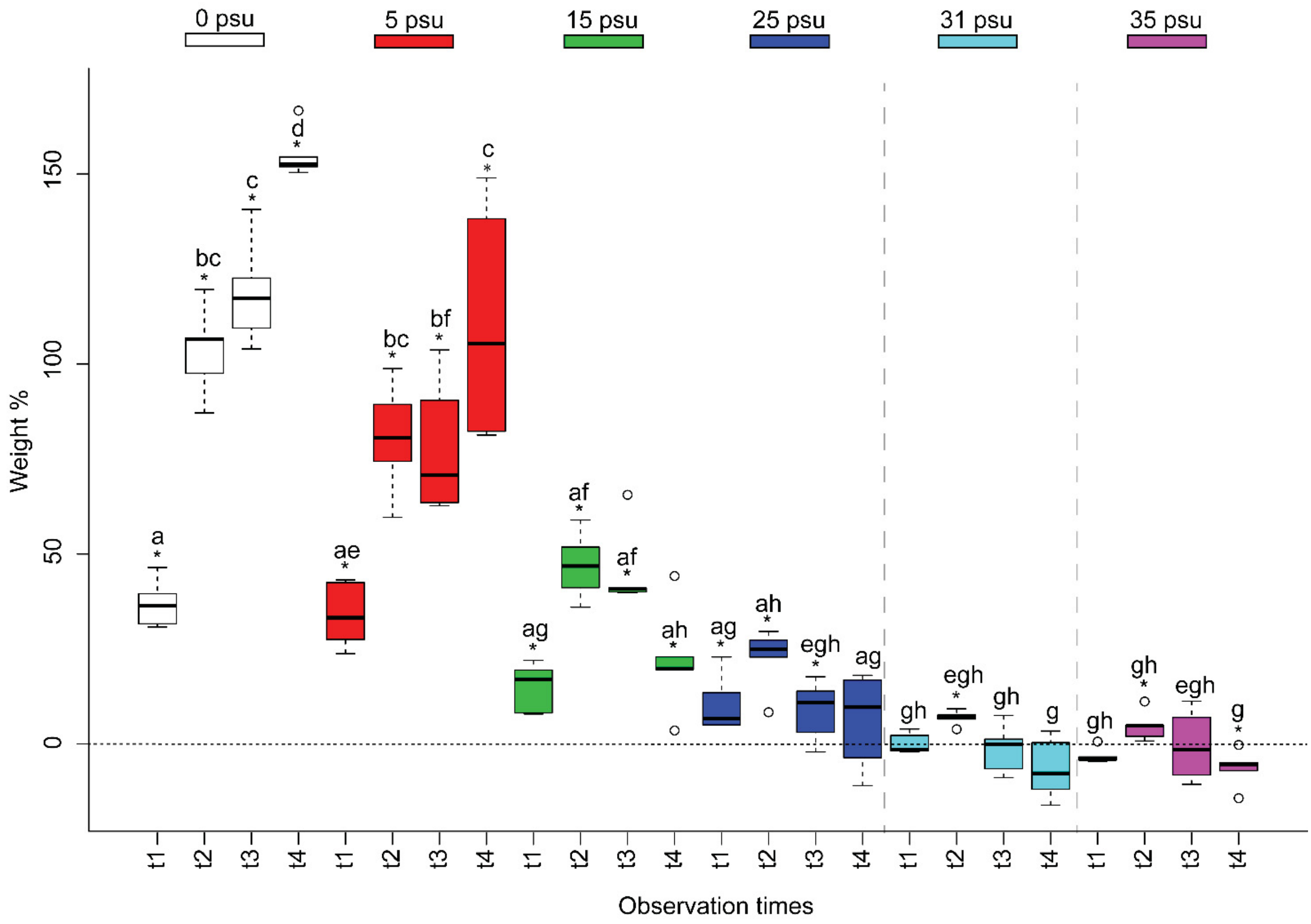


Fig. 2. Percentage of variation in body weights in the interaction between salinity and time for the annelid *Laeonereis culveri*. Reference salinity delimited by dashes. Horizontal dashed lines indicate w_{t0}. Colours referring to different salinities: white – 0 psu, red – 5 psu, green – 15 psu, blue – 25 psu, light blue – 31 psu, purple – 35 psu. T1 = 1 h, t2 = 6 h, t3 = 12 h, t4 = 24 h. Asterisks showing treatment with weights significantly different from time zero. Letters refer to the results of the pairwise Tukey HSD test.

3.1.2 Species with narrower salinity niches

The best model retrieved for *Nephtys fluviatilis* included salinity and time independently. Nevertheless, the second-best model, with only salinity as a factor, had a difference in the delta of less than 2 and was chosen as the most parsimonious (Table S1).

No significant weight variation was recovered for the reference salinity (i.e. 3 psu), and salinities lower than 15 psu over 24 h (Fig. 3-S1.3). The weight decreased significantly from the initial levels only at 25 and 35 psu (Figs. 3-S1.3). The best model explaining the mortality was the one with only the time (i.e. after 24 hours) with deceased specimens at 0 psu, the reference, and at 35 psu (Fig. S2).

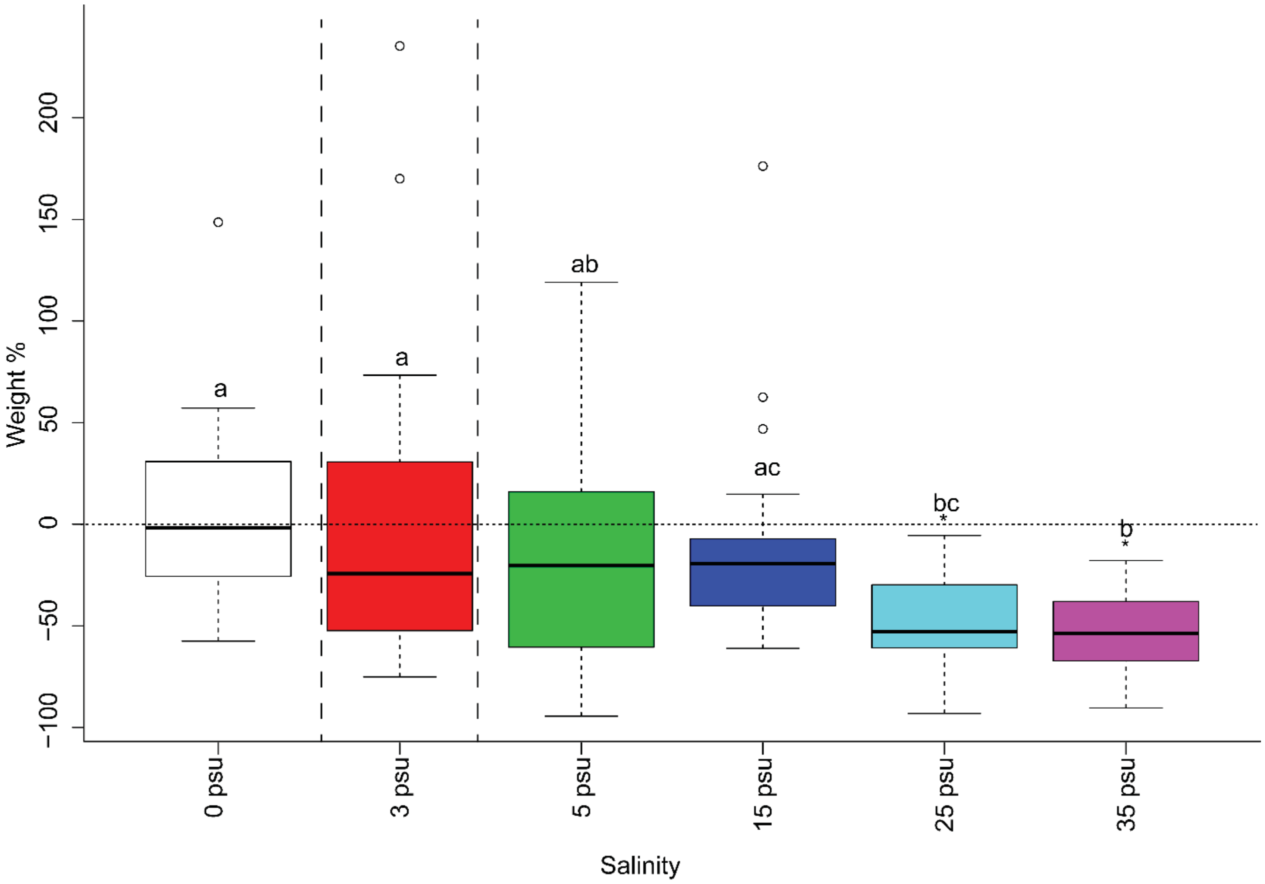
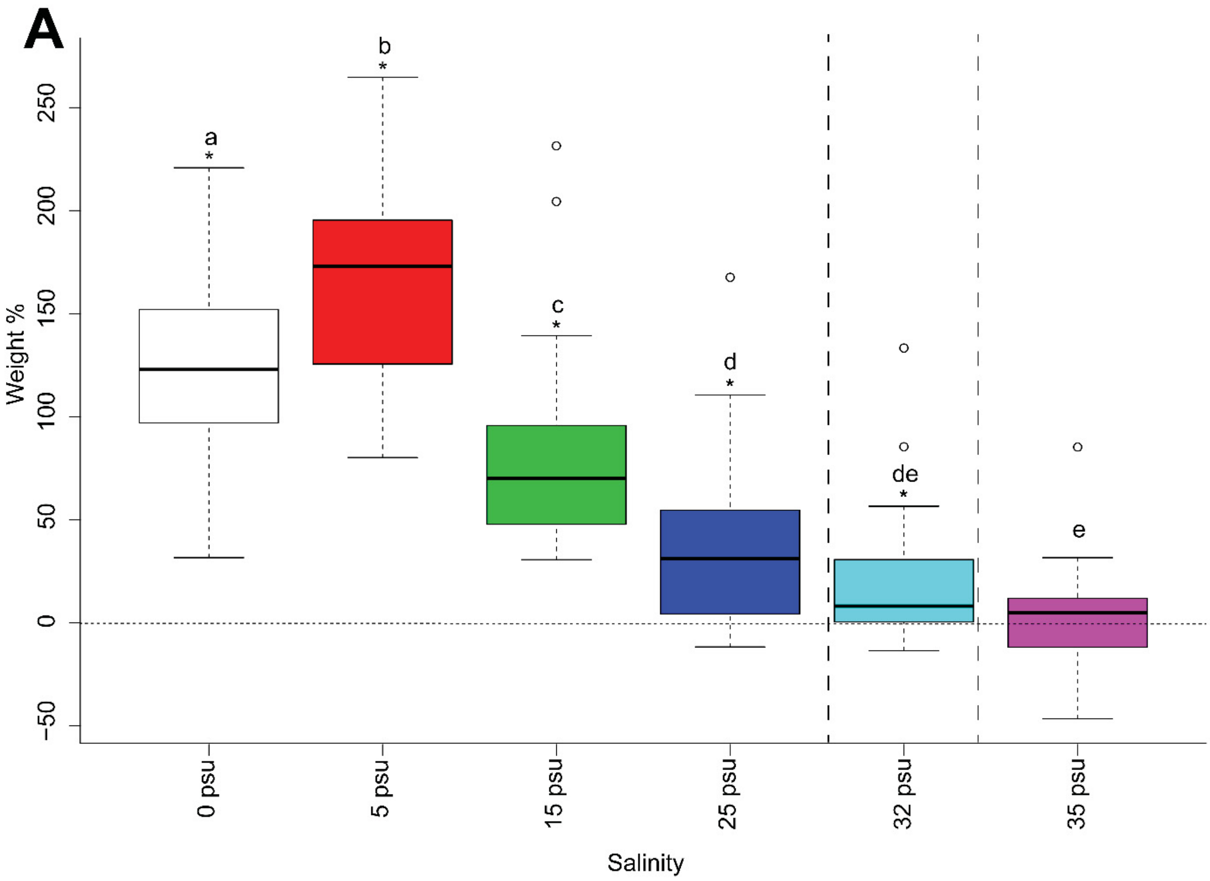


Fig. 3. Percentage of variation in body weights in *Nephtys fluviatilis* for the factor Salinity. Reference salinity delimited by dashes. Horizontal dashed lines indicate w_{t0}. T1 = 1 h; t2 = 6 h; t3 = 12 h; t4 = 24 h. Asterisks showing treatment with weights significantly different from time zero. Letters refer to the results of the pairwise Tukey HSD test.

The best model retrieved for *Isolda pulchella* included salinity and time independently (Table S1). Weight changed at each salinity (Fig. 4A) and differed significantly from its original value throughout the experiment (Fig. S1.4). The variation of weights of the reference (i.e. 32 psu) was similar only to the other higher salinities (i.e. 25 and 35 psu), even if the specimens at 25 psu showed significantly higher weights than worms placed at 35 psu. The largest variation in weight (increment) was at 5 psu, higher than 0 psu. Taking into account the time, the species showed a general gain of weight, with the highest increase after 1 h, followed by a continuous decrease and a stabilization after 12 hours (Fig. 4B). Mortality was retrieved for every salinity and time, but the GLM recovered the best model with salinity and time as independent factors. Most of the deaths occurred at 0 and 5 psu, respectively, at levels significantly higher than those at the other salinities (Fig. S2).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Salinity

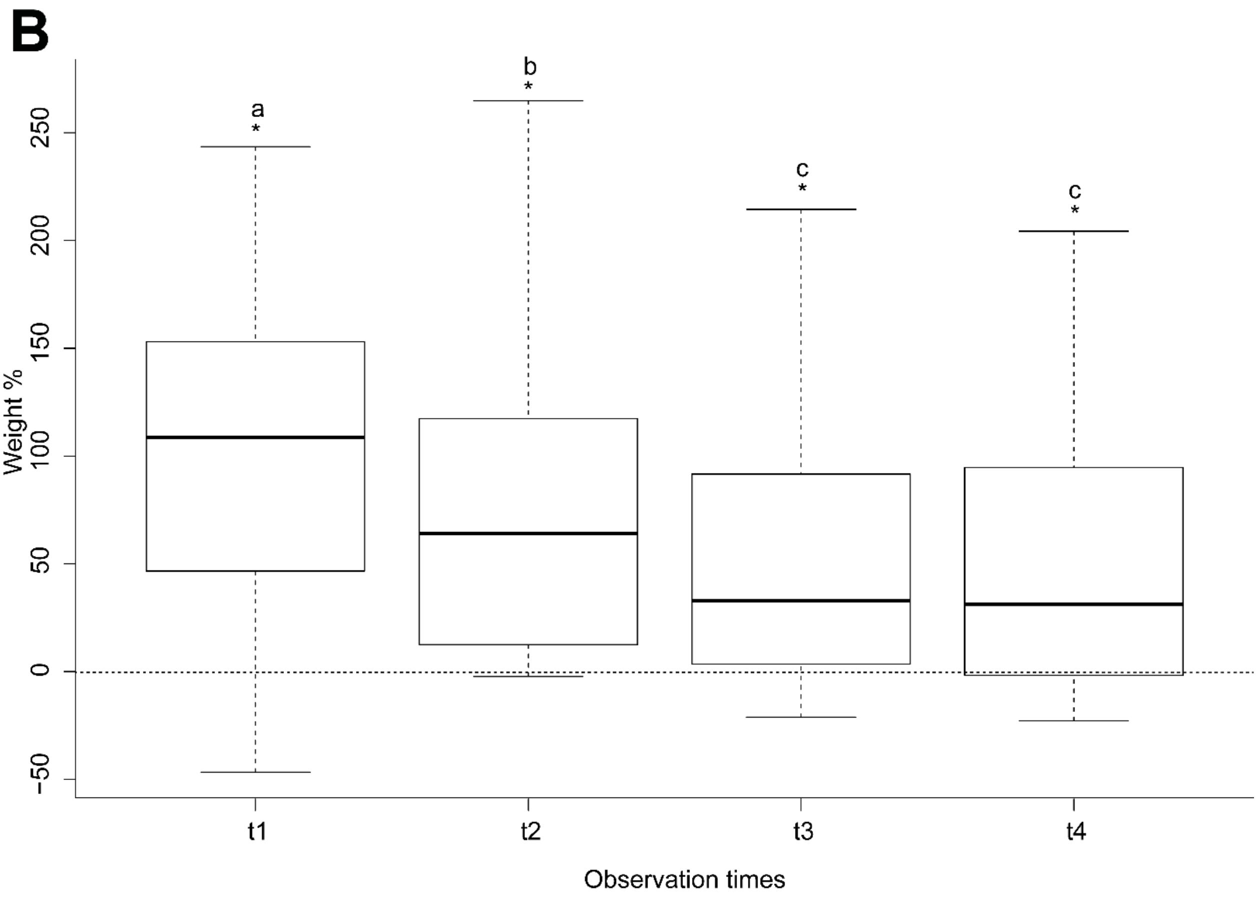

Fig. 4. Percentage of variation in body weights in *Isolda pulchella* for the factors Salinity (A) and Time (B). Reference salinity delimited by dashes. Horizontal dashed lines indicate w_{t0}. T1 = 1 h; t2 = 6 h; t3 = 12 h; t4 = 24 h. Asterisks showing treatment with weights significantly different from time zero. Letters refer to the results of the pairwise Tukey HSD test.

3.2 Histological analysis

The integument of all the species consists of a cuticle covering the epidermis (Fig. 5 A-D). The integument of *A. succinea* consists of a monostratified epithelium, with homogeneous stained blue cells covered by a thick cuticle (cuticle/width = 3.3; Fig. 5 A). The integument of *L. culveri* is pseudostratified, alternating secretory (i.e. glands) and non-secretory cells (i.e. epithelium). Gland cells show intense purple metachromasia, covered with a thick cuticle, comparably thinner than *A. succinea* (cuticle/width = 2.6; Fig. 5 B). *Nephtys fluviatilis* integument consists of a very thin layer of monostratified epithelium, with homogeneously stained blue cells. The cuticle appears as a thin layer (cuticle/width = 1.5; Fig. 5 C). *Isolda pulchella* consists of a monostratified integument, with homogeneously stained blue cells covered with a thin cuticle (cuticle/width = 1.8; Fig. 5 D).

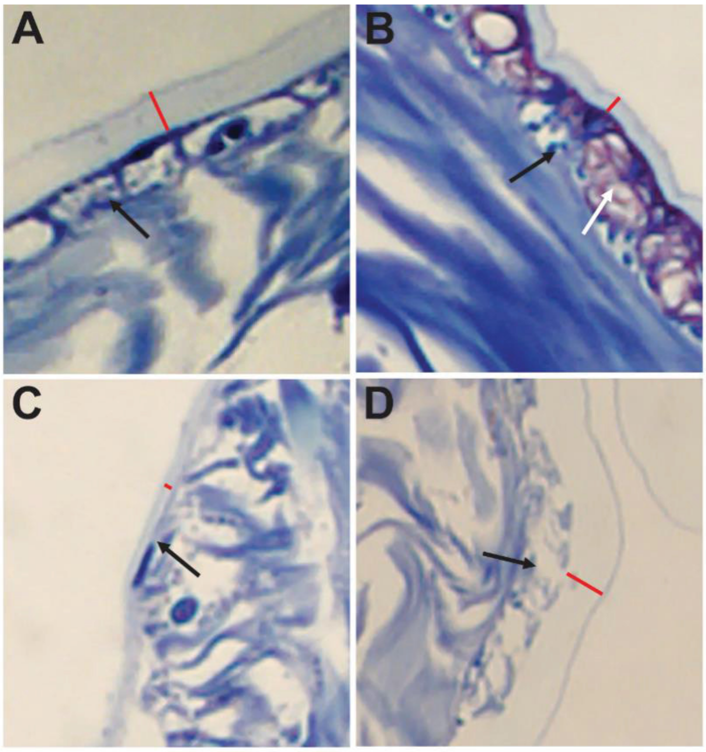

Fig. 5. Sagittal sections of the integument of the annelids *Alitta succinea* (A), *Laeonereis culveri* (B), *Nephtys fluviatilis* (C), and *Isolda pulchella* (D) stained with toluidine blue. Red bars indicate the thickness of the cuticle: (A) 11.5 µm, (B) 8.1 µm, (C) 2.1 µm, (D) 4.2 µm. Black arrows indicate epithelial cells; white arrow refers to gland cells.

4. Discussion

Our central hypothesis was partially supported. Depending on the amplitude of deviation from their respective reference salinity, each target species showed distinct responses in weight change and mortality rates, reflecting distinct physiological adaptations. Both the more euryhaline nereidids displayed morphophysiological adaptations to salinity fluctuations, including marked weight regulation and a more complex integument. Conversely, the thinner integument of the less euryhaline species, i.e. the nephtyid and the melinnid, may suggest that they rely on autecological strategies (Preston, 2009; Tait et al., 1981), including avoidance abilities in the first or tube building in the latter.

Both nereidids displayed a significant capacity for body water control after an initial shock. Nereidids include a number of freshwater species and may display a variety of adaptations to face any potential stress or disturbance related to marked and fast salinity changes (Glasby and Tarmo, 2008). They display a strong capacity for cell volume regulation, involving both a variation in the concentration of free amino acids (FAAs) and active/passive extrusion of ions such as Na^+, Cl^- and Mg^{++} (e.g.

Dykens and Mangum, 1984; Freel et al., 1973; Oglesby, 1965a, 1968). Mucciolo et al. (*under rev*) recently showed that this might be the case, since the NKCC cotransporter is abundant and widespread along the bodies of both nereidids.

Alitta succinea, after a first osmotic shock that led to a change in weight (i.e. swelling or shrinking, depending on the salinity), started to regulate, consequently stabilizing the weight and reverting the initial disturbance in volume/weight. Hypoosmotic stress may prompt an initial swelling for 1-2 h, reaching a *plateau* characterised by cell volume regulation, finally readjusting their cell volume and resuming original levels of volume/weight after 24 h (Oglesby, 1965a, 1965b, 1978; Oglesby et al., 1982). Interestingly, the final volume/weight is occasionally higher than the initial value (Dykens and Mangum, 1984). Indeed, *A. succinea,* when exposed to a hypoosmotic shock at salinity 5 psu, appeared to regulate the volume but maintained a weight higher than the initial one. On the contrary, worms were able to resume their initial weight at higher salinities, after a transient weight/volume loss. Thus, *Alitta* is clearly a marine species, dealing much better with high than low salinities (Lee and Bell, 1999). The death of individuals at 0 psu was the consequence of an excessive influx of water and the inability to decrease internal volume, which led to cell/body burst, as already reported by Oglesby (1969).

Laeonereis culveri showed a similar, albeit not identical, pattern when compared to *A. succinea*. Although both species were definitely very much harmed by freshwater, *L. culveri* was more affected by salinity 5 psu than *A. succinea*. *Laeonereis culveri* showed progressive swelling (increased weight) at 5 psu, with ~100% increase in weight at the last time of measurement. In contrast, *A. succinea* showed stability at ~50% weight increase in 5 psu, with less evidence of a time effect. Anyway, the high euryhalinity of *L. culveri* has already been reported (Koenig et al., 1981; Oglesby, 1965a, 1965b). Similarly, Castellano et al. (2020) found lower swelling or shrinkage than expected from the magnitude of the challenges, measuring cell volume in isolated cells from this species under hypo- and hyperosmotic conditions (50% changes relative to the isosmotic condition). In particular, Castellano et al. (2020) observed a distinct regulatory volume decrease response using isolated cells of *L. culveri*, upon a 50% hypoosmotic shock, which nicely fits the behaviour of this same species here (whole organism) at a salinity 15 psu. As in the case of *A. succinea*, *L. culveri* showed mortality in fresh water from continuous and uncontrolled swelling. These results highlighted how critical freshwater (salinities < 0.5 psu) may be for the survival of marine annelids if persistent in time.

The diverse responses to salinity changes over time displayed by the two nereidids result from accordingly diverse lifestyles. In fact, *A. succinea* is more able to swell and resist, maintaining more constant weight, which seems more fitting for animals from hard substrates with potentially fewer places, such as galleries and burrows, to buffer salinity decrease. In contrast, *L. culveri* is a tube dweller occurring in soft bottoms, known to “escape” from salinity changes burrowing itself deeper at the onset of a stressful condition (Oglesby, 1969). Such active burrowing behaviour allows for more suitable microhabitats, thus reducing and buffering osmotic stress, and allowing for sufficient volume regulation after 24 h (Preston, 2009). Additional evidence given by the differentiated distribution of the cotransporter Na^+-K^+-$2Cl^-$ (NKCC), a membrane protein involved in cell volume regulation (Mucciolo et al., *under rev*), along their bodies, as a reflection of their distinct lifestyles. NKCC is significantly more abundant along the integument of *L. culveri* than along the integument of *A. succinea*.

The greater or lesser ability to deal with reduced salinities is clearly reflected in the distribution patterns of *A. succinea* and *L. culveri* along the estuarine gradient. Despite their overlapping distribution, *A. succinea* is more abundant in mesohaline sectors, while *L. culveri* is more restricted to poly- and euhaline sectors. This same pattern was revealed by Oglesby (1965a), who reported on Atlantic estuaries of North America. The euryhalinity of *A. succinea* and *L. culveri* is also supported by their thick cuticle and epidermis as the correlation between their thickness and lifestyles (i.e. errant and burrower) are known for annelids from distinct saline regimes. A reduced or absent cuticle was reported for tube-building annelids (Storch, 1988), whereas infaunal annelids from sandy bottoms display a thick cuticle with epicuticular projections along the whole body (Gustavsson, 2001). A thick and less permeable integument is often associated with species from freshwater and estuarine environments (Preston, 2009). We propose that the thick and less permeable cuticle may contributes to a higher tolerance to low salinities by *A. succinea* when compared to *L. culveri*. In fact, within the nereidids, the relative thickness of the cuticle of *A. succinea* was of 3.3, and that of *L. culveri* was of 2.6. Notwithstanding, the ability of the latter species to occupy a greater variety of habitats (e.g. sandy and muddy substrates, oligohaline to polyhaline areas of estuaries) may also be explained by the presence of numerous gland cells, suggesting the production of acidic mucopolysaccharides (i.e. mucus). Acidic and neutral mucus secretions have already been described for either sessile or errant annelid species (e.g. Mastrodonato et al., 2005, 2006), and depending on its composition, different roles are associated, such as structural stability of membranes, electrolyte homeostasis, mechanical and chemical defence, or reproduction (Hausen, 2005; Mastrodonato et al., 2005; Stabili et al., 2009; Storch, 1988). Thus, this mucus may help *L. culveri* buffer low-term (less than 24 h) salinity changes, even better than *A. succinea* (also at 0 psu).

Nepthys fluviatilis and *Isolda pulchella* were distinctly more comfortable at either low or high salinities, respectively, according to their distribution along the estuarine gradient of the bay. Although displaying a putatively narrower salinity niche than the nereidids, *N. fluviatilis*, it should be remarked, was the species, among the four, with the lowest values of weight changes across the salinities tested. Although with mortality recorded at 0-3 psu, *N. fluviatilis* tolerates lower salinities, while *I. pulchella* tolerates high salinities. The nephtyid *N. fluviatilis* was able to control its weight from fresh water up to salinities ≤ 15 psu, which is typical of oligohaline organisms. This response of not showing change in weight of the whole organism at low salinities, seen here, fits well with the data from Castellano et al. (2020) with isolated cells from this species; these cells also did not swell upon a 50% hypoosmotic shock. Another feature of the adaptation of *N. fluviatilis* to oligohaline environments detected in Castellano et al. (2020) was a significant hyperosmotic state (by ~120 mOsm/kg H_2O) of its body fluids at a salinity of 15 psu. This is a significant signal for osmoregulatory capacity (AER), especially for such a small animal, member of a group of typically osmoconforming invertebrates (Deaton, 2009). A similar pattern was described for the nereidid *Hediste limnicola* (Oglesby, 1965a), which, like *N. fluviatilis*, is also known from low water salinities. Hyperregulation may result from active salt uptake, as displayed by some onuphids and nereidids (Ebbs and Staiger 1965; Fretter 1955), or the presence of a less permeable integument, as in the case of brackish water invertebrates when compared to their marine relatives, in general (Evans, 2009). While 15 psu was considered the ecological salinity limit for *N. fluviatilis*, which usually occurs in the muddy bottom of oligohaline sectors (Castellano et al., 2020; Giménez et al., 2005; Lana, 1986; Passadore et al., 2007), higher salinities did not significantly affect the survival of this species. In fact, even if it switched from osmoregulation to osmoconformation at higher salinities (i.e. ≥ 25 psu), its weight was approximately stable across tested salinities. The variation in weight did not change significantly until

5 psu (i.e. different from 3 and 0 psu), with *N. fluviatilis* maintaining, after some "readjustments", a similar weight over time. Thus, our results show how *N. fluviatilis* may tolerate salinities of ~> 25 psu for at least 24 hours; its restricted ecological distribution may reflect the results of other driving factors, such as interspecific competition, feeding or reproductive habits (e.g. Gale and Proctor, 2011; Lemieux et al., 1997).

The melinnid *I. pulchella* was the less euryhaline, and possibly the most osmoconformer, among the four studied species. In fact, the proportional gain of water was more than twofold depending on the salinity; its water permeability was apparently much higher than that of the other three species, a typical feature of marine osmoconformers (Deaton, 2009). Moreover, very low salinities (i.e. 0 and 5 psu) dramatically decreased its survival. Accordingly, *I. pulchella* is usually restricted to poly- and euhaline estuarine sectors, and its sessile lifestyle, and consequent low motility, may not allow for avoidance behaviour to cope with salinity variations. The ability to successfully respond to environmental stresses, such as hypoxia, thermal variation or salinity changes, is hampered by the sessile habitus and by the soft body of annelids (Rivera-Ingraham and Lignot, 2017). For instance, the terebellomorphs *Terebellides parvus* Solis-Weiss, Fauchald & Blankensteyn, 1991 and *Loimia medusa* (Savigny, 1822), usually restricted to the poly/euhaline sectors, such as *Isolda*, exhibited a similar tolerance to osmotic stress with increasing mortality rates when exposed to lower salinities (Ferraris et al., 1994; Krishnamoorthi, 1962). Moreover, like *I. pulchella*, both showed little capacity to adjust cell or body water/volume when salinity-challenged, displaying changes in weight according to the salinity (i.e. increasing in hypoosmotic media), emphasizing their supposed marine origin, and more limited estuarine distribution (Foster et al., 2010). The late volume regulation recorded for *I. pulchella*, which was able to maintain its weight after 12 h, is similar to the response of both *T. parvus* and *L. medusa*. Evidence of their high sensitivity to salinity variations is also provided by the restricted distribution of the NKCC, expressed in the body portions in direct contact with the external environment (Mucciolo et al., *under rev*).

Since the thick integument is the first trait to face salinity changes, as shown for the nereidids, the two species with narrower salinity niches, having a very thin cuticle and epidermis, may have other strategies for osmoregulation. Actually, besides the thickness, obviously the composition of the cuticle also interferes with its osmotic and ionic permeability (e.g. Hausen, 2005; Richards, 1984). In addition, the species may also express ion transporters that are capable of generating the significant osmotic gradient detected previously (Castellano et al., 2020). Moreover, nephtyids are mostly marine burrowers, occurring in sandy/muddy intertidal and subtidal bottoms, from shallow to abyssal waters (Rouse and Pleijel, 2001). Therefore, active avoidance of salinity fluctuations may be sufficient to buffer or escape osmotic stress. In fact, *N. fluviatilis* almost reaches the surface of the sediment, avoiding the freshwater below, during low tides, when salinity is lower. Conversely, it burrows to deeper layers of the sediment when the tide rises, thereby increasing salinity (Mucciolo, pers. obs.). Moreover, a thinner and more flexible integument is expected from burrowers (Gustavssnon, 2001). Thus, the thin integument is probably not associated with osmoregulation but is rather a shared trait within the nephtyids, suggesting phylogenetic constraints for a vagile, burrowing life in soft bottoms, as hypothesized for other clitellates (Gustavssnon, 2001).

The thin integument of the tubicolous *I. pulchella* was compatible with the results of our experiment as well as the initial hypothesis of having a low degree of euryhalinity, restricted to higher salinity environments. Thinner integuments are also associated with tube building, which provides advantages, such as sheltering, efficient feeding and respiration, and potential buffering to salinity shock (Fox, 1938; Kakui and Hiruta, 2017). Likewise, the tubicolous *Branchiomma luctuosum* (Grube, 1870), a sabellid occurring in brackish and marine waters (Mastrodonato et al., 2005), and *Aulodrilus pluriseta* (Piguet, 1906), a tubicolous freshwater clitellate (Gustavssnon, 2001), compensate for a thin integument with their tubes in stressful salinity conditions. Moreover, serpulids of the genus *Pomatocerus* also have a thicker cuticle in the exposed than in the less exposed body regions surrounded by the tube (Bubel, 1983), supporting the hypothesis that a thicker integument may provide protection against wide or abrupt salinity changes. Accordingly, our results suggest that living in a tube may represent one of the few traits allowing *I. pulchella* to tolerate salinity changes, even if they are lesser than the other species. In fact, *I. pulchella* occasionally occurs at salinities of approximately 25 psu along the estuarine gradient (Mucciolo, pers. obs.), while in our experiment, it displayed a twofold increase in volume when removed from the tube, showing only late and limited cell volume regulation.

5. Conclusion

This is the first study to couple field evidence, laboratory experiments and histological analyses to investigate salinity tolerance in annelids. Our target species displayed varying responses to osmotic stress and diverse integument patterns, mostly reflecting their life strategies and preferred habitats. As expected, the two nereidids confirmed their high degree of euryhalinity, showing strong cell volume regulation but, in both cases, being unable to tolerate fresh water. The nephtyid was distinctly less euryhaline but tolerated low salinities and fresh water. Finally, the melinnid presented a marine stenohaline behaviour and did not tolerate seawater dilution. Even though estuarine annelids are generally considered osmoconformers, they are remarkably euryhaline and have shown a good capacity for body water regulation. The ability to fast, efficient cell volume regulation upon salinity changes is crucial to achieve euryhalinity in unsteady habitats such as estuaries, especially for osmoconformers. The novel combination of approaches adopted here provided a better understanding of the osmoregulatory physiology in soft body, estuarine invertebrates, underlining how their distribution is modelled by adaptations that include both autecological and biological traits.

6. Acknowledgements

This work was supported by the Brazilian Agencies CAPES (Doctorate fellowship to SM) and CNPq (Doctorate fellowship to AD, #141565/2017-9, and Research grants to CAF, #302829/2015-6 and PL, #441439/2017-9).

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CHAPTER II

FIRST REPORT OF THE NA$^+$-K$^+$-2CL$^-$ COTRANSPORTER (NKCC) IN ESTUARINE ANNELIDS AND ITS VARIABLE EXPRESSION ALONG THEIR BODIES

Manuscript formatted following the guidelines of the journal: Tissue & Cell
ISSN: 0040-8166
Impact Factor: 1.837
Qualis: A4

First report of the Na^+-K^+-$2Cl^-$ cotransporter (NKCC) in estuarine annelids and its variable expression along their bodies

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Abstract

The expression of the Na^+-K^+-$2Cl^-$ cotransporter (NKCC), widely associated with cell volume regulation, was never directly demonstrated in annelids. We first searched for its presence *in silico* retrieving two annelid sequences from GenBank database, belonging to one marine and one freshwater annelid. Then, with the immunofluorescence, we confirmed for the first time the occurrence of this ubiquitous membrane carrier protein in different tissues of four species of estuarine annelids, regularly subjected to salinity fluctuations. We tested two euryhaline species (wide tolerance), the nereidids *Alitta succinea* and *Laeonereis culveri* (habitat salinity: ~10-28 psu), and two stenohaline species (restricted tolerance), the nephtyid *Nephtys fluviatilis* (habitat salinity: ~6-10 psu) and the melinnid *Isolda pulchella* (habitat salinity:

~28-35 psu). All four species showed a positive signal for NKCC expression. However, the expression of NKCC was not homogeneous among them. The free-living/burrowers (both euryhaline nereidids and the stenohaline nephtyid) displayed a widespread signal for NKCC along their bodies, in contrast to the stenohaline sedentary melinnid, in which the signal was restricted to the branchiae and the internal tissues of the body. The results are compatible with NKCC involvement in cell volume, especially in annelids that face wide variations in salinity in their habitats.

Key-words: Invertebrates. NKCC cotransporter. Osmoregulation. Physiology. Polychaetes.

1. Introduction

Annelids occupy a wide range of environments (e.g. seawater, freshwater, soil), thanks to their high body plasticity and life strategies (Rouse and Pleijel, 2001). One of the biggest requirements to survive in different habitats is the capability to regulate the bodily internal osmotic pressure in the fluids. For instance, marine annelids are generally in osmotic equilibrium with the saltwater, adjusting their internal osmotic pressure with the surrounding environment. Differently, freshwater and terrestrial animals usually maintain a moderate/ high internal osmotic pressure and need to face water gain and desiccation stress, respectively (Preston, 2009). Phylogenetic hypotheses, and the derived classifications of annelids, are constantly changing, due to both their diversification and adopted approaches. According to one of the most accepted arrangements (fig. 1A), Annelida are divided into two major clades, Sedentaria and Errantia, and five sister branches: Sipuncula, Amphinomidae, Chaetopteridae, Magelonidae, and Oweniidae (Weigert and Bleidorn, 2016). Regarding the osmoregulation, no correlation appears between annelid classification and osmoregulatory strategies, displaying either osmoconformity or osmoregulation along the whole phylum. Sedentaria, for instance, includes mostly osmoconformer annelids, with an exception of the Clitellata, animals performing mostly osmoregulation and occurring in freshwater and terrestrial environments. On the contrary, Errantia, as well as the remaining clades, group together mostly marine osmoconformer annelids with sporadic examples of species capable of establish osmotic gradients with the external environment (e.g. Castellano et al., 2020; Rouse and Pleijel, 2001; Preston, 2009). The diverse osmoregulatory strategies may be reflected also in the ecological and morphological differences that Sedentaria and Errantia present (fig.1 B-C). The tubicolous lifestyle of Sedentaria is reflected in reduced parapodia which, on the contrary, are mostly well developed for their intense locomotory activity in Errantia (Fig.1 B-C). Nonetheless, besides their main role in the locomotion, parapodia are involved in ions and gas exchanges (Preston, 2009). Different organization in the excretory system, such as the type and localization of nephridia (along the body in Errantia while restricted to the first few anterior segments in tubicolous Sedentaria), may influence higher osmotic tolerance and osmoregulatory capability (Fig.1 B-C) (Bartolomaeus and Quast, 2005; Goodrich, 1945).

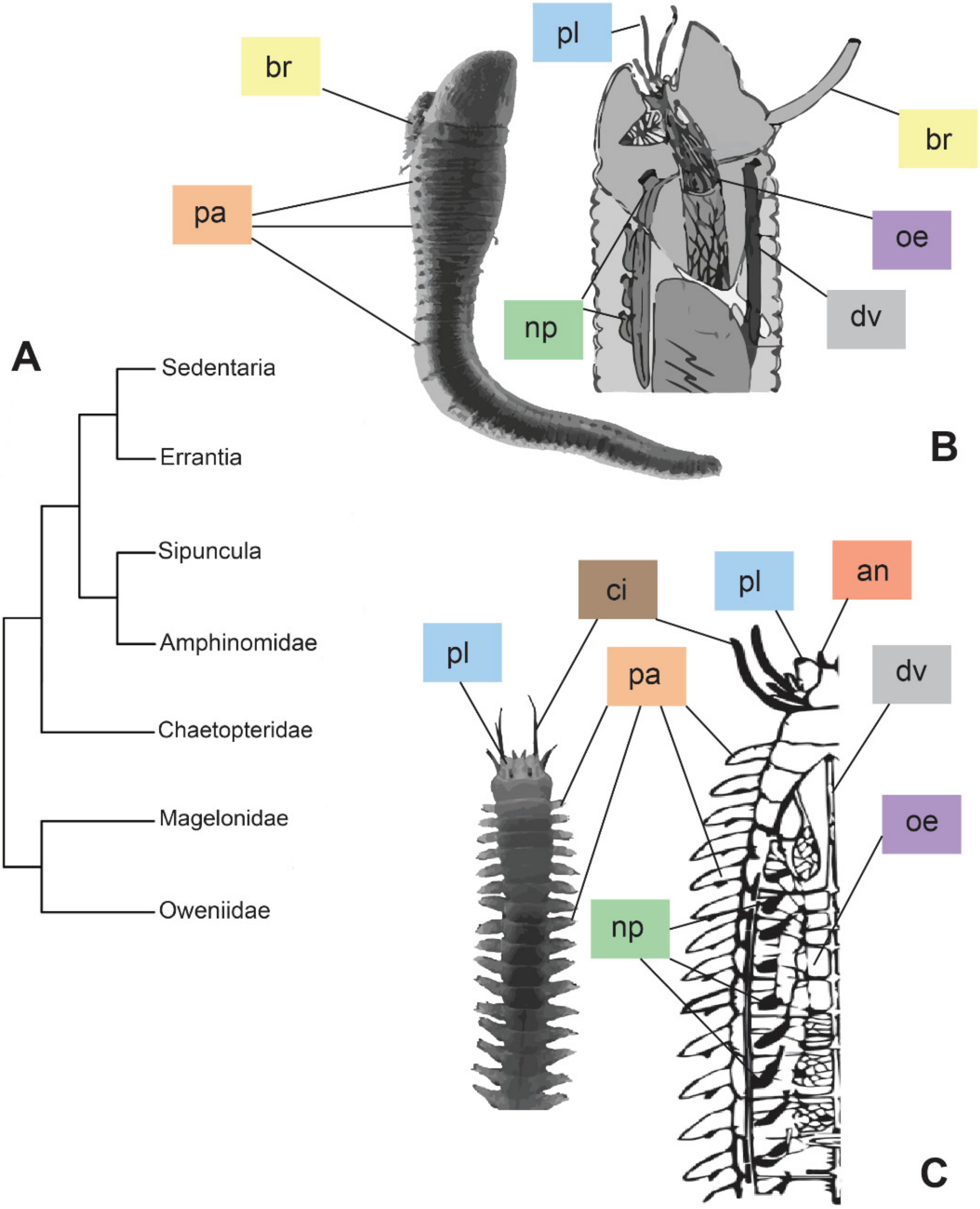

Figure 1. Phylogeny modified from Weigert and Bleidorn (2016) based on the molecular approach (A) and comparison between morphologies of the two major clades, Sedentaria (B) and Errantia (C). B= entire individual of Artacama sp. (Terebellidae) and sagittal section of an Alvinellidae anterior part. Images modified from Natural History Museum (2014) and Tzelin and Filippova (2005), respectively. C= entire individual of Neanthes sp. (Nereididae) and frontal section of a Nereididae. Images modified from Glasby et al. (2011) and Brown (1950), respectively. Abbreviations: an= antennas; br= branchiae; ci= cirri; dv= dorsal vessel; oe= oesophagus; np= nephridia; pa= parapodia; pl= palps. Colours of the labels referring to the different organs.

At the cellular level of body organization, different membrane proteins work together in order to regulate the cell volume, performing ions and water exchanges

(Russell, 2000). The Na^+-K^+-$2Cl^-$ (NKCC) cotransporter belongs to the family of cation-chloride cotransporters and transports sodium, potassium and chloride ions across the cell membrane under electroneutral conditions: 2 cations and 2 anions. Two isoforms are known in the literature, NKCC1 and NKCC2, the former occurring in the basolateral membrane of secretory epithelia and the latter only in the apical absorptive kidney epithelia (Haas and Forbush, 1998; Russell, 2000). NKCC1 plays a crucial role in vectorial salt excretion in vertebrates, as in the gills of marine teleosts, in the rectal gland of elasmobranchs, or in the salt gland of marine birds and reptiles (Clauss, 2001; Okada, 2004; Russell, 2000). The absorptive NKCC2, in turn, occurs in the apical membrane of the thick ascending limb of the loop of Henle in the mammalian kidney, where, actually, this cotransporter was discovered (Gamba et al., 1994; Payne and Forbush, 1994). In addition to its occurrence in epithelia, NKCC1 is also widely reported in cells in general, acting effectively in volume control (Haas and Forbush, 1998; Russell, 2000). In vertebrates, it has been specifically related to regulatory volume increase (RVI) activated by cell shrinkage, allowing for significant solute influx, which is followed by water influx and cell restoration (Haas and Forbush, 2000; Russell, 2000). Conversely, NKCC seems to be activated in case of cell swelling, performing the efflux of osmolytes during Regulatory Volume Decrease (RVD), due to distinct ionic electrochemical gradients expected in osmoconforming invertebrates, such as anemones and echinoderms (Amado et al., 2011; Castellano et al., 2016; Scemes and Cassola, 1995). As a transporter that relies on electrochemical gradients, it may operate reversibly, depending on the prevailing driving forces. In fact, depending on the anisosmotic or isosmotic conditions, it may operate in RVI or RVD, respectively, even in mammalian cells (Russell, 2000).

Marine annelids are frequently euryhaline, thus tolerating wide and fast salinity changes in their habitats meaning that their cells are intensely challenged with respect to their volume (Deaton, 2009; Foster et al., 2010). Studies on the presence and functions of NKCC are scarce and only hypothesized in annelids. For example, Dykens and Mangum (1984) pointed out how the presence of the Na^+/K^+-ATPase, occurring only in the superficially vascularized part of the parapodia, was not sufficient to explain the cell volume regulation widespread in the whole body. The potential presence and functionality of NKCC in osmoregulation/cell volume regulation of annelids was proposed for the leech *Hirudo medicinalis* Linnaeus, 1758, based on the effects of its inhibitor furosemide in primary urine formation (Clauss, 2001; Zerbst-

Boroffka et al., 1997). Nevertheless, no study has clearly demonstrated the presence of this membrane protein in the epithelia of annelids. Therefore, the aim of this work was to reveal the expression and localization of the NKCC cotransporter through immunofluorescence in the bodies of marine annelids that live under variable salinity challenges in estuarine habitats.

2. Materials and methods

2.1. In silico *search*

To check for the presence of the NKCC in the annelids *in silico*, a preliminary search on internet database and a phylogenetic reconstruction was conducted. An amino-acid sequence of the NKCC per phylum was firstly selected from the comprehensive phylogenetic analysis on the entire CCC family carried out by Hartmann et al., (2013). Multiple blastp (Altschul et al., 1990) were performed on GenBank (Benson et al., 2015; last accessed June 12, 2020) using each selected sequence as query against the Annelida database (taxid 6340). For each protein, only sequences with an e-value of 0.0 were downloaded. All accession numbers are listed in the supplementary data (tab. S1). One sequence per species was aligned using MUSCLE (Edgar, 2004) as implemented in MEGA7 (Kumar et al., 2016). A maximum likelihood (ML) tree was built using PhyML (Guindon et al., 2010), and the robustness of the nodes was estimated by 1,000 bootstrap replicates. Sequences of the choanoflagellate *Monosiga brevicollis* KCC and CIP1 were used as root of the phylogeny.

2.2. Immunofluorescence

Sampling was carried out along the Paranaguá Estuarine Complex (PEC) in southern Brazil, one of the most preserved coastal areas along the southwestern Atlantic, despite increasing port and tourist activities (fig.2). The estuary is considered mixed, with seasonal pattern variations of salinity and temperature and an increasing salinity gradient going from the inner to the outer sectors (Marone et al., 2005). Species were chosen considering their occurrence along the estuary and expected salinity tolerance: a) the nereidids *Alitta succinea* (Leuckart, 1847), usually associated with hard and human-made substrates, and *Laeonereis culveri* (Webster, 1879), in soft bottoms, both abundant in meso/polyhaline sectors of the estuaries habitat salinity range: ~10-28 psu), and putatively euryhaline; b) the nephtyid *Nephtys fluviatilis*

Monro, 1937, from the oligohaline sectors of the estuary (habitat salinity range: ~6-10 psu), and the melinnid *Isolda pulchella* Müller, 1858, usually related to poly/euhaline sectors of the bays (habitat salinity range: ~28-35 psu), both putatively stenohaline (fig. 2). Animals were sampled using a shovel or grasping the buoys, depending on the species. Authorization for animals sampling was provided by "Sistema de Autorização e Informação em Biodiversidade" (SISBIO, permit # 36255-1).

In the laboratory, the animals were acclimated for 48 h in plastic containers of 1 l with water, and 3-4 cm of sediment collected from each sample area, under constant temperature (~20°C), aeration and natural photoperiod, and fed with common aquarium flocks. Individuals of *A. succinea* and *L. culveri* were fixed in 2% paraformaldehyde diluted in 1% PBS for 2 h, while *I. pulchella* and *N. fluviatilis* were fixed in 2% paraformaldehyde in 1% PBS for 1.5 h because of their smaller size. Animals were then washed in 1% PBS, incubated in 1% PBS with 5% sucrose for 2 h and finally in 1% PBS with 15% sucrose overnight at 4°C. Specimens were embedded in Fisher Healthcare™ Tissue Plus™ O.C.T. compound and stored at -20°C. Blocks were sectioned in the cryostat Leica CM1850, Heidelberger, Germany at -25°C, with sections of 15-20 µm thickness for the species *A. succinea* and *L. culveri*, 5-8 µm for the species *I. pulchella* and *N. fluviatilis*, and placed on glass slides previously prepared with a solution of 2% 3-aminopropyltriethoxysilane diluted in acetone for section adhesion. Sections washing and incubation followed the protocol from Prodocimo and Freire (2006) using the primary antibody anti-NKCC1 (T4, anti-NKCC1 cotransporter from human colonic crypt, T84 cell, developed by Lytle et al. (1995), produced in mouse and obtained from the Development Studies Hybridoma Bank of the Department of Biological Sciences of the University of Iowa, USA), and the secondary antibody anti-mouse IgG (Fab specific) −FITC antibody, produced in mouse (Sigma Aldrich). Negative controls for each species, incubating sections only with the secondary antibody, were carried out to confirm the specificity of the fluorescent signal and the binding of the primary antibody with the NKCC cotransporter along the body of annelids. Slides were observed and photographed under a Nikon A1R MP+ multiphoton confocal microscope. Finally, depending on the species, the fluorescence intensity of a selected area of each picture from different parts of the body was quantified by using Fiji software (Schindelin et al., 2012) on 5-7 specimens per species. To test where the fluorescence was higher (i.e. higher abundance of NKCC) and if it was significantly different from the negative control, we took the five highest gray values/pixel² per area selected and used them as replicates. The nonparametric Kruskal-Wallis test (Kruskal and Wallis, 1952) was used to test for significant differences, in each species and in the same confocal session, between the negative control and the tissues treated with the primary antibody and among different body parts. Afterwards, the non-parametric Wilcoxon test (Wilcoxon, 1945) was applied as post-hoc analysis to reveal which parts were significantly more fluorescent. To better understand the anatomy of the studied annelids and allow for a more accurate interpretation of the immunofluorescence pictures, histological slides of sagittal sections of specimens of each species were taken using staining with periodic acid Shiff (PAS) and Mayer’s hemalum solutions following the protocol Scillitani et al. (2011).

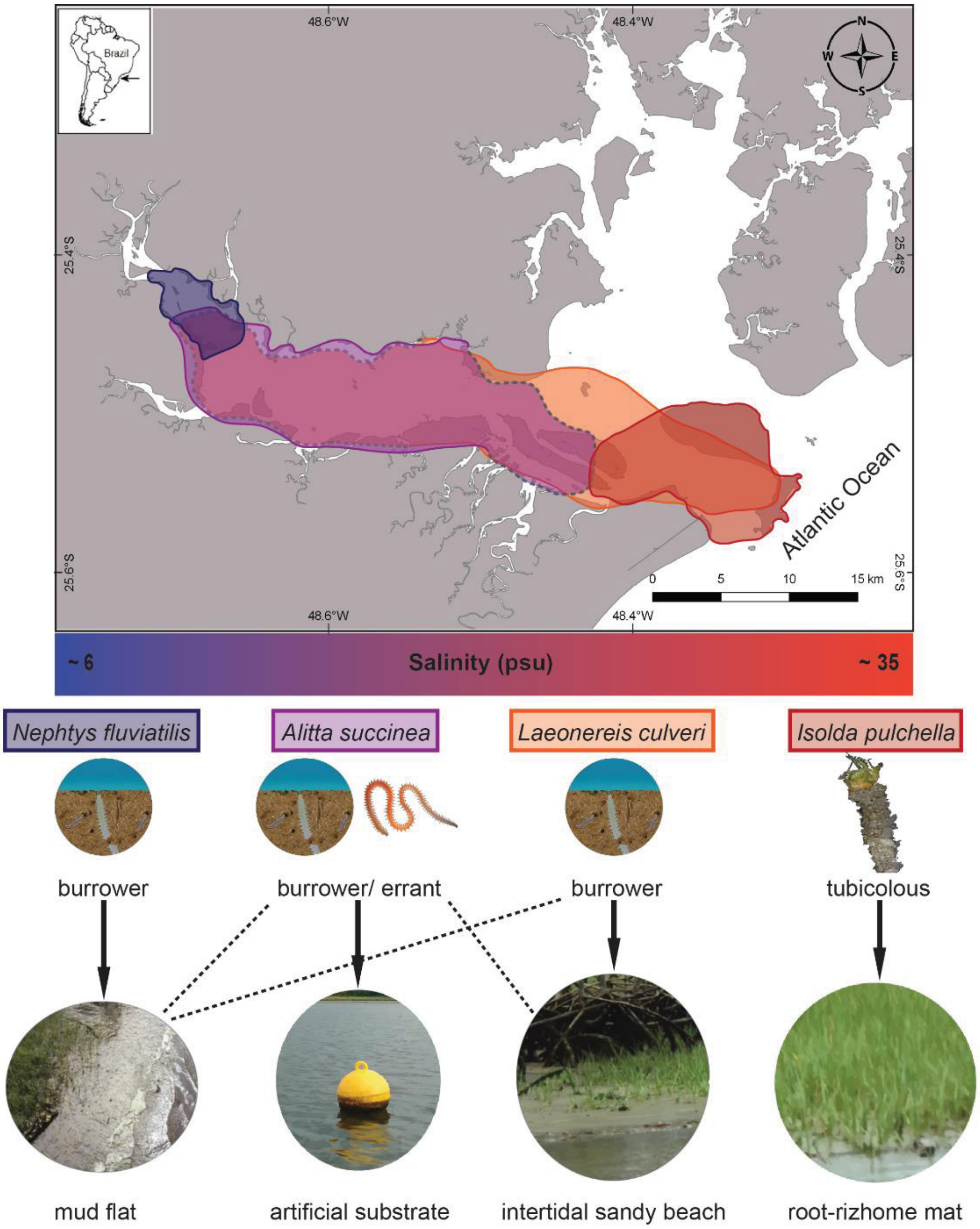

Figure 2. Map representing the occurrence of the studied species and the gradient of salinity along Antonina and Paranaguá Bays, life strategies and types of habitat. Colours in the map referring to the area of occurrence: blue *N. fluviatilis*, violet *A. succinea*, orange *L. culveri*, red *I. pulchella*. Dashed lines in the map and in the types of habitat indicate occasional occurrences.

3. Results and discussion

The blastp search retrieved sequences of two annelids: *Capitella teleta* Blake, Grassle & Eckelbarger, 2009, a widespread annelid usually occurring in intertidal and shallow-water habitats (Blake et al., 2009) and *Helobdella robusta* Shankland, Bissen & Weisblat, 1992, a freshwater leech (Shankland et al., 1992). Both species belong to the Sedentaria clade (Weigert and Bleidorn, 2016). The phylogeny reflected the actual reconstruction of metazoan, positioning the annelids together with a mollusc (i.e. *Lottia gigantea* Sowerby, 1834) (Dunn et al., 2014) (Fig.3).

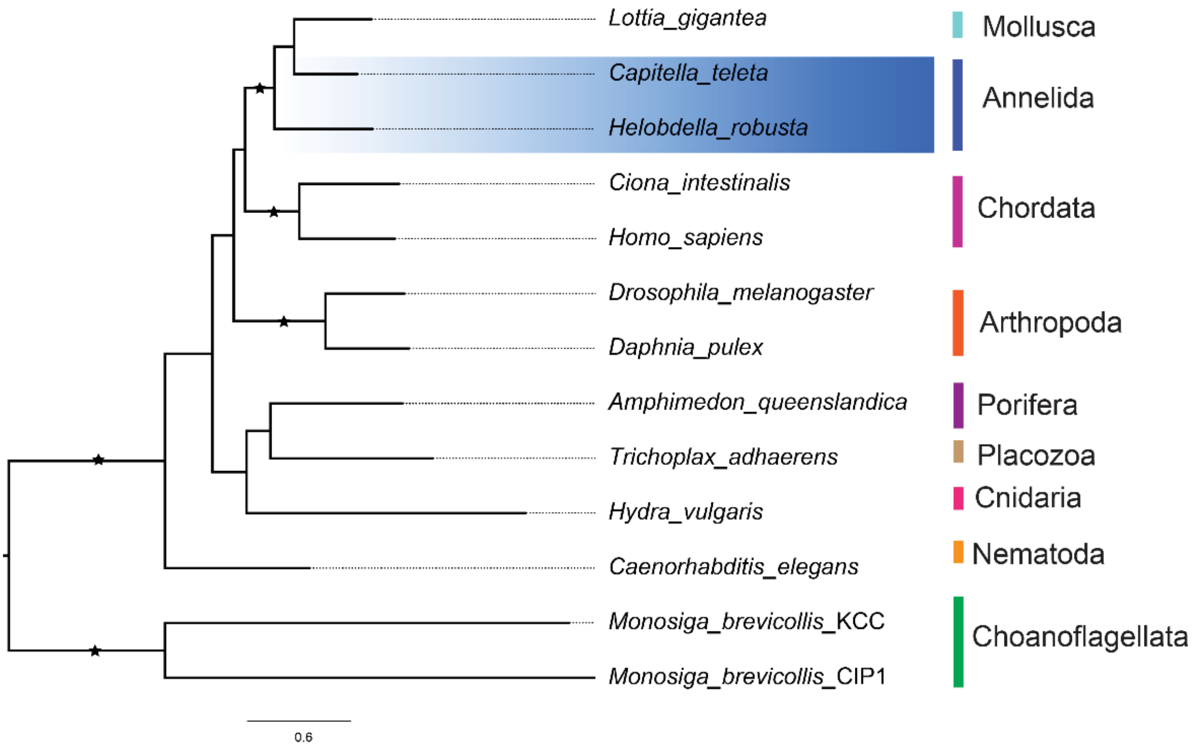

Figure 3. Phylogenetic relationships of NKCC among metazoans. Maximum-likelihood (ML) analysis with 1,000 bootstrap replicates; tree rooted with the choanoflagellate sequences. Stars on branches referring to bootstrap values >85%. Annelida sequences in blue-shaded box. Colours relative to different phyla.

With the immunofluorescence, the presence of the NKCC cotransporter was revealed along the body of the two nereidids and the nepthyid (Errantia) and the melinnid (Sedentaria). Each species showed a different pattern of immunolocalization and expression of the cotransporter (figs. 4-5), suggestive of physiological adaptations to their specific estuarine habitats and salinity regimes. In both *A. succinea* and *L. culveri*, the signal was retrieved in either the muscles, integument, or internal epithelia (fig. 4A-B, D), and in *A. succinea*, also in cephalic appendages (fig. 4C). In *A. succinea*, the integument and the muscles of the parapodia displayed a significantly higher signal when compared with the segments (tab. S2, fig. S1). Parapodia of some annelids (e.g. nereidids, capitellids) are involved in ionic, gas, and dissolved organic matter exchanges (Abele et al., 1998; Da Rosa et al., 2005; Dykens and Mangum, 1984; Preston, 2009), suggesting a potential role in salt and water fluxes of this species parapodia. Moreover, parapodia are highly vascularized, bearing also cirri known to have sensory functions, together with cephalic appendages (Smith, 1957). The involvement of the NKCC1 was already reported in sensory organs of some invertebrate taxa, e.g. molluscs, spiders and flies (Bukanova et al., 2005; Pfeiffer et al., 2009; Stenesen et al., 2019), by modulating the effect of GABAergic transmissions with the transport of Cl^-, important for synaptic transmissions (Schulte et al., 2018).

L. culveri, the euryhaline species occurring in the poly- and euhaline sectors of the estuary, showed a higher signal of the NKCC along the integument than in its muscle bundles (tab. S3, figs. 4F-H, S2). The higher abundance of NKCC along the integument may be due to its position directly in contact with the external medium and, consequently, facing salinity fluctuations and volume changes. In fact, it is obvious that the main function of the cuticle is protective, being influenced in composition and complexity by the environment and the animal lifestyle (Richards, 1984). *L. culveri* also showed a slightly higher signal in the parapodium integument than in the body (fig. S2). The distinct location of the NKCC along these two nereidids may be related to their varying life strategies and reflect their tolerance and responses to salinity variations, since *A. succinea* is usually found in hard substrata, while *L. culveri* is a tube-dweller of sandy/muddy bottoms. Previous studies on the effects of osmotic stress in these two species pointed out a stronger cell volume regulation and higher euryhalinity of *L. culveri* in comparison with other estuarine and marine annelids (Castellano et al., 2020; Oglesby, 1981).

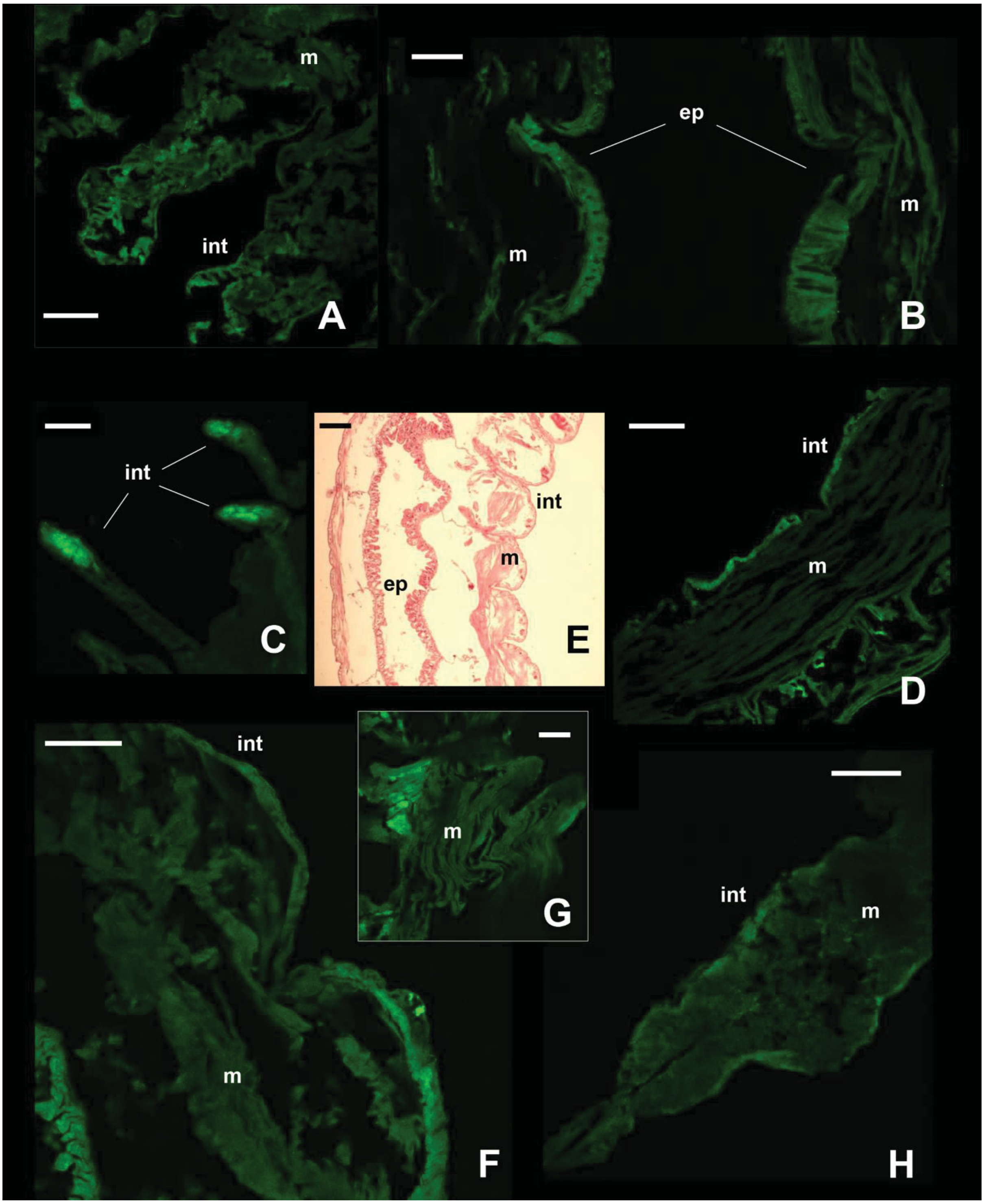

Figure 4. Signal of NKCC in the two euryhaline annelids *A. succinea* and *L. culveri.* Immunofluorescence signal in *A. succinea* is shown in A-D; in *L. culveri*, it is shown in F-H. Abbreviations: ep= internal epithelium; int= integument; m= muscles. E= Sagittal histological section of a specimen of *L. culveri* for comparison with immunofluorescence pictures. *A. succinea*: A= parapodia; B= digestive trait; C= tentacular cirri; D= segments. *L. culveri*: F= segment; G= detail of the muscles; H= parapodium. Scale bars (A-C, D-H): 160 μm; scale bar (E): 500 μm.

The NKCC signal in the nephtyid *N. fluviatilis* was significantly higher in the integument, muscles, and peritoneum than in the negative controls but did not vary among tissues or body parts (tab. S4, fig. 5A-C). Considering the size of the parapodia, which increases from the head to the pygidium, the animals were roughly divided into anterior, central and posterior parts. Interestingly, the intensity of the fluorescence displayed by the posterior portion was statistically the highest among them, while the central portion was the lowest (fig. S3). Taking into account the life strategies of this species, such a pattern may reflect the varying exposure of each body part to varying osmotic stress. In fact, these burrowing animals perform regular vertical movements in the galleries they dig, depending on the tide (Pérez-Torrijos et al., 2009). Thus, they may leave their extremities more exposed to salinity fluctuations than the middle portion of their bodies. At the same time, a higher intensity in both the anterior and posterior parts may suggest other roles for this membrane protein in addition to osmoregulation. It may be potentially involved in processes regarding growth and regeneration, both related to the posterior part of the body (Clark, 1968; Seaver et al., 2005). Moreover, the anterior part of the body bears the brain as well as some sensory organs, such as the nuchal organs, that may require the presence of this protein for neurotransmission. In fact, besides neurotransmission and osmoregulation, a higher expression of NKCC1 was reported for neurogenesis of murines, body development (e.g. connective and olfactory tissues, early embryonal stages of developing heart), and brain injuries in mice (Haering et al., 2015; Hubner et al., 2001; Liang and Huang, 2017).

In contrast with the other species, the intensity of the fluorescence in *I. pulchella* was significantly higher than that in the negative controls only in the branchiae and the internal tissues of either the thorax or the abdomen (fig. 5E-G). This may be the result of both its lifestyle and anatomy. *I. pulchella* is a tube-building suspension feeder that usually occurs among the roots and rhizomes of salt marshes in the polyhaline and euhaline sectors of estuaries. Like other terebellomorphs, the head and the branchiae - located in the first segments of the thorax – are more exposed to salinity fluctuations than other body parts because they are usually placed outside the tube to catch food (Hernández-Alcántara and Solís-Weiss, 2009). The production of mucus may act in this case in a contrasting way. From a physiological point of view, *I. pulchella* may rely on mucus production to buffer the effects of salinity fluctuations along the length of its body, which would explain the absence of the NKCC in the integument. The mucous secretions were already found as an osmoregulatory response in other animals, such as oligochaetes and gastropods (Grimm-Jørgensen et al., 1986; Heredia et al., 2008). On the other hand, its production and presence along the body may have led to a bias in this work. Natural fluorescence was reported for the mucus secreted by some other annelid species (Heredia et al., 2008; Verdes and Gruber, 2017), and the potential autofluorescence of the mucus of *I. pulchella* may have hindered the signal induced by the presence of the NKCC. Supporting this hypothesis, the intensity of the fluorescence of the negative controls of the integument was significantly higher than that of the treatments (fig. S4). Assuming the occurrence of autofluorescence, these results may suggest that the intensity of the NKCC signal was either lower than the mucus signal or completely absent and that the treatments interfered with the autofluorescence itself. However, potential autofluorescence may also be related to the cuticle itself, which is composed of collagenous fibres (Richards, 1984), known for the typical autofluorescence seen mostly throughout the green wavelengths (Jenvey and Stabel, 2017). The highest fluorescence signal was recovered in the inner tissues of the thorax (tab. S5, figs. 5F, S4), which may reflect the position of the excretory system in this family. As with other species belonging to sedentary families (e.g. Sabellidae, Terebellidae), few pairs of protonephromixia are restricted to the anterior part of the body (Goodrich, 1945). In addition, the first nephridiopore in *Isolda* is located in the IV segment between the branchiae (Hernández-Alcántara and Solís-Weiss, 2009; Mcintosh, 1922), highlighting why the signal of the immunofluorescence may have been higher in the first segments of the thorax. Indeed, the isoform NKCC2 was already associated with the excretion system in vertebrates, restricted only to the apical membrane of kidney epithelial cells from the thick ascending limb of Henle’s loop (Russell, 2000). Finally, the NKCC was retrieved as well in the digestive epithelia located in the abdomen (fig. S4), supporting once again the similarity of functions (i.e. uptake of Cl^-) and position of this membrane protein in other taxa, either vertebrates or invertebrates (Castellano et al., 2016; D’Andrea-Winslow et al., 2001; D’Andrea et al., 1996).

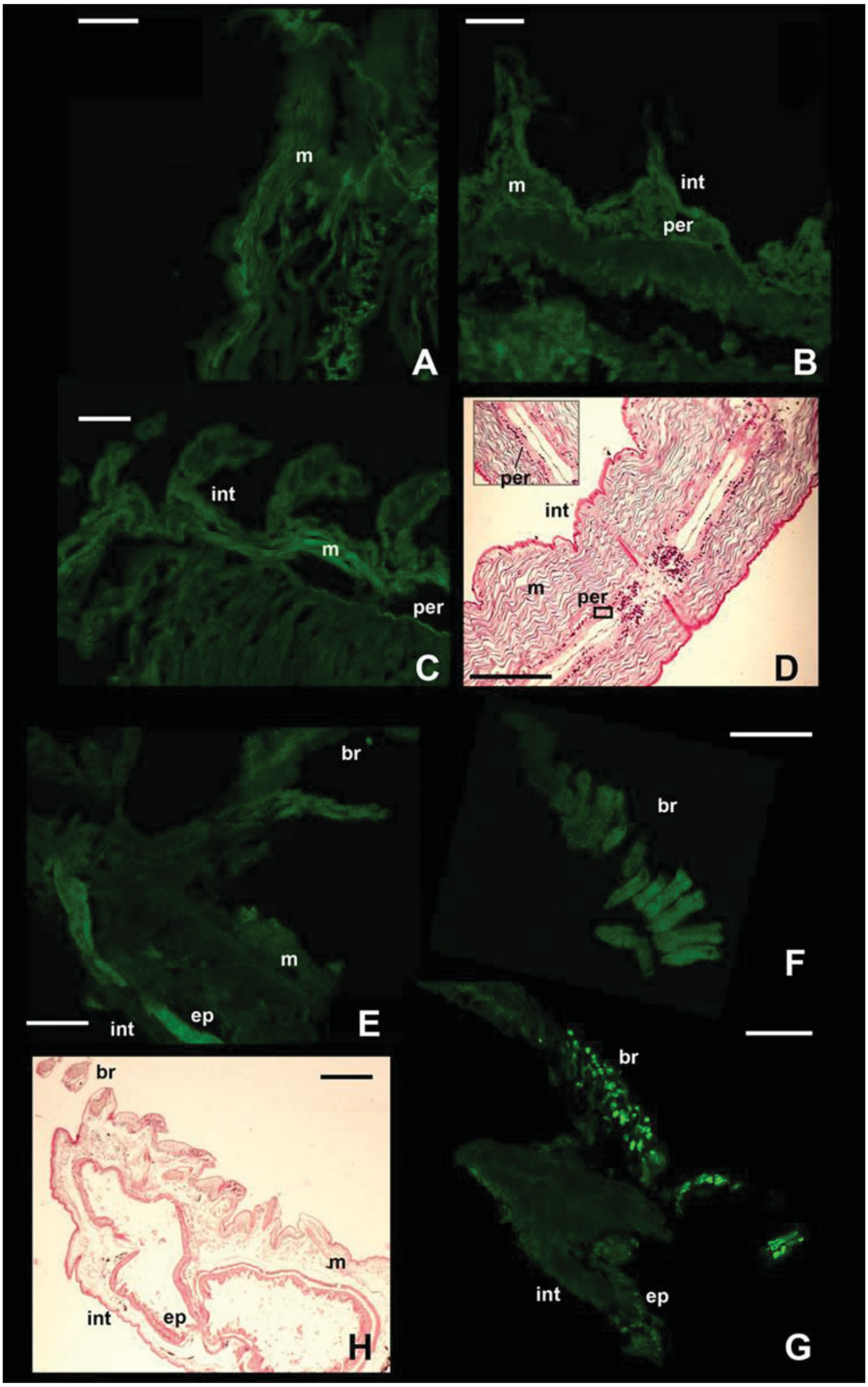

Figure 5. Signal of NKCC in the two stenohaline annelids *N. fluviatilis* and *I. pulchella.* Immunofluorescence signal in *N. fluviatilis* shown in A-C; in *I. pulchella* in E-G. Abbreviations: br= branchiae; ep= internal epithelium; int= integument; m= muscles; per= peritoneum. D, H= Sagittal histological sections of a specimen of *N. fluviatilis* and *I. pulchella*, respectively, for comparison with immunofluorescence pictures. *N. fluviatilis*: A= detail of a segment; B= posterior segments with parapodia; C= central segments with parapodia and peritoneum. *I. pulchella*: E, G= head and first segments of the thorax; F= detail of the branchia. Scale bars (A-H): 160 µm.

4. Conclusions

NKCC, retrieved *in silico* in two species of annelids, has been immunocytochemically confirmed and localized in the bodies of four other species of estuarine annelids, balancing the number of confirmed annelids with this cotransporter between Sedentaria and Errantia (i.e. three species for each clade). Its expression was not homogeneous among the species examined, putatively reflecting their specific ecological challenges with respect to cell volume regulation. The free-living/burrowers (both nereidids and the nephtyid) displayed a more widespread signal for NKCC, in contrast to the stenohaline and sedentary melinnid, which displayed a more limited signal to a few regions of the body. Moreover, the variety of tissues in which the signal was retrieved suggests its centrality to the crucial process of cell volume regulation in essentially conforming invertebrates that dwell in waters of potentially fluctuating salinities, stressing the need for further physiological studies to fully understand the role played by this membrane protein in annelids.

5. Funding

This work was supported by the Brazilian Federal Government Sponsor Agencies CAPES (Doctorate fellowship to SM) and CNPq (Doctorate fellowship to AD, #141565/2017-9, and Research grant to CAF, #302829/2015-6).

6. Acknowledgments

The first author wishes to thank MSc Ricardo Castro Álvarez for helpful suggestions on the biology of the target species. The authors thank the Centro de Tecnologias Avançadas em Fluorescência of Universidade Federal do Paraná for allowing access to their confocal microscope.

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CHAPTER III

FINDING AQUAPORINS: AN *IN SILICO* ASSESSMENT AND A CASE STUDY FOR ANNELIDS

Manuscript formatted following the guidelines of the journal: Biology of the Cell
ISSN: 0248-4900
Impact Factor: 3.922
Qualis: A3

Finding aquaporins: an *in silico* assessment and a case study for annelids

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Abstract

Background Information: Estuarine animals display a variety of biological adaptations to face marked salinity changes, which are a major driving force in these environments. At the cellular level, the presence of major intrinsic proteins group (MIPs), commonly called aquaporins (AQPs), plays a pivotal role in physiological processes mediating across cell transport of water and small solutes. Such water channels are widespread throughout living organisms, and their structure is conserved. Despite their relevance, the presence of MIPs in annelids has only been mentioned in broad eukaryotes reviews. Herein, we provide the phylogenetic signal and discuss the putative physiological function of MIPs in estuarine annelids based on *in silico* and experimental approaches.

Results: Phylogenetic reconstructions clustered the annelid aquaporins within both orthodox and unorthodox clades but also in aquaglyceroporins. Seven paralogs of orthodox aquaporins were also recovered from the assembled transcriptome of *A. succinea* publicly available, and their presence was experimentally confirmed by RT-PCR. Putative orthologs of the vertebrate AQP1-like, AQP8 and the AQP11-like subfamilies were recovered.

Conclusion: Similarities between annelids and human aquaporins were found in the “key” residues along the sequences, as well as in their three-dimensional structure. We suggested the presence of a most recent common ancestor in each aquaporin subfamily and recurrent independent expansions that greatly diversified the structures and functions of the annelid MIPs.

Significance: We provided evidence on the presence and diversification of the MIPs in annelids. The putative similarity of annelid and vertebrate MIPs may encourage further studies using annelids as novel model organisms in order to better understand osmoregulation in estuarine habitats.

Key-words: Annelids. MIPs phylogeny. Aquaporins. *Alitta succinea.* Osmoregulation.

1. Introduction

Estuaries are highly dynamic systems characterised by large salinity fluctuations due to regular or stochastic events, such as pluviosity, rivers, tides, waves and storms (Potter et al., 2015). In this environment, salinity variation is a major ecological and evolutionary challenges to be faced because of the fast water movements inside or outside animal bodies to maintain or restore the osmotic balance (Velasco et al., 2019). Thus, estuarine organisms generally exhibit a variety of biological adaptations at different levels of organization. For instance, complex excretory systems and less permeable cuticles are common (Goodrich, 1945; Oglesby, 1965; Preston, 2009). At the cellular level, membrane or channel proteins work together to regulate the cell volume. Carrier proteins, for instance, are entitled to transport ions through the plasma membranes. Among them, the Na-K-2Cl (NKCC) cotransporter and Na^+/K^+-ATPase are already known in the literature to play an important role in osmoregulation (Russell, 2000; Castellano et al., 2016; Mucciolo et al., *under rev*). Water permeates the cells by simple diffusion, passing through the lipid bilayer membranes, in the paracellular pathway, but its movement may also be facilitated by aquaporin water channels in the transcellular pathway (Madsen et al., 2015). In fact, the existence of these channels was suggested after comparing the varying permeability of the plasma membrane of red blood cells and renal tubules (Denker et al., 1988; Preston et al., 1992).

The commonly called aquaporins, considered the plumbing system of the cells (Agre et al., 1998), belong to this major intrinsic proteins group (MIPs) and are involved in the transport of water and/or small solutes, such as mainly glycerol but also ammonia, urea, metalloids and carbon dioxide (Laloux et al., 2018). They are present in almost all living organisms, and their tertiary and quaternary structures are highly conserved among taxa (Shapiguzov, 2004; Abascal et al. 2014). Aquaporins associate together along the cell membranes, forming an assemblage of four homotetramers, each one composed of six transmembrane helices, forming a pore (Wang and Tajkhorshid, 2007). Each tetramer presents two main restrictions that act as selectivity filters, the two highly conserved NPA motifs (asparagine-proline-alanine) and the ar/R restriction (three aromatic amino acids and one arginine). These filters are located in different parts of the protein, and the role is to react with the water molecules and to regulate the substrate specificity (Hub and De Groot, 2008). Notwithstanding these shared structural features, their overall primary structure is poorly conserved, displaying only ~30% identity with each other (Ishibashi, 2006). These differences are reflected in the high diversity of the MIP family, which is in constant expansion due to the increase in publicly available complete genomes and transcriptomes. The high diversification of these membrane proteins is thought to be the result of a combination of factors, such as single gene or whole genome duplications, and horizontal gene transfers (Abascal et al., 2014). Reconstructions of phylogenetic relationships among all the existing MIP subfamilies have already been carried out, always retrieving four groups: i) the classical or orthodox AQPs (AQP0, 1, 2, 4, 5, 6) that are associated with water transport, ii) the aquaammoniaporins (AQP8) sometimes included in the orthodox AQPs, iii) the aquaglyceroporins (AQP3, 7, 9, 10), and iv) the unorthodox AQPs (also called superaquaporins) (AQP11–12), found only in vertebrates (e.g. Soto et al., 2012; Abascal et al., 2014; Finn et al., 2014; Finn and Cerdà, 2015; Laloux et al., 2018). Certainly, the diversification of the MIPs appeared to depend on the taxa. For instance, the majority of bacteria present a single copy of only one member of the MIP or one paralog per aquaporin and aquaglyceroporin, while specific subfamilies were retrieved in plants (i.e. PIPs, NIPs, TIPs, SIPs, XIPs, HIPs). Vertebrates present seven paralogs of aquaporins (i.e. 0, 1, 2, 4, 5, 6, 8), four of aquaglyceroporins (i.e. 3, 7, 9, 10), and two of unorthodox aquaporins (i.e. 11, 12). In invertebrate taxa, aquaporins have already been characterized in arthropods (e.g. Yanochko and Yool, 2002; Duchesne et al., 2003; Kaufmann et al., 2005; Ball et al., 2009), nematodes (e.g. Huang et al., 2007), and molluscs (Pieńkowska et al., 2014; Kosicka et al., 2016). It is currently assumed that invertebrate orthologs show high similarity with vertebrates AQP1 and AQP4 (Tomkowiak and Pieńkowska, 2010).

The presence of MIPs was only indirectly suggested in annelids by assessing the change in the body weight of the nereidid *Perinereis* sp. with a water flux regulator (i.e. angiotensin II and angiotensin III) and an aquaporin inhibitor, i.e. tetrachloroaurate (III) (Satou et al., 2005). Another hint was found in clitellate annelid *in silico* (Abascal et al., 2014) but never confirmed later.

Scarce taxon sampling may hide the real abundance and diversity of MIPs in invertebrates. The high diversity of MIPs, and their relevance in the physiological process mediating the transport of water and various small solutes across the cells (Laloux et al., 2018), might play a role in annelid adaptations to salinity variation in estuarine systems. For instance, aquaporins are known to be involved in H_2O_2 transport, a molecule that works as a messenger in many biological processes, such as cell proliferation and differentiation, tissue repair, inflammation, circadian rhythm, and ageing (Sies, 2014). Moreover, other aquaporins are able to facilitate the transport of ammonia and urea, potentially relevant for some osmoregulation processes (Zeuthen et al., 2009), metalloids, ions and gases that can be involved in salt and water uptake in the nephrons (Ortiz and Garvin, 2002; Laloux et al., 2018).

In this work, we investigated the presence of aquaporins in annelids through an *in silico* approach against online databases and experimentally confirmed it in a target estuarine annelid. Considering estuarine annelid diversity and their morphological plasticity, we expect to assess the correlation between MIPs diversification and annelid physiological adaptations.

2. Results and discussion

The blast (i.e. blastp and tblastn) and the textual search of “aquaporin” against Annelida database, retrieved putative MIP sequences of a number of annelid species, i.e. *Capitella teleta* Blake, Grassle and Eckelbarger, 2009 (Capitellidae), the nereidids *Perinereis aibuhitensis* (Grube, 1878), *Platynereis dumerilii* (Audouin and Milne Edwards, 1833) and *Alitta succinea* (Leuckart, 1847), *Helobdella robusta* Shankland, Bissen & Weisblat, 1992 (Clitellata: Glossiphoniidae), to be added to the already known AQP paralogs of the clitellates *Eisenia andrei* (Bouché, 1972) and *Lumbricus rubellus* Hoffmeister, 1843, recovered by Abascal et al. (2014), in a phylogenetic reconstruction of the metazoan MIP family.

The phylogenies of each MIP subfamily reflected the current metazoan tree (Dunn et al., 2014), with two clades: Protostomia, with annelids, molluscs, bryozoans and brachiopods, branching within Lophotrochozoa (fig. 1), and Deuterostomia. No MIP paralogs were retrieved for Ctenophora, Chaetognata, Gnathostomulida, Micrognathozoa, Gastrotricha, Phoronida, Entoprocta, Cycliophora, Rhombozoa, Nematomorpha or Loricifera. No genomes/transcriptomes were found for Onychophora or Kynorincha.

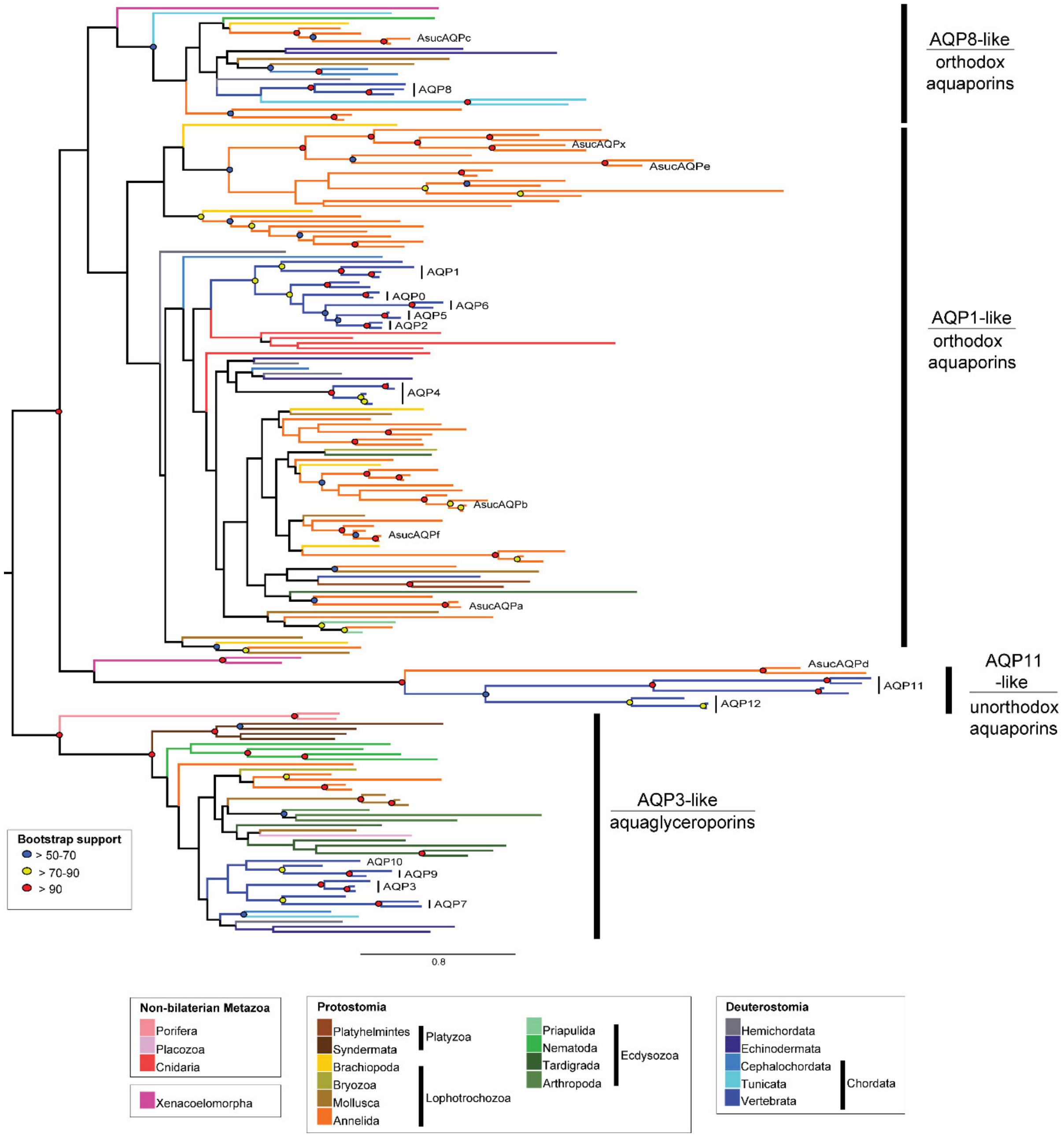


Figure 1. Maximum likelihood phylogenetic reconstruction of metazoan MIPs. Branches of the tree coloured according to taxonomy in the accompanying legend. Labels in the tree refer to *Alitta succinea* AQPs (AsucAQPa, b, c, d, e, f, x) and to vertebrate MIPs (AQP0-12). Blue circles at the nodes indicate bootstrap support > 50-70; yellow circles indicate bootstrap support > 70-90; red circles indicate bootstrap support > 90.

A total of sixty-five MIPs paralogs and/or orthologs were recovered in a number of annelid species, implementing the metazoan phylogeny of Abascal et al. (2014), which reported only three paralogs (figs. 1-2). To date, the only annelid aquaporins included in previous works, *Eisenia andrei* (CAX48970.1) and *Lumbricus* *rubellus* (CAX48991.1 and CAX48991.1), clustered within the orthodox AQP1-like subfamily, such as most of those belonging to other invertebrates (figs. 1-2) (Abascal et al., 2014; Pieńkowska et al., 2014). Indeed, the structure, function and body localization of the aquaporins of insects and molluscs have already been recognised as putative orthologs of mammalian AQP1 and AQP4 (Tomkowiak and Pieńkowska, 2010; Pieńkowska et al., 2014). Their wide distribution along the bodies and the more basal position of these aquaporins in the clade grouping all the vertebrate orthodox ones (i.e. AQP0, 1, 2, 4, 5 and, 6), may explain the similarity between vertebrate AQP1 and AQP4 with the invertebrate ones.

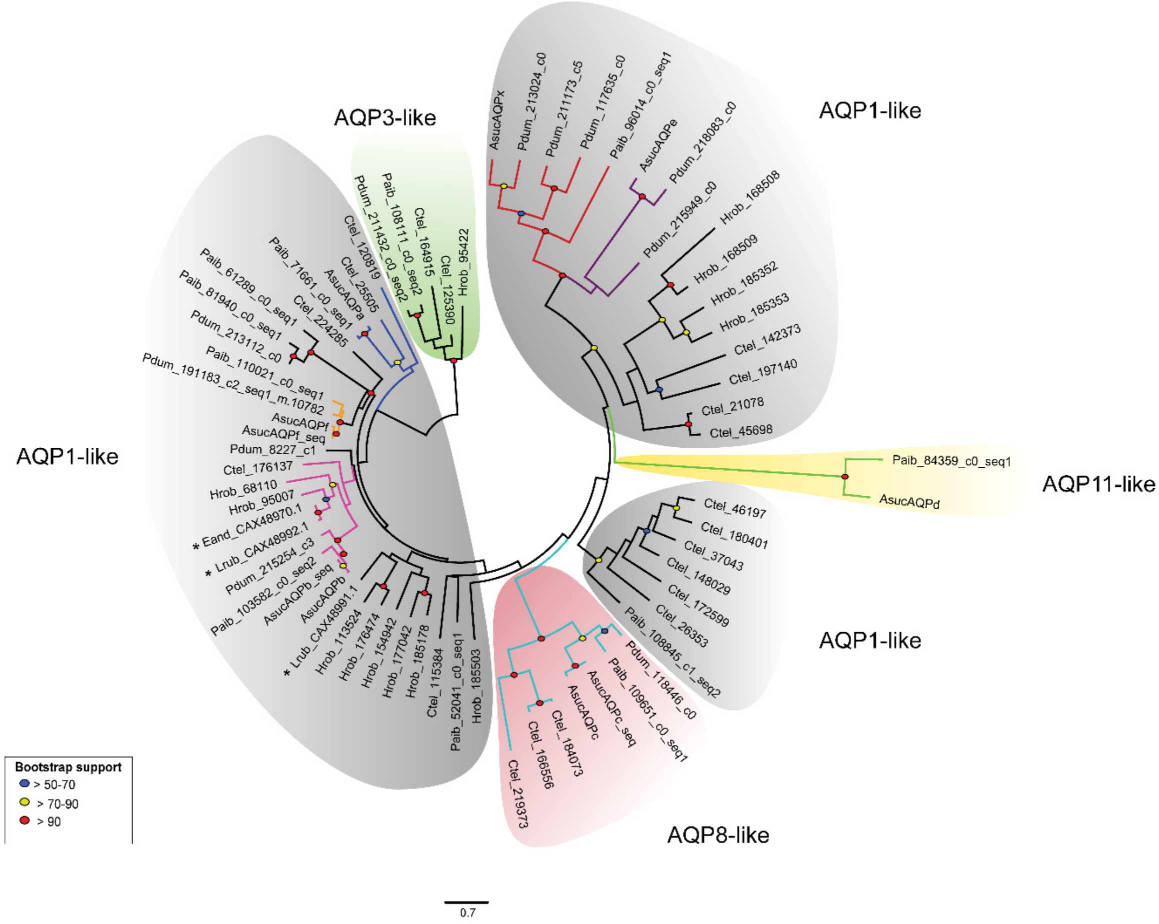
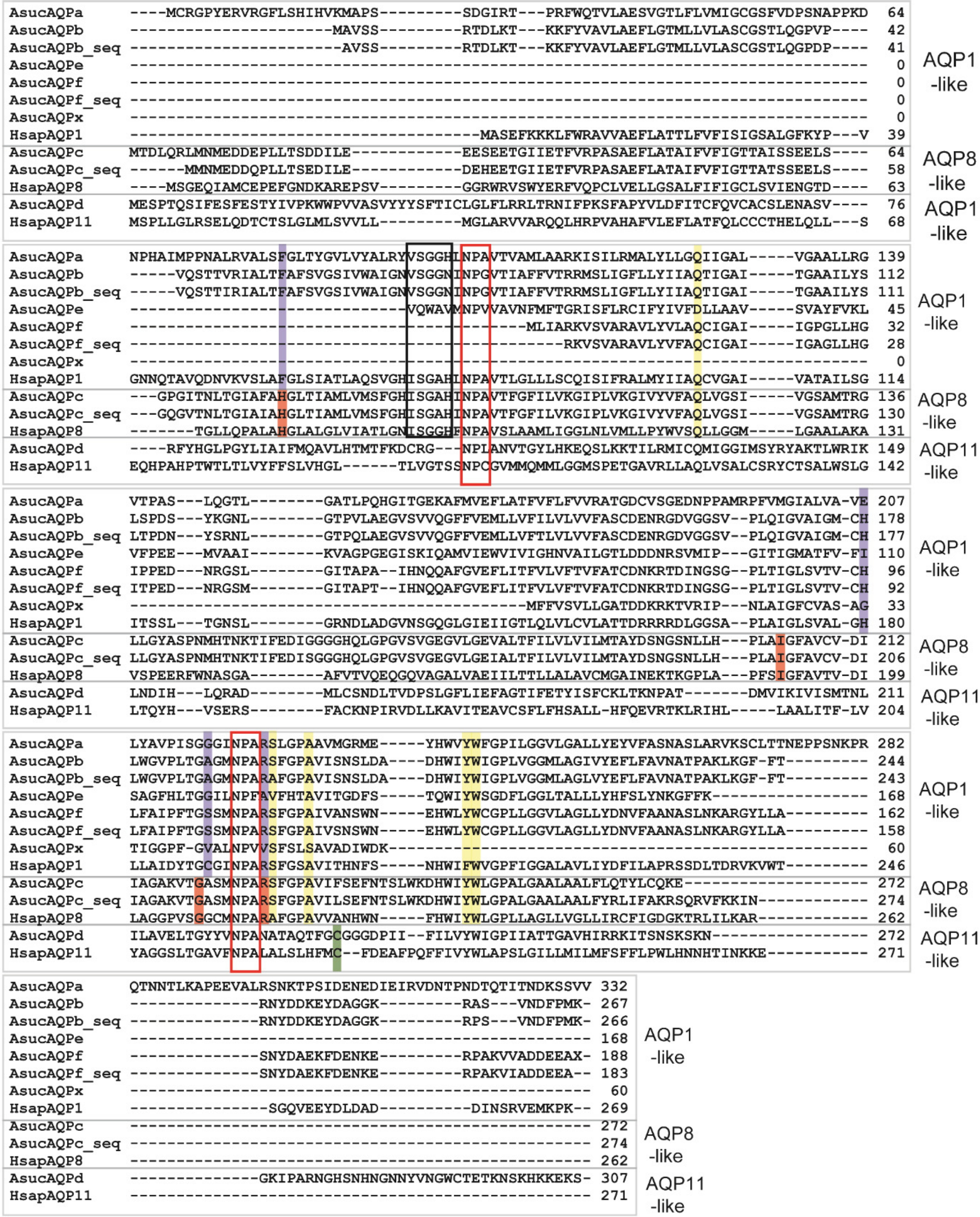


Figure 2. Maximum likelihood phylogenetic reconstruction of MIPs in annelids. Coloured branches represent clades of each AQP recovered in *Alitta succinea* transcriptome. Blue circles at the nodes indicate bootstrap support > 50-70; yellow circles indicate bootstrap support > 70-90; red circles indicate bootstrap support > 90. Asterisks refer to annelids included in MIPs phylogeny of Abascal et al. 2014. Asuc = *A. succinea*; Ctel = *Capitella teleta*; Eand = *Eisenia andrei*; Hrob = *Helobdella robusta*; Lrub = *Lumbricus rubellus*; Paib = *Perinereis aibuhitensis;* Pdum = *Platynereis dumerilii*. Accession numbers in table S1.

A total of seven putative aquaporin paralogs were assembled in the transcriptome of *A. succinea* from Kocot et al. (2016) (fig. 3). Our findings reflected the subdivision of MIPs into four groups, as proposed by Soto et al. (2012) (figs. 1-2). Accordingly, animal MIPs may be distinguished depending on their sequence identity in: i) orthodox aquaporins or AQP1-like (vertebrate AQP0, 1, 2, 4, 5 and 6; *A. succinea* AsucAQPa, b, e, f and x), ii) aquaamoniaporins, or AQP8-like (i.e. vertebrate AQP8; *A. succinea* AsucAQPc), iii) aquaglyceroporins, or AQP3-like (vertebrate AQP3, 7, 9 and 10; annelids e.g. Hrob95422), iv) superaquaporins, or AQP11-like (vertebrate AQP11 and 12A/B; *A. succinea* AsucAQPd). Most of the recovered annelid aquaporin paralogs (i.e. AsucAQPa, b, e, f and x) clustered in the orthodox subfamily as sister groups of deuterostomes, suggesting episodes of whole genome duplication at the early stages of their evolutionary history (Abascal et al., 2014).



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AsucAQPa     ----MCRGPYERVRGFLSHIHVKMAPS---------SDGIRT---PRFWQTVLAESVGTLFLVMIGCGSFVDPSNAPPKD  64
AsucAQPb     ----------------------MAVSS---------RTDLKT---KKFYVAVLAEFLGTMLLVLASCGSTLQGPVP----  42
AsucAQPb_seq -----------------------AVSS---------RTDLKT---KKFYVAVLAEFLGTMLLVLASCGSTLQGPDP----  41
AsucAQPe     --------------------------------------------------------------------------------   0
AsucAQPf     --------------------------------------------------------------------------------   0
AsucAQPf_seq --------------------------------------------------------------------------------   0
AsucAQPx     --------------------------------------------------------------------------------   0
HsapAQP1     --------------------------------------MASEFKKKLFWRAVVAEFLATTLFVFISIGSALGFKYP---V  39
AsucAQPc     MTDLQRLMNMEDDEPLLTSDDILE------------EESEETGIIETFVRPASAEFLATAIFVFIGTTAISSEELS----  64
AsucAQPc_seq ------MMNMEDDQPLLTSEDILE------------DEHEETGIIETFVRPASAEFLATAIFVFIGTTATSSEELS----  58
HsapAQP8     ----MSGEQIAMCEPEFGNDKAREPSV---------GGRWRVSWYERFVQPCLVELLGSALFIFIGCLSVIENGTD----  63
AsucAQPd     MESPTQSIFESFESTYIVPKWWPVVASVYYYSFTICLGLFLRRLTRNIFPKSFAPYVLDFITCFQVCACSLENASV----  76
HsapAQP11    MSPLLGLRSELQDTCTSLGLMLSVVLL---------MGLARVVARQQLHRPVAHAFVLEFLATFQLCCCTHELQLL---S  68

AsucAQPa     NPHAIMPPNALRVALSFGLTYGVLVYALRYVSGGHINPAVTVAMLAARKISILRMALYLLGQIIGAL-----VGAALLRG 139
AsucAQPb     -----VQSTTVRIALTFAFSVGSIVWAIGNVSGGNINPGVTIAFFVTRRMSLIGFLLYIIAQTIGAI-----TGAAILYS 112
AsucAQPb_seq -----VQSTTIRIALTFAFSVGSIVWAIGNVSGGNINPGVTIAFFVTRRMSLIGFLLYIIAQTIGAI-----TGAAILYS 111
AsucAQPe     ------------------------------VQWAVMNPVVAVNFMFTGRISFLRCIFYIVFDLLAAV-----SVAYFVKL  45
AsucAQPf     -------------------------------------------MLIARKVSVARAVLYVLAQCIGAI-----IGPGLLHG  32
AsucAQPf_seq -----------------------------------------------RKVSVARAVLYVFAQCIGAI-----IGAGLLHG  28
AsucAQPx     --------------------------------------------------------------------------------   0
HsapAQP1     GNNQTAVQDNVKVSLAFGLSIATLAQSVGHISGAHINPAVTLGLLLSCQISIFRALMYIIAQCVGAI-----VATAILSG 114
AsucAQPc     ---GPGITNLTGIAFAHGLTIAMLVMSFGHISGAHINPAVTFGFILVKGIPLVKGIVYVFAQLVGSI-----VGSAMTRG 136
AsucAQPc_seq ---GQGVTNLTGIAIAHGLTIAMLVMSFGHISGAHINPAVTFGFILVKGIPLVKGIVYVFAQLVGSI-----VGSAMTRG 130
HsapAQP8     -------TGLLQPALAHGLALGLVIATLGNISGGHFNPAVSLAAMLIGGLNLVMLLPYWVSQLLGGM-----LGAALAKA 131
AsucAQPd     ----RFYHGLPGYLIAIFMQAVLHTMTFKDCRG---NPLANVTGYLHKEQSLKKTILRMICQMIGGIMSYRYAKTLWRIK 149
HsapAQP11    EQHPAHPTWTLTLVYFFSLVHGL------TLVGTSSNPCGVMMQMMLGGMSPETGAVRLLAQLVSALCSRYCTSALWSLG 142

AsucAQPa     VTPAS---LQGTL--------GATLPQHGITGEKAFMVEFLATFVFLFVVRATGDCVSGEDNPPAMRPFVMGIALVA-VE 207
AsucAQPb     LSPDS---YKGNL--------GTPVLAEGVSVVQGFFVEMLLVFILVLVVFASCDENRGDVGGSV--PLQIGVAIGM-CH 178
AsucAQPb_seq LTPDN---YSRNL--------GTPQLAEGVSVVQGFFVEMLLVFTLVLVVFASCDENRGDVGGSV--PLQIGVAIGM-CH 177
AsucAQPe     VFPEE---MVAAI--------KVAGPGEGISKIQAMVIEWVIVIGHNVAILGTLDDDNRSVMIP---GITIGMATFV-FI 110
AsucAQPf     IPPED---NRGSL--------GITAPA--IHNQQAFGVEFLITFVLVFTVFATCDNKRTDINGSG--PLTIGLSVTV-CH  96
AsucAQPf_seq ITPED---NRGSM--------GITAPT--IHNQQAFGVEFLITFVLVFTVFATCDNKRTDINGSG--PLTIGLSVTV-CH  92
AsucAQPx     -------------------------------------------MFFVSVLLGATDDKRKTVRIP---NLAIGFCVAS-AG  33
HsapAQP1     ITSSL---TGNSL--------GRNDLADGVNSGQGLGIEIIGTLQLVLCVLATTDRRRRDLGGSA--PLAIGLSVAL-GH 180
AsucAQPc     LLGYASPNMHTNKTIFEDIGGGGHQLGPGVSVGEGVLGEVALTFILVLVILMTAYDSNGSNLLH---PLAIGFAVCV-DI 212
AsucAQPc_seq LLGYASPNMHTNKTIFEDISGGGHQLGPGVSVGEGVLGEIALTFILVLVILMTAYDSNGSNLLH---PLAIGFAVCV-DI 206
HsapAQP8     VSPEERFWNASGA--------AFVTVQEQGQVAGALVAEIILTTLLALAVCMGAINEKTKGPLA---PFSIGFAVTV-DI 199
AsucAQPd     LNDIH---LQRAD--------MLCSNDLTVDPSLGFLIEFAGTIFETYISFCKLTKNPAT-------DMVIKIVISMTNL 211
HsapAQP11    LTQYH---VSERS--------FACKNPIRVDLLKAVITEAVCSFLFHSALL-HFQEVRTKLRIHL-----LAALITF-LV 204

AsucAQPa     LYAVPISGGGINPARSLGPAAVMGRME-----YHWVYWFGPILGGVLGALLYEYVFASNASLARVKSCLTTNEPPSNKPR 282
AsucAQPb     LWGVPLTGAGMNPARSFGPAVISNSLDA----DHWIYWIGPLVGGMLAGIVYEFLFAVNATPAKLKGF-FT--------- 244
AsucAQPb_seq LWGVPLTGAGMNPARAFGPAVISNSLDA----DHWIYWIGPLVGGMLAGLVYEFLFAVNATPAKLKGF-FT--------- 243
AsucAQPe     SAGFHLTGGILNPFAVFHTAVITGDFS-----TQWIYWSGDFLGGLTALLLYHFSLYNKGFFK----------------- 168
AsucAQPf     LFAIPFTGSSMNPARSFGPAIVANSWN-----EHWLYWCGPLLGGVLAGLLYDNVFAANASLNKARGYLLA--------- 162
AsucAQPf_seq LFAIPFTGSSMNPARSFGPAIVSNSWN-----EHWLYWCGPLLGGVLAGLLYDNVFAANASLNKARGYLLA--------- 158
AsucAQPx     TIGGPF-GVALNPVVSFSLSAVADIWDK----------------------------------------------------  60
HsapAQP1     LLAIDYTGCGINPARSFGSAVITHNFS-----NHWIFWVGPFIGGALAVLIYDFILAPRSSDLTDRVKVWT--------- 246
AsucAQPc     IAGAKVTGASMNPARSFGPAVIFSEFNTSLWKDHWIYWLGPALGAALAALFLQTYLCQKE-------------------- 272
AsucAQPc_seq IAGAKVTGASMNPARSFGPAVILSEFNTSLWKDHWIYWLGPALGAALAALFYRLIFAKRSQRVFKKIN------------ 274
HsapAQP8     LAGGPVSGGCMNPARAFGPAVVANHWN-----FHWIYWLGPLLAGLLVGLLIRCFIGDGKTRLILKAR------------ 262
AsucAQPd     ILAVELTGYYVNPANATAQTFGCGGGDPII--FILVYWIGPIIATTGAVHIRRKITSNSKSKN----------------- 272
HsapAQP11    YAGGSLTGAVFNPALALSLHFMC--FDEAFPQFFIVYWLAPSLGILLMILMFSFFLPWLHNNHTINKKE----------- 271

AsucAQPa     QTNNTLKAPEEVALRSNKTPSIDENEDIEIRVDNTPNDTQTITNDKSSVV 332
AsucAQPb     --------------RNYDDKEYDAGGK---------RAS---VNDFPMK- 267
AsucAQPb_seq --------------RNYDDKEYDAGGK---------RPS---VNDFPMK- 266
AsucAQPe     -------------------------------------------------- 168
AsucAQPf     --------------SNYDAEKFDENKE---------RPAKVVADDEEAX- 188
AsucAQPf_seq --------------SNYDAEKFDENKE---------RPAKVIADDEEA-- 183
AsucAQPx     --------------------------------------------------  60
HsapAQP1     ---------------SGQVEEYDLDAD----------DINSRVEMKPK-- 269
AsucAQPc     -------------------------------------------------- 272
AsucAQPc_seq -------------------------------------------------- 274
HsapAQP8     -------------------------------------------------- 262
AsucAQPd     --------------GKIPARNGHSNHNGNNYVNGWCTETKNSKHKKEKS- 307
HsapAQP11    -------------------------------------------------- 271
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Figure 3. Multiple alignment of predicted MIPs in *Alitta succinea* and human AQP1 (P29972), AQP8 (XP_011544124.1) and AQP11 (Q8NBQ7). In red boxes the two NPA motifs. The black box indicates the ISSGH sequence, typical of the AQP1-like subfamily. The ar/R regions of both the AQP1-like and AQP8-like subfamilies are shaded in blue and red, respectively. The Cys in the C-terminal NPA boxes of the AQP11-like subfamily are shaded in green. The positions P1-P5 of the AQP1-like subfamily are shaded in yellow.

AsucAQPa, AsucAQPb, and AsucAQPf, together with the majority of the aquaporins belonging to the protostomes analysed here, grouped within the clade of the vertebrate AQP1-like subfamily (fig. 1). These aquaporins, as most orthodox ones, present a very conserved ar/R region with a typical aromatic phenylalanine (Phe or F), ISSGH sequence and P1-P5 residues, which all play an important role in their structures and functions (Froger et al., 1998; Calvanese et al., 2013; Pieńkowska et al., 2014). AsucAQPa and AsucAQPb presented the ISGGH sequence located upstream of the first NPA motif with a substitution in the first position with the valine (Val or V) (fig. 3). Analysis of the five polypeptides chain (P1-P5) retrieved the residues glutamine-serine-alanine-phenylalanine-tryptophan (Q/S-A-F-W) (fig. 3). The ar/R region of these aquaporins displayed substitutions in the second and third residues in comparison with mammalian AQP1 (fig. 3). AsucAQPa was formed by the amino acids (aa) Phe, glutamine (Gln or Q), cysteine (Cys or C), and arginine (Arg or R), presenting a substitution in the second position compared with mammalian AQP1, which displays a histidine (His or H) (fig. 3). The AsucAQPb ar/R filter was formed by Phe, His, glycine (Gly or G), and Arg, while mammalian AQP1 has Cys in the third position (fig. 3). Similar types of substitutions in aquaporins of plants or animals such as *Arabidopsis thaliana* (L.) Heynh and *Homo sapiens* Linnaeus 1758 were found in the ISSGH sequences, like for the Gln, which was retrieved in this study as the residue in P1, and usually coexists with the threonine (Thr or T) instead (Pieńkowska et al., 2014). Some freshwater pulmonated gastropods, such as *Lymnaea stagnalis* (Linnaeus, 1758), *Stagnicola palustris* (O.F. Muller, 1774), and *Ladislavella occulta* (Jackiewicz, 1959) showed similar substitutions among amino acids with similar chemical nature, with Gln instead of Thr (both polar aa) in their putative aquaporins (i.e. LsAQP1, SpAQP1 and CoAQP1), which were already classified as paralogs of the AQP1-like subfamily, suggesting that these replacements do not affect the functioning of the water channel (Pieńkowska et al., 2014).

AsucAQPb and AsucAQPf were shared only among annelids, while AsucAQPe and AsucAQPx were recovered in lophotrochozoan taxa, as sister groups of the orthodox aquaporin clade (fig. 1). Nevertheless, it is not rare to report the existence of aquaporin paralogs exclusive to some taxa. For instance, there are aquaporins described as typical of molluscs, such as Maqp and Mglp (Kosicka et al., 2016), or of insects, such as DRIP, PRIP, and BIB (Campbell et al., 2008). Considering the diversity of the Lophotrochozoa clade, inhabiting marine, freshwater, or terrestrial environments, with different functional traits and body plans (Halanych, 2016), it is tempting to say that the high AQPs diversification is related to the physiological adaptation, such as osmoregulation and desiccation. Moreover, considering osmoregulation, contrarily to vertebrates, which rely on the nephronic system, Lophotrochozoa usually displays different protective mechanisms, such epidermis, nephridia, and Malpighian tubules, which may lead to further specialization of some proteins (Campbell et al., 2008). To support the hypothesis that the annelid AQPs play a role in the adaptations to the salinity variation in the estuarine system, the aquaporins recovered in the present work displayed a high number of substitutions in the NPA motifs, and the NPG box in the N-terminal (shared only in annelids and previously known only for plants in the C-terminal), found in AsucAQPb, which may influence the pore size of the aquaporin and its substrate selectivity (Ishibashi et al., 2011)

Phylogenetic reconstructions placed AsucAQPc as the ortholog of vertebrate AQP8 (fig. 1). The ar/R region of AsucAQPc was formed by His, isoleucine (Ile or I), Gly, and Arg, which are also the same aa present in mammalian AQP8 (fig. 3). Structurally different from either orthodox or aquaglyceroporins, this aquaporin transports other substrates than water, such as free radicals, such as H_2O_2 (e.g. Liu et al., 2006). Moreover, the contribution of this protein to ammonia transport may be fundamental for osmoregulation, helping in regulating the bodily salt/water balance of the annelids. For instance, this protein was also reported to increase the water permeability of membranes by redistributing intracellular vesicles to the plasma membrane in vertebrates (Garcia et al., 2001). The invertebrate *Caenorhabditis elegans* (Maupas, 1900) (Nematoda) showed as well AQP8 involvement in response to either hyper- or hypoosmotic stress by increasing vesicle docking to the lumen of excretory cells and promoting water and osmotic active substance transport to maintain intracellular homeostasis (Igual Gil et al., 2017). Putative orthologs of AQP8 have already been found in plants (i.e. TIPs). In fact, some authors have already debated whether the molecular similarities of these proteins were synapomorphies, supporting the hypothesis of a most recent common ancestor for both animal and plant aquaporins (Soto et al., 2012), or if this was the result of analogous functions in animals and plants that led through natural selection to minimal sequence convergence (Abascal et al., 2014). Unlike Abascal et al. (2014), who recovered among the invertebrates only the nematodes within the clade of AQP8, our study showed how orthologs of this aquaporin are present in almost every analysed taxon.

This suggests that this aquaporin was missing in other invertebrate taxa because of a bias due to lack of taxonomic representation in molecular databases, prompting the need of a better coverage.

AsucAQPd was retrieved as a putative ortholog of vertebrate AQPs11-like (fig. 1), with which shared Cys in the ninth position after the second NPA motif, considered the signature of this subfamily (Ishibashi et al. 2011). Nonetheless, its presence in annelids is debatable. The long branches and a sister group position in the phylogeny considering all the non-aquaglyceroporins reflect the high divergence of vertebrate unorthodox aquaporins from the other aquaporins in structure and function. The similarity of these aquaporins with intracellular SIPs – an aquaporin clade typical of plants, and a potential ancestral gene common to both clades was suggested (Soto et al., 2012). However, when analysing the sequences of the SIPs, no conserved residues of AQP11 were found, pointing out that the association within the same clade is more likely a long branch artifact rather than a real relationship of orthology (Abascal et al., 2014). On the other hand, high variability in the overall aquaporin primary structures and substitutions within the NPA motifs is common in invertebrates (Kosicka et al., 2020), thus potentially explaining the great difference of our AsucAQPd compared with the already known AQP11 and 12. Moreover, a few studies have also suggested the presence of these orthologs in other invertebrates, such as molluscs and nematodes (Ishibashi et al., 2011; Kosicka et al., 2016).

No aquaglyceroporins were recovered in the *A. succinea* transcriptome; however, their presence was retrieved in both the nereidids *Perinereis aibuhitensis* and *Platynereis dumerilii,* as well as in the other annelids and invertebrates. Their presumed absence may be related to sequencing errors in their transcriptome. Indeed, the presence of glycerol facilitators was associated with an adjustment of membrane water permeability during hypoosmotic stress (Luyten et al., 1995; Beese et al., 2009). The importance of these water channels is also highlighted by their presence in a wide range of taxa from bacteria and archaea through all domains and kingdoms.

The presence of three aquaporins was then confirmed in *A. succinea* by sequencing, namely, AsucAQPb, AsucAQPc, and AsucAQPf. The amplification of these genes occurred only in fed animals. The starvation faced by some of our annelids, coupled with their potentially different life stages, may have influenced the expression of the MIPs, explaining why we were able to recover three aquaporins by RT-PCR, only in fed animals (Skowronski et al., 2016; Chou et al., 2018). The entire open reading frames (ORF) that encoded for AsucAQPb was of 266 aa. A comparison of this AQP with the assembled one, recovered seven aa of difference, i.e. 95.86% of aa sequence identity (fig. 2-3). The length of the entire ORF of AsucAQPc was of 274 aa, showing the presence of 24 aa substitutions when compared to the *in silico* one, i.e. 95.08% aa sequence (fig. 2-3). Finally, the partial ORF that encoded for AsucAQPf was 183 aa in length, differing for aa substitutions in seven different sites, i.e. 95.86% aa sequence identity compared with the assembled one (fig. 2-3).

High similarity among the tertiary structures of the annelid aquaporin paralogs was recovered when compared with the vertebrate AQP4. They consist of six transmembrane domains plus two additional membrane embedded α-helices, displaying the typical hourglass shape of MIPs (fig. 4).

Finally, the diversity of the annelid MIPs as well as the presence of more than one annelid paralog within the same clade, retrieved by analysing their transcriptomes as *P. dumerilii* within AsucAQPe and x clades, may be the result of several recent gene duplications. These events are common in adaptive processes and may lead to co-option and to a consequently functional diversification of the genes through changes in gene regulatory levels or in parts of the amino acid sequence not required for the current function (True and Carroll, 2002). This is the case of the ortholog AQP4 of some insects (i.e. Holometabola), which evolved in a glycerol transporter, after a single mutation of His174 from the ar/R region with Ala174 (Finn et al., 2015).

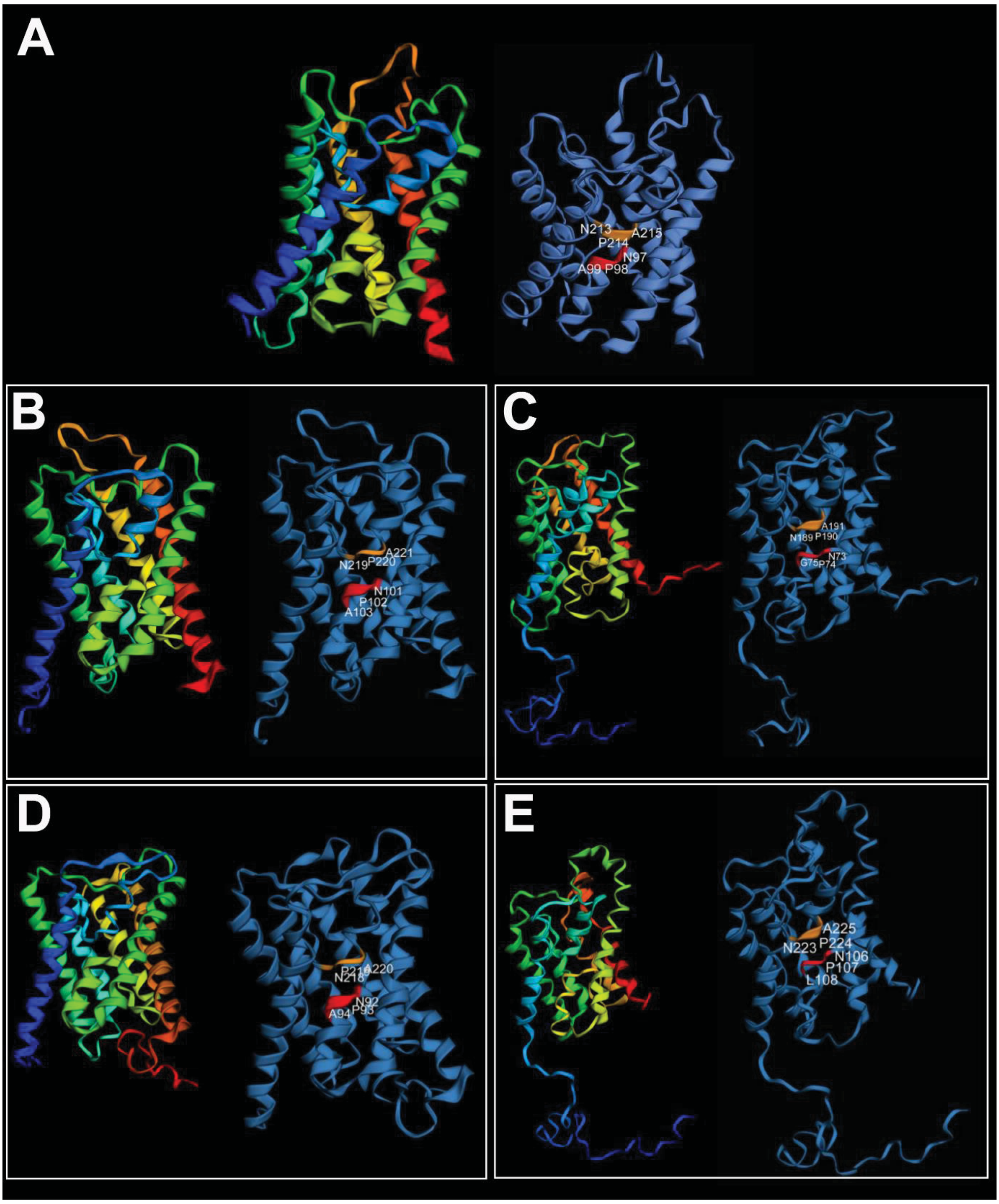

Figure 4. Three-dimensional structure of: (A) the *Homo sapiens* AQP4 (PDB ID: 3GD8), used as template; (B) AsucAQPa; (C) AsucAQPb; (D) AsucAQPc; (E) AsucAQPd. On the left, aquaporin structures coloured in rainbow indicate N-C terminal, from blue to red. On the right, NPA motifs are indicated in red and orange.

3. Conclusion

The widespread presence and abundance of MIPs are the result of their fundamental role in a variety of biological processes, such as body water homeostasis. We reconstructed, for the first time, the phylogeny of annelid MIPs, and confirmed the presence of three putative AQPs previously recovered *in silico* by RT-PCR. Our findings were congruent with previous studies concerning the phylogeny of MIPs across metazoans, supporting the hypothesis of a most recent common ancestor in each subfamily between invertebrates and vertebrates, emphasized by the presence of highly conserved "key" aa sequences in common with vertebrate MIPs. Here, we inferred the physiological roles of annelid aquaporins on the basis of models already known or applied to vertebrates. However, further studies on the water channels of invertebrates are highly advisable to explore how different combinations of aa along the sequence may influence the permeability of aquaporins and to better understand the osmoregulatory physiology of these soft-body animals.

4. Materials and Methods

4.1 Databases mining and phylogenetic analyses

Textual search of "aquaporin" using the gene search tool and blasts, i.e. blastp and tblastn (Altschul et al., 1990), using the sequences of all the human MIPs as query (tab. S1), were performed against Annelida on JGI (Grigoriev et al., 2012; visited between November 2018 and January 2019).

Vertebrate MIPs sequences were selected from Abascal et al. 2014. Then, human MIPs were used as queries to perform also blastp and tblastn against each metazoan taxon (including Annelida) on various online databases present in GenBank: nr, proteins, and TSA (Benson et al., 2015; visited between January 2019 and November 2020). Several tblastn against the *Alitta succinea* transcriptome retrieved from Kocot et al. (2016) were performed using both the human and annelid *Capitella teleta* putative MIPs as queries. MIPs from *Homo sapiens* and *A. succinea* were used to recover sequences of the annelid *Platynereis dumerilii* on PdumBase (Chou et al., 2018). Sequences with an e-value of at least 10^{-2} were downloaded. Invertebrate sequences were then reverse blasted against Annelida on GenBank proteins to increase the chance of obtaining more paralogs of annelid MIPs. All accession numbers of the sequences used are listed in the supplementary material (tab. S1).

Transcriptome assemblies of *A. succinea* were performed using Geneious Prime (http://www.geneious.com) assembling the fragments from the overlapping parts or using the putative aquaporin of *Perinereis aibuhitensis* as a model (GenBank TSA Paib_110021_c0_seq1). Multiple sequence alignments of only annelid MIPs and of metazoan MIPs were carried out with MUSCLE (Edgar, 2004) as implemented in SeaView X (Gouy et al., 2010) and manually checked. The N- and C-terminal parts of the sequences were trimmed to the last well-aligned stretch. Multiple sequence alignments of the metazoan and annelid MIP sequences are shown in supplementary data S2-S3. Maximum likelihood (ML) trees were inferred using PhyML (Guindon et al., 2010), and the robustness of the nodes was estimated by 100 bootstrap replicates. The best substitution model was tested with the SMS routine in PhyML using both AIC and BIC as optimality criteria (Lefort et al., 2017), LG +G+F was selected for the metazoan alignment, and LG +G+I+F for the annelids. The trees were edited with FigTree (Rambaut, 2010). In accordance with previous phylogenetic analyses, the phylogenies were rooted using aquaglyceroporins, which formed a highly supported (100% bootstraps) monophyletic group (Zardoya, 2005; Abascal et al., 2014).

4.2 Sampling

The target species was the estuarine *Alitta succinea* (Annelida: Nereididae), which is usually associated with hard and human-made substrates (Villalobos-Guerrero and Carrera Parra, 2015). Sampling was carried out in Lesina Lake, located on the coast of the southwestern Adriatic Sea in Italy. The lake is subjected to marked salinity variations due to the limited connections with seawater and periodic freshwater inputs, with a salinity range of 11 - 34 psu and a temperature range of 7 – 26°C depending on the season (Spagnoli and Andresini, 2018, Manini et al., 2005). Thirty specimens were sampled in mud sediment using an Ekman manual grab sampler. In the laboratory, half of the animals were acclimated for 48 h under constant temperature (~20°C), aeration and natural photoperiod in two plastic containers of 1 l with the water and the sediment collected in the sampling area. The remaining half was separated into 2.5 ml falcon tubes with the same water but without substrate (i.e. food).

4.3 Cloning of A. succinea AQPs

Total RNA was extracted from the central portion of the body of frozen worms, homogenizing two/three specimens (~100 mg of tissue) in Trizol (Invitrogen), following the manufacturer's protocol. Reverse transcription of the total RNA was performed with Super Transcript III (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. Amplifications of the cDNA were carried out using primers designed *ad hoc* starting from the *A. succinea* assembled transcriptome of Kocot et al. (2016) (tab. 1). Amplifications were performed in a 25 µl reaction volume containing 20 mM PCR buffer, 0.5 µM forward and reverse primers, 0.2 mM of each dNTP, 0.25 U/µl DreamTaq (Thermo Fisher Scientific), 3-8 µl of template cDNA, and nuclease-free water to bring the mix to a final volume of 25 µl. The amplification conditions for each primer pair are listed in the table 2. The amplicons obtained were ligated into the pcrII TOPO vector by TOPO TA cloning kit (Thermo Fisher Scientific) and cloned into One Shot TOP10 *Escherichia coli* competent cells (Thermo Fisher Scientific). Vectors from positively screened clones were isolated and sequenced according to the Sanger method at Eurofins Genomics (Ebersberg, Germany) or Microsynth AG (Switzerland). Sequence quality checks, assembly, and comparisons to the predicted gene annotations were carried out with Geneious Prime (http://www.geneious.com).

Table 1. Primers list and the relative PCR thermal conditions for cloning *A. succinea* AQPs. *Primers not designed because the AQP prediction was < 300 bp.

Gene	**Primer**		**bp predicted amplified fragments**	**PCR thermal cycling conditions**
AsucAQPa	Afor	CCATCAGTCACTTGTTGCCC	1116	1) 95°C (3 min), 2) 30 cycles: 95°C (30 s), 54°C (30 s), 72°C (2 min), 3) 72°C (10 min)
	Arev	CTGAACTTTTATCATTGGTGATAAGG		
AsucAQPb	Bfor	GCCCTCATCATCAGCTAAAATG	1042	1) 95°C (3 min), 2) 30 cycles: 95°C (30 s), 54°C (30 s), 72°C (2 min), 3) 72°C (10 min)
	Brev	GACATCCCTCCCTGGTGATT		
AsucAQPc	Cfor	GAGGGGTGTCACAGTCTAATG	1022	1) 95°C (3 min), 2) 30 cycles: 95°C (30 s), 55°C (30 s), 72°C (2 min), 3) 72°C (10 min)
	Crev	CCATTCAGCAATGCGGAGTAAC		
AsucAQPd	Dfor	CTGCTACTAATGGACAAAACTGTCA	933	1) 95°C (3 min), 2) 30 cycles: 95°C (30 s), 55°C (30 s), 72°C (2 min), 3) 72°C (10 min)
	Drev	CTTCTGATATGGACAGCTCCTG		
AsucAQPe	Efor	CAATGGGCAGTCATGAATCCTG	462 (incomplete)	1) 95°C (3 min), 2) 30 cycles: 95°C (30 s), 55°C (30 s), 72°C (2 min), 3) 72°C (10 min)
	Erev	GAGCAGAGCTGTAAGGCCTC		
AsucAQPf	Ffor	GCCGGGATCAATGCTTATCGC	641 (incomplete)	1) 95°C (3 min), 2) 30 cycles: 95°C (30 s), 56°C (30 s), 72°C (2 min), 3) 72°C (10 min)
	Frev	CGCCCTCTAGTGTTGTGAGC		
AsucAQPx	*			

4.4 Three-dimensional structural predictions

The tertiary structure of *A. succinea* aquaporins was predicted using Phyre2 (Kelley et al., 2015), considering only the best hit models with a confidence of 100% and a coverage of at least 80%. When possible, the sequences obtained from the cloning were selected for this analysis; otherwise, those assembled from the public databases were used.

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CHAPTER IV

CONNECTIVITY OF ESTUARINE POLYCHAETES FROM SOUTHERN BRAZIL: THE INFLUENCE OF CONTEMPORARY AND HISTORICAL DRIVERS ON FOUR ANNELID SPECIES

Manuscript formatted following the guidelines of the journal: Marine Biology
ISSN: 0025-3162
Impact Factor: 2.050
Qualis: A2

Connectivity of estuarine polychaetes from southern Brazil: the influence of contemporary and historical drivers on four annelid species

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Abstract

The genetic composition of marine and estuarine populations is the result of geological events, oceanographic factors as well as life history strategies. Using the COI molecular marker, we investigated how the current genetic diversity of a selected set of annelid species was shaped by varying historical and contemporary drivers in three estuaries of southern Brazil. Species were chosen according to their degree of euryhalinity and their occurrence along the estuaries: the nereidids *Alitta succinea* and *Laeonereis culveri*, both with high a degree of euryhalinity and broader salinity niches; the nephtyid *Nephtys fluviatilis* and the melinnid *Isolda pulchella*, both with a low degree of euryhalinity and narrower salinity niches. The genetic variations among the estuaries reflected their life-history and their tolerance to salinity fluctuations, showing high connectivity among the populations of the nereidids, segregation for the oligohaline nephtyid and no segregation for the melinnid. Both nereidids displayed two different MOTUs. A putative new species for science (*Alitta* sp. A), occurs in the three estuaries, while the “cosmopolitan” *A. succinea* occurs only in the estuaries with international ports. The two MOTUs of *L. culveri*, congruent with recent studies about this species complex, were *L. pandoensis* along the three estuaries, and *L. acuta* occurring only in the PEC and Guaratuba Bay. Our results indicate that the current genetic composition and distribution patterns of the target annelid populations result indeed from both historical drivers – which have led to panmixia or isolation, depending

on the species – and contemporary factors, such as the potential for larval dispersal and the tolerance to salinity changes.

Key-words: Annelids. Subtropical estuaries. Populations connectivity. *Alitta* species complex. *Laeonereis* species complex.

1. Introduction

Current patterns of genetic variation within and among populations result from a combination of historical and contemporary factors that may influence both dispersal and gene flow (Garant et al. 2007). For instance, due to fast climate changes that influenced sea-level and related parameters worldwide, the Pleistocene strongly affected the distribution and the current genetic diversity of species belonging to different taxa (Hewitt 2000; Baker and Fritz 2015). There is a general consensus that the estuarine and coastal marine faunas were the most impacted by such changes, since regressive coastlines limited estuarine connectivity and caused the drying up of isolated, temporary lagoons (Hewitt 2000, 2004). These events potentially led to both the promotion of bottleneck events and population differentiation (Olson et al. 2009; Tschá et al. 2016).

The Southern and South-Eastern Brazilian coasts have been heavily shaped by marine transgressions and regressions during the Pleistocene (Angulo et al. 2006). Current estuarine fauna occurring in these areas probably originated from former populations that expanded into the newly created estuarine environments once they became inhabitable (Tschá et al. 2016). However, present-day population distributions and their current genetic diversity are shaped not only by ancient or more recent Pleistocene events, but also by modern processes related to local adaptation and life-history characteristics, such as salinity requirements, and dispersal abilities, which may also isolate populations and create effective barriers to gene flow (Manel et al. 2003; Orsini et al. 2013).

Estuaries provide highly dynamic habitats because of a continuous convergence of terrestrial, oceanographic and atmospheric processes, that affects abiotic variables, such as salinity, temperature, water turbulence (Potter et al. 2015). The regular and abrupt salinity changes represent a primary physiological limitation for many organisms, affecting their dispersion and connectivity in different ways, e.g. creating seasonal barriers, isolating or linking different environments (Oglesby 1965b;

Whitfield et al. 2012; Chang and Marshall 2016). Recruitment and dispersion in these environments depend on the combination of hydrodynamic processes and life-history strategies (Constanza et al. 1993; Robins et al 2013). Estuaries provide high level of dispersion and mobility for the resident organisms, due to the rapid hydrodynamic transport associated to the tides, wind and pressure (Constanza et al. 1993). Moreover, coastal and marine taxa usually display a planktonic larval stage, whose duration is species-specific (Pechenik 1999; Bhaud 2000). This larval phase is a key factor for benthic animals, allowing them to avoid inbreeding, to occupy new areas with less competitors, and to escape potentially adverse conditions (Bilton et al. 2001). The link between dispersal ability and genetic connectivity among the populations has already been highlighted (Chust et al. 2016). For instance, panmixia among different populations is common in species with planktonic phases, while segregation is expected in species with short or no planktonic phase (e.g. Palumbi 1994; Sanvicente-Añorve et al. 2011; Fobert et al. 2019; Van der Stocken et al. 2019). On the other hand, dispersion may lead to some disadvantages, such as a decrease of survival because of the inability to locate a suitable new site, predation, and low reproductive success (Bilton et al. 2001; Garant et al. 2007). Thus, some species retain lecitotrophic larvae, by reducing the dispersion with mucus, or by alternating the type of larvae according to prevailing environmental conditions (Young and Eckelbarger 1994; Dorresteijn and Westheide 1999; Tsutsumi 2005).

Molecular tools allow to investigate the connectivity pattern of marine species, inferring about the gene flow and the potential driven-factors involved in these processes (López-Duarte et al. 2012), Moreover, their use may also be focused on unravelling the presence of potential species complexes/ cryptic species. In fact, an increasing number of studies indicate that the high intraspecific heterogeneity and the cosmopolitan distribution usually assigned to a single species name actually correspond to complexes of cryptic species (e.g. Simon et al. 2017; Nygren et al. 2018; Teixeira et al. 2019).

Annelids seem to be particularly good models to test hypotheses related to the relative importance of historical and contemporary drivers in modulating the current genetic structure of estuarine fauna. They numerically dominate estuarine environments, being represented by either infaunal or epibenthic and sessile or errant species (Venturini et al. 2011; Dafforn et al. 2013). Their high population densities give them a central functional role in most benthic communities influencing and creating a complex mosaic of micro- and macro- environments relevant for the control of ecosystem functioning (Pischedda et al. 2008).

Herein, we explored how the connectivity among populations of four annelid species along three subtropical estuaries was affected by past and contemporary drivers. We expected high genetic flow for the nereidids *Alitta succinea* (Leuckart, 1847) and *Laeonereis culveri* (Webster, 1879), since both display a high degree of euryhalinity and a broad occurrence along the estuaries. Segregation was expected for the is nephtyid *Nephtys fluviatilis* Monro, 1937, which has a lower degree of euryhalinity, and usually restricted to oligohaline, more confined sectors. Panmixia was expected among the populations of the melinnid *Isolda pulchella* Müller, 1858, usually restricted to the euhaline and outer sectors of the estuaries, thus facilitating its dispersion between estuaries.

2. Materials and methods

2.1 Sample collection

Sampling was carried out in three large estuarine systems along the southern Brazilian coast, all of them subjected to similar geological and oceanographic conditions during the Pleistocene: the Paranaguá Estuarine Complex (PEC), Guaratuba Bay and Babitonga Bay.

The Paranaguá Estuarine Complex (PEC) is one of the largest (612 km²) and most preserved coastal areas along the southern American coast, despite increasing port and tourist activities in the last decades. It is ~40 km far from Guaratuba Bay, which is ~15 km long (W-E axis) and 5 km wide (N-S axis), with a surface area of 50.19 km² (Bigarella 2001; Marone et al. 2006). Babitonga Bay, ~40 km south of Guaratuba Bay, and ~80 km from the PEC, is an estuarine complex at the northern coast of the state of Santa Catarina, with a surface area of 160 km² (IBAMA 1998). The three bays share a microtidal regime, predominantly semidiurnal, with spring tides that may reach up to 2 m in the inner estuarine sector (Marone et al. 2006). Salinity fluctuations are high (~0-35 psu), with horizontal and vertical highly stratified waters (Marone et al. 2005, 2006). Three sectors can be recognized: euhaline (30-40 psu) near the inlets, mesohaline (5-20 psu) in the middle, and the inner oligohaline sector (0-5 psu), which display greater riverine influence (Lana and Bernardino 2018).

Target species were chosen taking into account both their occurrence along the estuarine gradient and their expected salinity tolerance. Animals were sampled using a shovel or scraping the fishermen's buoys, depending on the species.

The nereidids *Alitta succinea* - usually related to hard, natural or human-made substrates - and *Laeonereis culveri* - occurring in mud-sandy bottoms – are both abundant in meso/polyhaline sectors (5 – 30 psu) of the estuaries, euryhaline. The nephtyid *Nephtys fluviatilis*, from the oligohaline sectors (0 – 5 psu) of the estuary, and the melinnid *Isolda pulchella*, usually related to euhaline sectors (30 – 40 psu) of the bays, both stenohaline. For practical reasons, we used *a priori* the epithets most commonly assigned in the regional literature to local populations of the four species. However, we were aware of potential genetic differentiation and nomenclature problems at the beginning of the study, mainly in the cases of *Alitta succinea* and *Laeonereis culveri,* already suspected to be species complexes.

The reproductive biology of each species was also checked to assess the potential dispersal rate of their larvae. According to the literature, pelagic planktotrophic larvae are expected for *A. succinea* (Hardege et al. 1990), while *L. culveri* has benthic lecithotrophic larvae (Mazurkiewicz 1975). No data about the larvae of *N. fluviatilis* are available, but other nephtyids have a pelagic planktotrophic larva (Pérez-Torrijos et al. 2009). Finally, there are no published ontogenetic data about *I. pulchella* but the larvae of the close genus *Melinna* are pelagic planktotrophic (Hernández-Alcántara and Solís-Weiss 2009).

2.2 DNA isolation, amplification, sequencing and alignment

In laboratory, animals were preserved in ethanol 96%, in a freezer at – 4° C. When available, the DNA was isolated from 10-15 specimens per species, from each estuary. Total DNA was extracted from the central segments of their body, using NaOH. The protocol consisted of: 1) addition of 300 µl of 50 mM NaOH to a vial containing the annelid segment previously reduced in small fragments; 2) incubation at 95ºC for 60 min; 3) addition of 300 µl of Tris–HCl 1 M, pH 8; 4) centrifugation at 12,000 rpm for 5 min. The DNA was quantified from the supernatant with Nanodrop 2000 (Thermo Fisher Scientific).

Amplifications of the mtDNA cytochrome c oxidase subunit I (COI) were carried out using the universal primers LC01490 (5' GGTCAACAAATCATAAAGATATTGG 3') and HC02198 (5'

TAAACTTCAGGGTGACCAAAAAATCA 3’) (Folmer et al. 1994), or PolyLCO (5’ GAYTATWTTCAACAAATCATAAAGATATTGG 3’) and PolyHCO (5’ TAMACTTCWGGGTGACCAAARAATCA) (Carr et al. 2001). Amplifications were performed in a 20 µl reaction volume containing 4 µl 1 x PCR buffer (1.5 mM $MgCl_2$), 0.8 µl forward and reverse primers (0.5 µM), 0.8 µl of each dNTP (0.2 mM), 0.1 U/µl GoTaq G2 DNA Polymerase (Promega), 1-3 µl template DNA, and nuclease-free water to bring the mix to a final volume of 20 µl. The PCR thermal cycling conditions for both the COI primer pairs consisted of an initial denaturation at 94ºC for 3 min, followed by 35 cycles of 95ºC for 30 s, primer annealing at 49-51°C for 15 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were purified with Exonuclease I and Shrimp Alkaline Phosphatase (rSAP) (New England Biolabs), following the manufacturer’s protocol. Sequencing of amplified segments were performed with Big Dye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific) using the Applied Biosystems 3500xL Genetic Analyzer (Applied Biosystems) at the Bioquímica e Biologia Molecular department, in the Universidade Federal do Paraná (UFPR).

The consequent trace files were checked manually; unreadable zones and primers were removed and ambiguous bases corrected. The sequences were aligned using Clustal W (Thompson et al. 1994) implemented in MEGA 7.0 (Kumar et al. 2016), and inspected for eventual stop codons and indels to prevent inclusion of pseudogenes in the analyses. Alignments of different length were obtained for each species: 595 bp for *A. succinea*, 587 bp for *L. culveri*, 587 bp for *N. fluviatilis*, 579 bp for *I. pulchella*. Several blastn (Altschul et al. 1990) were performed in NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for sequence control. DNA barcode data were updated in the dataset “DS-ESPBR” in the Barcode of Life Data System (BOLD; Ratnasingham and Hebert 2007). Detailed information on the specimens and sequences are provided in Table S1.

2.3 Estimates of genetic diversity

The divergence within the species was calculated using Kimura-2-parameters (K2P; Kimura 1980) as implemented in BOLD in Distance Summary. Number of haplotypes (H), haplotype diversity (Hd) and nucleotide diversity (π) were calculated with the program DNASP 5.10 (Librado and Rozas 2009) to estimate the genetic diversity for each species.

2.4 Molecular-based species delineation

Several blastn were performed in BOLD to recover COI sequences of the species *Alitta succinea* and *Laeonereis culveri* publicly available. Molecular Operational Taxonomic Units (MOTUs) were defined applying three methods of molecular-based species delineation. The Automatic Barcode Gap Discovery (ABGD) species delineation tool was run on a web interface (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html; Puillandre et al. 2012), using default settings (i.e. K2P). This tool, based on the barcode gap detection (i.e. break between the distribution of intraspecific and interspecific distances of the barcode region), sorts the sequences into hypothetical species (Puillandre et al. 2012). Then, COI sequences were submitted to the BIN system implemented in BOLD. This approach clusters barcode sequences algorithmically to calculate MOTUs showing high concordance to species (Ratnasingham and Hebert 2013). Finally, the tree-based method applied was bPTP (bayesian Poisson Tree Processes; Zhang et al. 2013). The bPTP method incorporates the number of substitutions in the model of speciation and assumes that the probability that a substitution gives rise to a speciation event follows a Poisson distribution. The branch lengths of the input tree are supposed to be generated by two independent Poisson process classes, one corresponding to speciation and the other to coalescence (Zhang et al. 2013). For the input trees, maximum-likelihood (ML) method was used through PhyML (Guindon et al. 2010). Branch support was inferred by 1,000 bootstraps. The best substitution model (GTR+G+I for both *A. succinea* and *L. culveri* alignments) was tested with the SMS routine in PhyML using both AIC and BIC as optimality criteria (Lefort et al., 2017). The trees were edited with FigTree (Rambaut, 2010). Species delimitation analyses were done on the bPTP web server (available at: http://species.h-its.org/) with 1×10^6 iterations of Markov chain Monte Carlo and 25% burn-in. Molecular diagnostic characters were computed for the two MOTUs of *A. succinea* using Diagnostic Characters tool with default settings (i.e. K2P), implemented in BOLD.

2.5 Population structure and connectivity

To assess the genetic differentiation among the estuaries for each species and MOTUs (i.e. two MOTUs for both *A. succinea* and *L. culveri*), Fst estimations were performed using p-distances in Arlequin 3.5 (Excoffier and Lischer 2010). Significance of pairwise Fst values was determined by performing 10,100 permutations between locations, under the null hypothesis of no differentiation. Analysis of molecular variance (AMOVA) was also performed in Arlequin 3.5. A haplotype network for each species was computed using the COI data with the median-joining method (Bandelt et al. 1999) in the software Popart (v. 1.7).

Fu's Fs (1997), Tajima' D (1989) and mismatch distributions were computed in Arlequin 3.5 (Excoffier and Lischer 2010) to test the departure from neutrality. The mismatch distribution was tested against the predicted outcome of the models under demographic and spatial expansion in order to give further support to the neutrality tests. Finally, the Geographic Distance Correlation tool (Blagoev et al. 2016), using default settings (i.e. K2P) as implemented in BOLD, was performed to assess the correlation between genetic diversity and geographical distances.

3. Results

3.1 Estimates of genetic diversity

A total of 156 sequences were recovered, divided into *Alitta succinea* (37), *Laeonereis culveri* (34), *Nephtys fluviatilis* (44), *Isolda pulchella* (41) (Tab. S1). The maximum overall distance between species was of 20.93%. Distance within species varied from 21.07% in *A. succinea* to 1.04% in *I. pulchella; L. culveri* with 19.23% and *N. fluviatilis* with 2.08% (Tab. S2). The highest Hd was in *N. fluviatilis* (0.937) followed by *A. succinea* (0.929), although the nucleotide diversity was highest in *L. culveri* (0.085) followed again by *A. succinea* (0.069). PEC showed the highest number of haplotypes (and relative Hd), for all the target species, while Guaratuba Bay showed the lowest. Also, for the nucleotide diversity the PEC displayed the highest values, while the lowest varied according to the species, with no differences in *I. pulchella* (Tab. 1).

Table 1. Number of sequencies (N), number of haplotypes (H), haplotype diversity (Hd) and nucleotide diversity (π) based on COI in the four target species of this study.

Species	Region	N	H	Hd	π
Alitta succinea	All	37	19	0.929	0.069
	PEC	12	10	0.970	0.096
	Guaratuba Bay	14	8	0.890	0.003
	Babitonga Bay	11	9	0.964	0.009
Laeonereis culveri	All	37	17	0.909	0.085
	PEC	13	10	0.949	0.077
	Guaratuba Bay	12	5	0.758	0.008
	Babitonga Bay	9	5	0.722	0.037
Nephtys fluviatilis	All	44	21	0.937	0.007
	PEC	14	10	0.956	0.005
	Guaratuba Bay	15	6	0.800	0.005
	Babitonga Bay	15	7	0.724	0.001
Isolda pulchella	All	41	19	0.841	0.003
	PEC	15	8	0.838	0.003
	Guaratuba Bay	13	8	0.859	0.003
	Babitonga Bay	13	9	0.872	0.003

3.2 Molecular-based species delineation

Blastn performed against BOLD database using as query the COI sequences of each species recovered for *L. culveri* two different BINs: 17 specimens belonging to the BIN ACN4284 -100% identity with *Laeonereis pandoensis* (Monro, 1937), and 17 specimens belonging to the BIN ADR4231 - 99% identity with *Laeonereis acuta* (Treadwell, 1923). For *A. succinea* nine specimens matched the BIN AAL7714 (99%) comprising other specimens of the same species, although for the remaining 28 sequences the closest sequences belonged to the same BIN but with only 82% of identity, meaning no match. No previous *I. pulchella* and *N. fluviatilis* sequences were available in the nucleotide database; the query *I. pulchella* recovered 84% identity with *Melinna albicincta* Mackie & Pleijel, 1995 (ACH9309), and the query *N. fluviatilis* recovered 83% identity with *Aglaophamus agilis* (Langerhans, 1880) (AAW0075).

The three analyses of molecular species delimitation (BINs, ABGD, bPTP) recovered two different MOTUs for both *A. succinea* and *L. culveri* each species (Fig. 1). The analysis of molecular diagnostic characters performed on the two MOTUs of *Alitta,* recovered 95 diagnostic characters for the new MOTU found in this work (hereby called *Alitta* sp. A) and 91 for *A. succinea* (Tab. S3).

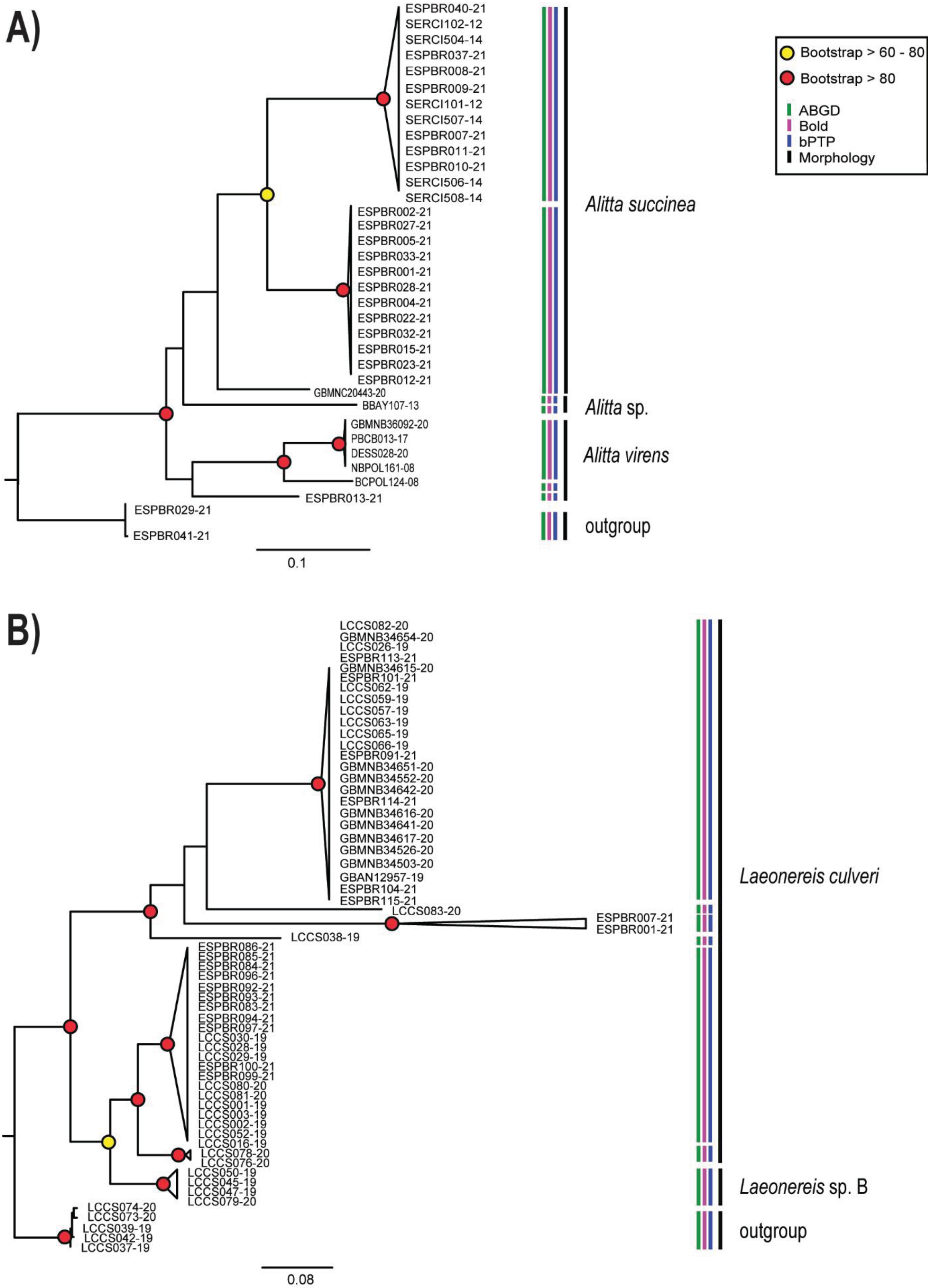

Figure 1. Maximum-likelihood phylogenies of the COI of A) *Alitta succinea* and B) *Laeonereis culveri.* Sequences from this work start with the code "ESPBR"*. Alitta* spp. tree was rooted with *Pseudonereis* sp. as outgroup. *Laeonereis* spp. tree was rooted with *Laeonereis* sp. A as outgroup.

3.3 Population structure and connectivity

The network of *A. succinea* was split into two groups with 95 mutations of difference: *Alitta* sp. A shared haplotypes among the three estuaries; *A. succinea,* not found in Guaratuba Bay, shared haplotypes only between PEC and Babitonga Bay (Fig. 2A). *Laeonereis culveri* showed a similar subdivision, with two groups displaying 93 mutations of difference. This pattern supports the results of a molecular delimitation carried out by Sampieri et al. (*in press*), simultaneous to our own study: *L. acuta* grouped haplotypes only belonging to PEC and Babitonga Bay (no shared haplotypes), while *L. pandoensis* shared haplotypes among the three estuaries (Fig. 2B). Populations of *N. fluviatilis* from Babitonga Bay presented only private haplotypes that grouped together, differing from PEC and Guaratuba Bay (Fig. 2C). Finally, no pattern was retrieved for *I. pulchella*, which showed multiple shared haplotypes among all the estuaries (Fig. 2D).

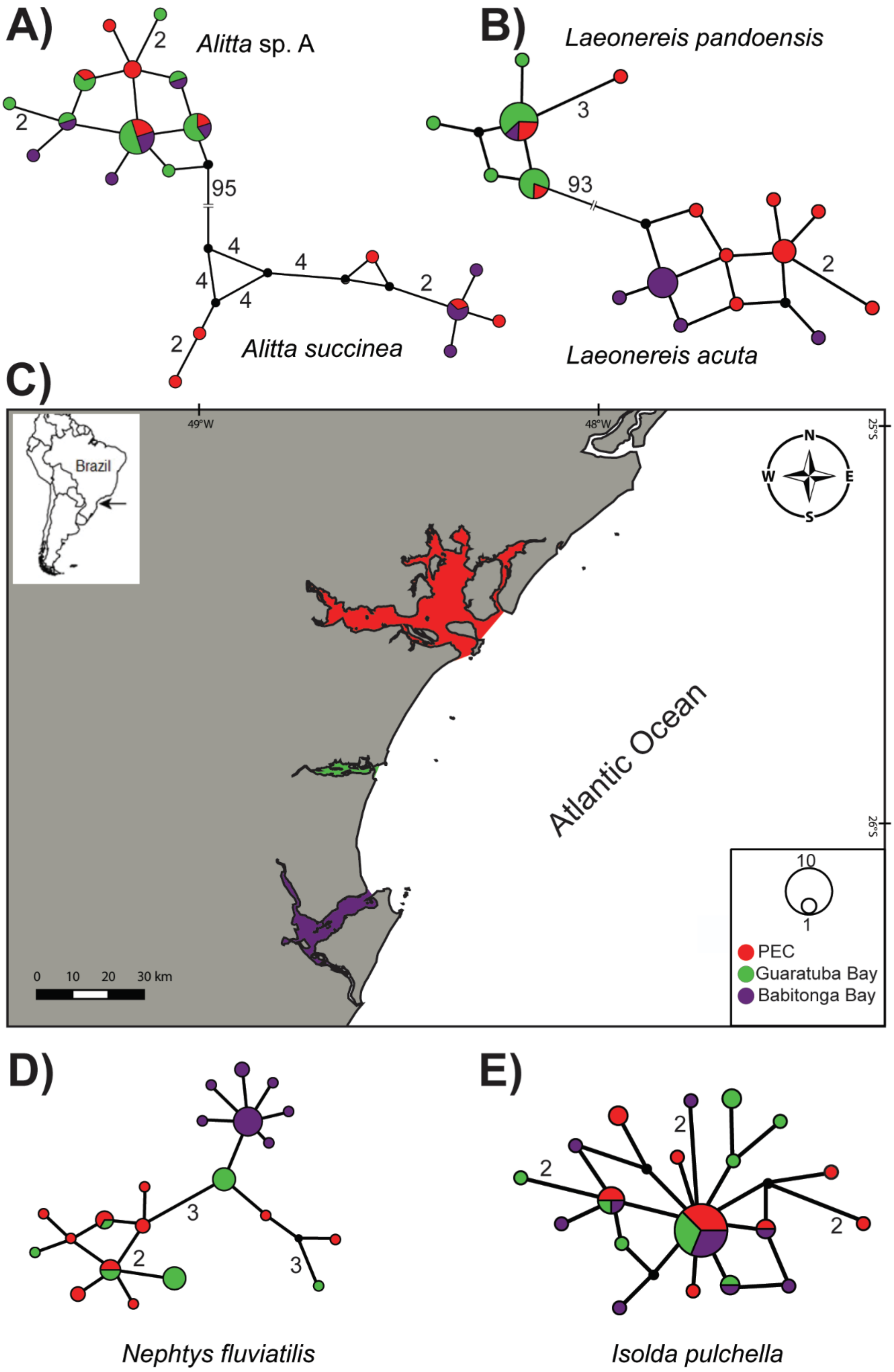

Figure 2. COI median joining haplotype networks and study area (C). A = *Alitta succinea sensu latu*, comprising the MOTUs *Alitta* sp. 1 and *Alitta succinea.* B = *Laeonereis culveri sensu latu*, comprising the MOTUs *L. pandoensis* and *L. acuta*. D = *Nephtys fluviatilis;* E = *Isolda pulchella.* Size of the circles are proportional to the number of specimens with relative haplotype. Black dots represent missing haplotypes, more than one mutation is reported with numbers. PEC= Paranaguá Estuarine Complex.

The populations' genetics (Fst) of *L. acuta* and *N. fluviatilis* differed significantly among the bays, showing a segregation of the ones in Babitonga Bay from the ones of the other two estuaries (that were not significantly different in *N. fluviatilis*; Tab. 2, S4).

Tajima's D values were negative and significant for *A. succinea* and *I. pulchella*, while Fu's Fs test showed a significant negative value for *L. culveri, N. fluviatilis* and *I. pulchella* (Tab. 3). Both the indices indicate an excess of rare haplotypes. The nereidids displayed a significant deviation from the null hypothesis of demographic and spatial expansion (Tab. 3). Correlation between genetic and geographic distances was significant only for *N. fluviatilis* and *L. acuta*. (Tab. S5).

Table 2. AMOVA results for each species. In bold the significant values.

	Source of variation	d.f.	Sum of squares	Variance components		Percentage of variation		d.f.	Sum of squares	Variance components		Percentage of variation
Alitta sp. A	Among populations	2	32	0.95090	Va	12.08	*Alitta succinea*	1	2.753	0.19637	Va	8.63
	Within populations	27	186.833	6.91975	Vb	87.92		5	10.398	2.07957	Vb	91.37
Laeonereis pandoensis	Among populations	2	0.943	-0.05414	Va	-8.71	*Laeonereis acuta*	1	4.996	0.49219	Va	**37.32**
	Within populations	14	9.455	0.67539	Vb	108.71		15	12.399	0.82662	Vb	**62.68**
Nephtys fluviatilis	Among populations	2	32.727	1.02025	Va	**42.02**	*Isolda pulchella*	2	2.566	0.02617	Va	2.75
	Within populations	41	57.717	1.40773	Vb	**57.98**		38	35.196	0.9262	Vb	97.25

Table 3. Tajima's D value, Fu's Fs, demographic distribution (SSD and raggedness index) for each species. In bold the significant values.

	D	Fs	SSD	Raggedness
Alitta sp. A	-1.804	2.300	**0.008**	**0.056**
Alitta succinea	**-1.658**	-0.004	**0.089**	**0.224**
Laeonereis pandoensis	-1.296	**-1.783**	**0.005**	**0.072**
Laeonereis acuta	-0.986	-6.644	**0.000**	**0.000**
Nephtys fluviatilis	-0.814	**-8.828**	0.009	0.021
Isolda pulchella	**-1.970**	**-15.634**	0.003	0.041

4. Discussion

Our results support the hypothesis that varying levels of genetic structure among the four target species are associated to their contemporary life strategies and to historical drivers. The more euryhaline nereidids displayed high connectivity, although larvae are pelagic for *Alitta succinea* and benthic for *Laeonereis culveri*. Historical drivers, such as the Pleistocene glaciations, may be responsible for either the segregation or the panmixia showed by the less euryhaline nephtyid and melinnid, respectively.

The overall molecular analyses of both the nereidids unveiled the presence of a hidden diversity. The initially called *Alitta succinea* showed two distinct MOTUs, with 20.30% of mean distance between them: the first is a potentially new species for science (*Alitta* sp. A), widespread in the three estuaries, whereas the proper *A. succinea* (BIN AAL7714) occurred only in the PEC and Babitonga Bays, both with two major ports, potential vectors for the introduction of widespread species. The molecular distance and the number of mutations between these two species of *Alitta* were higher than those of the two MOTUs of *L. culveri* which were recognised as two distinct species during the development of our own study (Sampieri et al. *in press*). High dispersal capacity, together with a high degree of euryhalinity and broad substrate preferences, are obviously related to the widespread distribution of *Alitta*. For instance, the first ontogenetic stages in nereidids, starting from the fertilization, are totally restricted to the water column (Ushakova and Saranchova 2003). No cases of reproduction in *A. succinea sensu latu* have ever been reported without epitoky, i.e the process characterized by deep bodily changes of male and female individuals, anticipating the migration to the surface of the water column and the release of the gametes (Aguiar and Santos 2017, and all the references therein). Moreover, a high degree of euryhalinity, recently described by Mucciolo et al. *under rev* (first chapter), may allow for *Alitta* to occur in a variety of coastal and estuarine habitats once the larva is settled, although it prefers oligo/mesohaline estuarine sectors. An “equilibrium” among the populations of this species was suggested by the neutrality tests, with no demographic or spatial expansion and without the presence of rare alleles, in contrast to the cosmopolitan *A. succinea*. The latter species showed contrasting results of D and Fs; however, the high abundance of rare alleles, together with its disjunct presence, limited to the bays with active ports, strongly suggest that multiple introductions have occurred via shipping. In fact, ports are well-known to act as “sink” of non-indigenous species (NIS), which are constantly supplied with new propagules from international shipping (Gollasch 2006, Seebens et al. 2013).

A similar pattern was recovered for *Laeonereis culveri,* which was split into two MOTUs, one from PEC and Babitonga Bay, and the other from Guaratuba Bay. Our results are congruent with a recent analysis of the species complex of *L. culveri* along the western Atlantic coast, which *L. acuta* from Rio de Janeiro, São Paulo and Paraná PEC) coasts, and *L. pandoensis* from Rio de Janeiro to Rio Grande do Sul coasts, except for Paraná (Sampieri et al. *under rev*). Our more exhaustive sampling along the Paraná (i.e. PEC and Guaratuba Bay) and Santa Catarina coasts (i.e. Babitonga Bay), allowed for the discrimination of more detailed distribution patterns for both species. They may even be found in sympatry, as at the Guaraqueçaba sampling site (PEC), suggesting even the possibility of interbreeding. Considering the wide use of the epithet *L. culveri* in ecotoxicological essays (e.g. Geracitano et al. 2002; Barros et al. 2018; Braga et al. 2018), our study points to the need of further studies to discern the diversity of this species complex. However, the life strategies of both sexes to spawn on the bottom, to brood embryos and larvae in special tubes constructed by the females, which are reflected in low dispersion rates of the larvae, may contribute to their effective genetic segregation. Their low larval dispersal capacity may also be the reason of a significant segregation between PEC and Babitonga Bay for *L. acuta*. Further studies should be addressed to widely assess the interbreeding potential of these two cryptic species at this location.

The high degree of euryhalinity of *L. culveri sensu latu* may also explain the “equilibrium” recovered among the populations of *L. pandoensis*, which occurs along the three estuaries, and with no demographic or spatial expansion nor the presence of rare alleles. Conversely, the presence of rare alleles in *L. acuta*, its absence in Guaratuba Bay and the correlation of the genetic distance with the geographic ones, suggest that a recent “sweep” process may have shaped its regional distribution, promoting bottlenecks and local extinctions. Several sea level fluctuations correlated to the Pleistocene glaciation, from ~2 mya to 11,700 ya (Ehlers and Gibbard 2011), may have influenced the recent distribution of *L. acuta,* as well as the genetic diversity of the other species herein. Taking into account the physiography of the estuaries investigated, Guaratuba appears rather small compared to the other estuaries (< 3 times Babitonga Bay and < 10 times PEC). Thus, the available area for settlement may be reduced, even more if the space might be already colonized by other species, following the “founder takes all” density-dependent process, where the first founders rapidly colonize a new habitat, while subsequent migrants are unable to successfully settle the space (Waters et al. 2013; Vieira et al. 2019).

Nephtys fluviatilis is restricted to the oligohaline, more internal and confined sectors of the bays. Such a spatial segregation, more evident for the population of Babitonga Bay, led to a reduced gene flow and to genetic structure in relation to populations from PEC and Guaratuba Bay. The population of Babitonga Bay is likely to have passed through a recent bottleneck, as supported by the molecular analyses, displaying: lower genetic diversity (i.e. nucleotides and haplotypes) in Babitonga when compared to the other bays, the presence of rare alleles. Our results fit the hypothesis that during Pleistocene events, the connections among the populations were more likely among adjacent estuaries; thus, the genetic flow to more distant populations probably happened through a stepping-stone dispersion mode (Baggio et al. 2017). No larval dispersion data are available for *N. fluviatilis*, but the larvae of other nephtyids are pelagic and may remain in the planktonic stage for 11-42 days (Caron et al. 1995). This long planktonic phase, together with the short-term tolerance (i.e. 24 h) to salinity changes of this species (Mucciolo et al. *under rev*; first chapter), may explain the high genetic connectivity between the closer PEC and Guaratuba Bay, which were connected during the last glaciation (Tschá et al. 2016). In fact, larval dispersion among adjacent estuaries could potentially occur in few hours or days, once the larvae have been flushed by tides into the sea (Smith et al. 2015).

Conversely, *I. pulchella* showed panmixia among the regional populations, a pattern that can be also explained by both historical and contemporary drivers. During the transgression phases of the sea, in which the basins were flooded with seawater, previously isolated populations may have likely fused into a large panmictic group (Tschá et al. 2016), allowing the populations to settle along the estuaries until reaching their salinity limit. Moreover, this species is the most “marine” of the four target species, occurring along the euhaline sectors of the three estuaries. No data about larval development are available for *I. pulchella*, but larvae of the close genus *Melinna* have a relatively short planktonic stage (i.e. few days), a time span that potentially would allow for gene flow among the estuaries through the shallow continental shelf. This interpretation is congruent with the negative results of D and Fs, the absence of genetic segregation and by the spatial and demographic expansion. *Isolda pulchella* occurs along the whole Brazilian coast (Ribeiro et al. 2018), and the presence of rare alleles may be correlated to some haplotypes coming from estuaries not investigated herein.

5. Conclusion

In our work we comparatively inferred the genetic connectivity of populations from four estuarine annelid species among three subtropical bays from southern Brazil. Our results show how the life strategies of each species may affect population connectivity and distribution along contemporary estuarine gradients, but we also recovered a strong genetic signal from past geological events, such as the Pleistocene regressions and transgressions. We revealed a hidden diversity for the species which were *a priori* assigned to *Alitta succinea* and *Laeonereis culveri*. Each species name corresponds in fact to two different MOTUs, which can be sympatric or allopatric. *Alitta succinea sensu strictu* is restricted to the PEC and Babitonga Bay. A putative new species of *Alitta*, yet to be described, occurs in the three estuaries. The initially called *L. culveri* corresponds to two MOTUs*, Laeonereis acuta* and *Laeonereis pandoensis*. Our findings provide a better understanding of patterns of connectivity in estuarine invertebrates, by underlining how their current distribution reflects the interplay of contemporary biological traits and historical drivers.

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GENERAL CONCLUSION

This work revealed how the current distribution and the genetic characteristics of four annelid species along three subtropical estuaries are shaped by contemporary and historical drivers, such as the tolerance to salinity changes and geological events. The main novelty of this thesis was the integrative approach adopted, which coupled "old-fashioned" - but still efficient - methodologies to more recent ones, in order to explore different biological organization levels, from the molecular structure to ecological processes. Moreover, a comparative approach, which considered species with different lifestyles and putative salinity tolerance, helped to disclose the diversity and plasticity of these fascinating animals.

Outcomes from each chapter were generally in accordance with the life strategy and the autecology of the target species here investigated. The experiment carried out in the first chapter confirmed that the two target nereidids display a high degree of euryhalinity, both showing strong cell volume regulation, but being both unable to tolerate fresh water. The nephtyid was distinctly less euryhaline but tolerated low salinities and fresh water, while the melinnid presented a marine stenohaline behaviour and did not tolerate seawater dilution. Moreover, the thickness of the integument of each species was not strictly related to the osmotic behaviour. These findings prompt as autecological traits, morphophysiological adaptations and evolutionary history of the species probably all play complementary roles in the distribution of the four annelid species.

The second chapter reported for the first time the occurrence and the expression of the NKCC along the body of the four target species, using immunofluorescence. Its expression was not homogeneous, and reflected the lifestyle of the annelids. The free-living/burrowers (both nereidids and the nephtyid) displayed a more widespread signal for NKCC, in contrast to the stenohaline and sedentary melinnid, which displayed a more limited signal restricted to the body regions directly exposed to the surrounding environment (i.e. branchiae) and the internal tissues of either the thorax or the abdomen.

We reconstructed the annelid MIPs phylogeny in the third chapter. Putative orthologs of the vertebrate AQP1-like, AQP8 and the AQP11-like subfamilies were recovered, and three paralogs of *Alitta succinea* were confirmed by RT-PCR. Similarities between annelids and human MIPs were found in the "key" residues along the sequences, confirming their MIP nature and opening new questions about their functioning.

Chapter four showed how the life strategy of each species influenced its population connectivity and distribution patterns along estuarine gradients, in response to geological events and contemporary drivers. According to their high euryhalinity, the nereidids displayed high genetic flow among the bays. The low degree of euryhalinity of the nephtyid and its limited distribution in the oligohaline sectors were reflected in the segregation of the populations from Babitonga Bay; while the “marine” behaviour and the restricted occurrence of the melinnid in the euhaline sectors explained their almost complete lack of genetic structure among estuaries. Moreover, we found a putative new species related to the complex *A. succinea*, and two distinct species, *Laeonereis acuta* and *Laeonereis pandoensis*, for the initially called *Laeonereis culveri.* Our results are congruent and add to recent molecular analysis of the genus *Laeonereis*.

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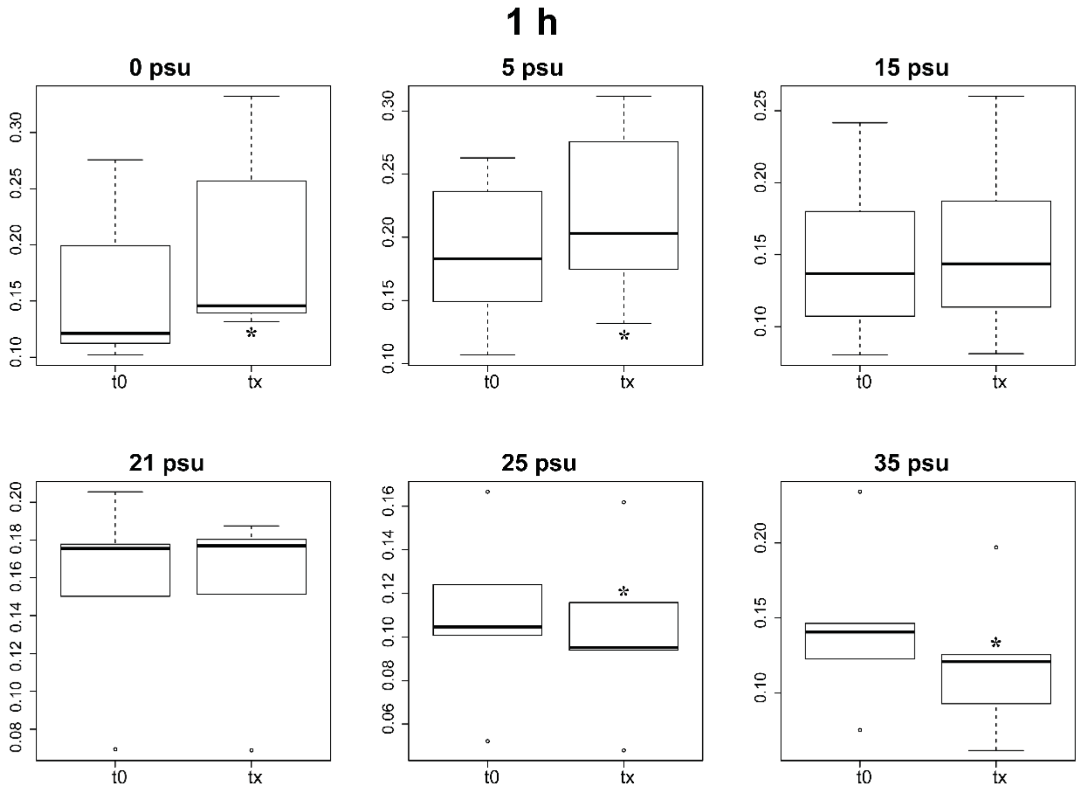
SUPPLEMENTARY MATERIAL

Chapter I

A



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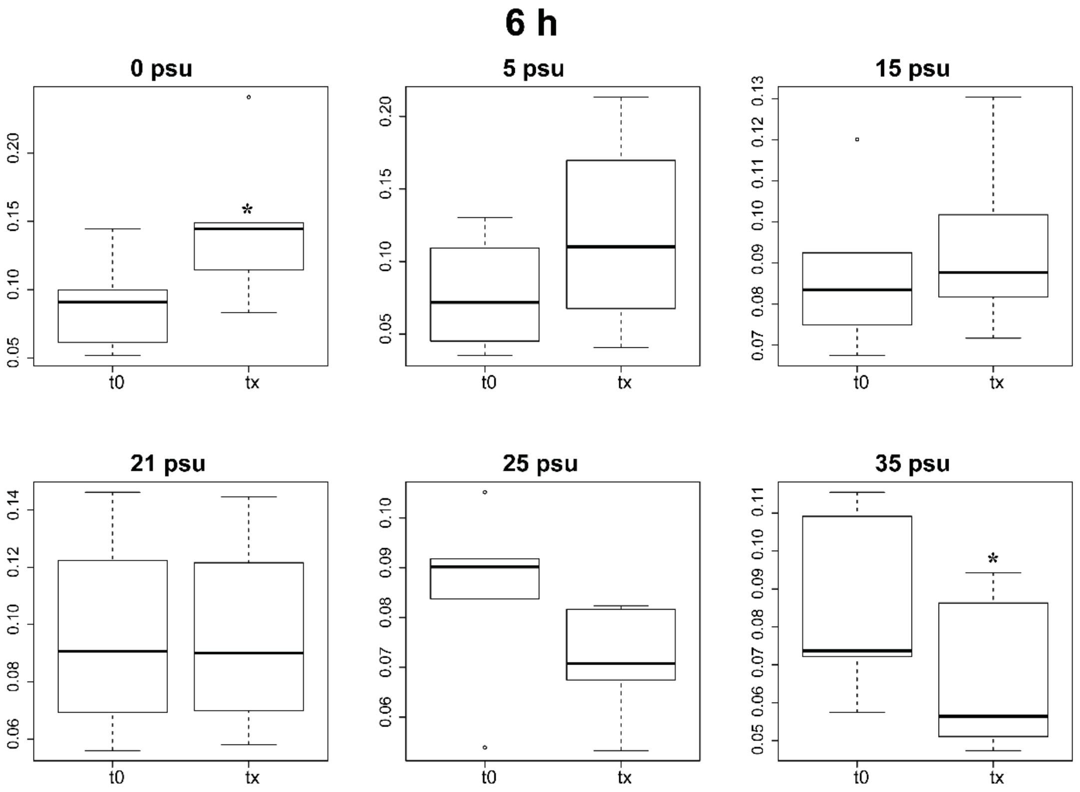


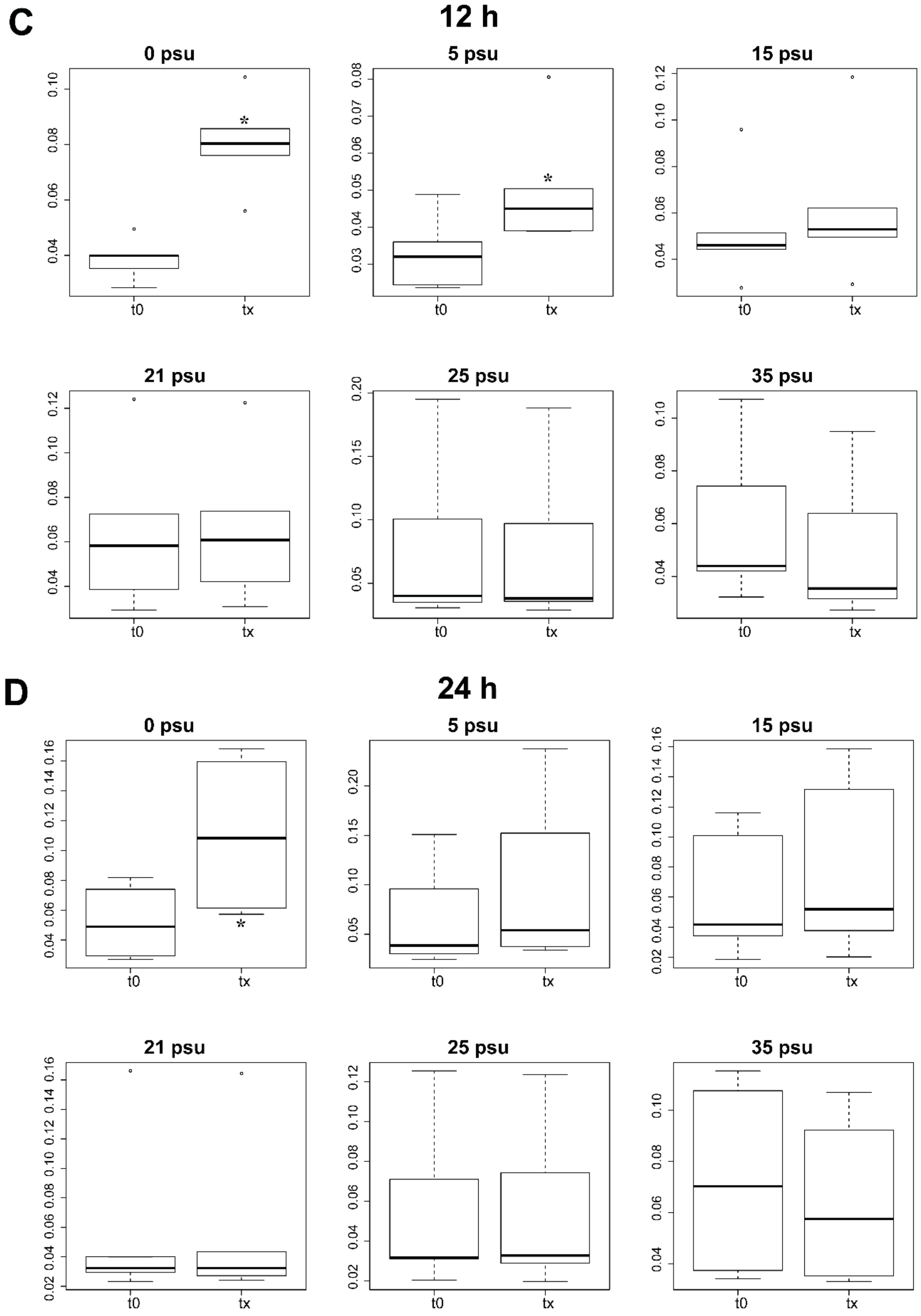
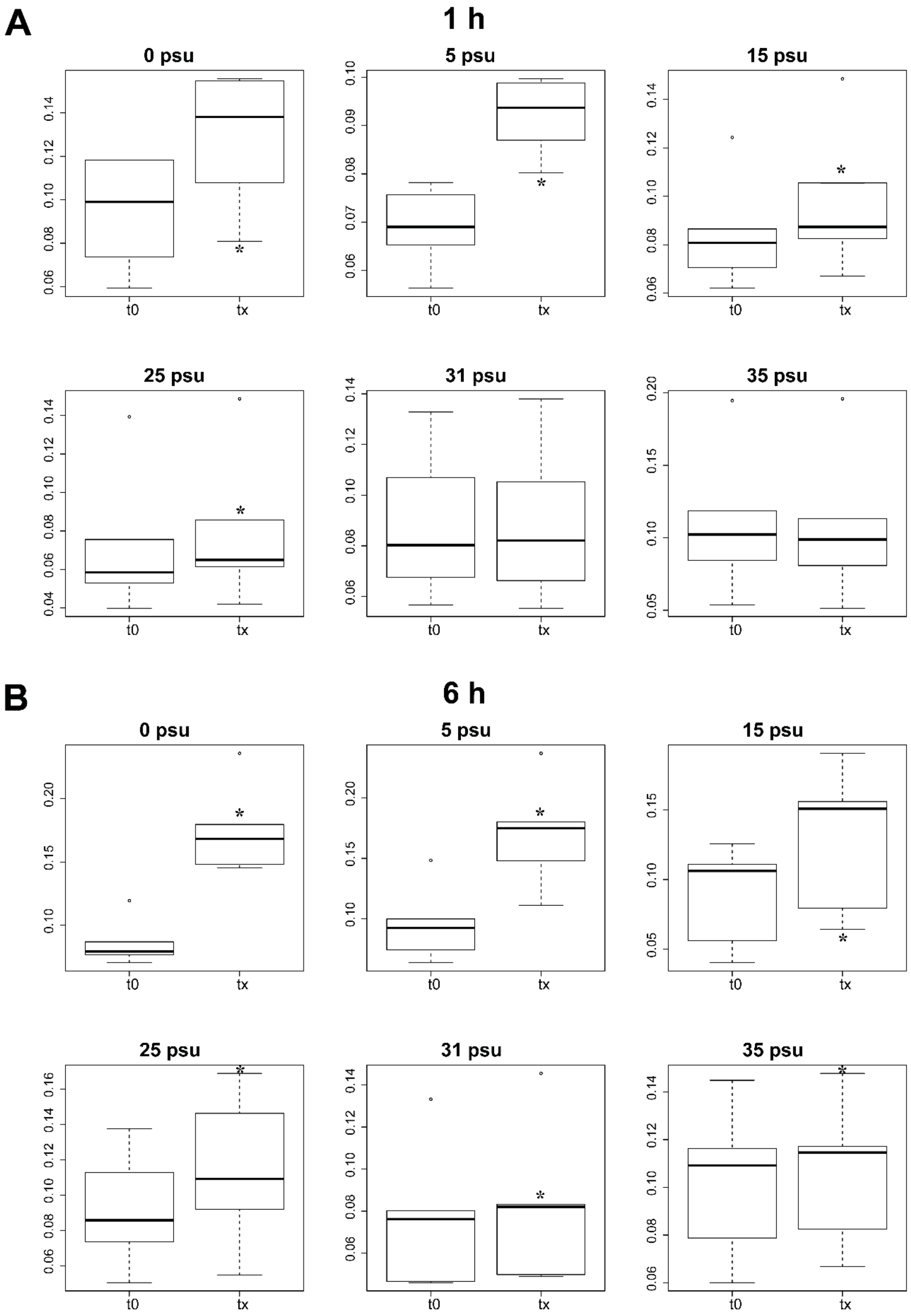


Fig. S1.1 Percentage of variation in body weight of the annelid *Alitta succinea* from the time zero (t0) to the second measurement (tx), in each observation time and salinity. Asterisks showing treatment with weights significantly different from time zero.


A
1 h
0 psu
0.06 0.08 0.10 0.12 0.14
5 psu
0.06 0.07 0.08 0.09 0.10
15 psu
0.06 0.08 0.10 0.12 0.14
25 psu
0.04 0.06 0.08 0.10 0.12 0.14
31 psu
0.06 0.08 0.10 0.12 0.14
35 psu
0.05 0.10 0.15 0.20
t0
tx
*
B
6 h
0 psu
0.10 0.15 0.20
5 psu
0.10 0.15 0.20
15 psu
0.05 0.10 0.15
25 psu
0.06 0.08 0.10 0.12 0.14 0.16
31 psu
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0.06 0.08 0.10 0.12 0.14

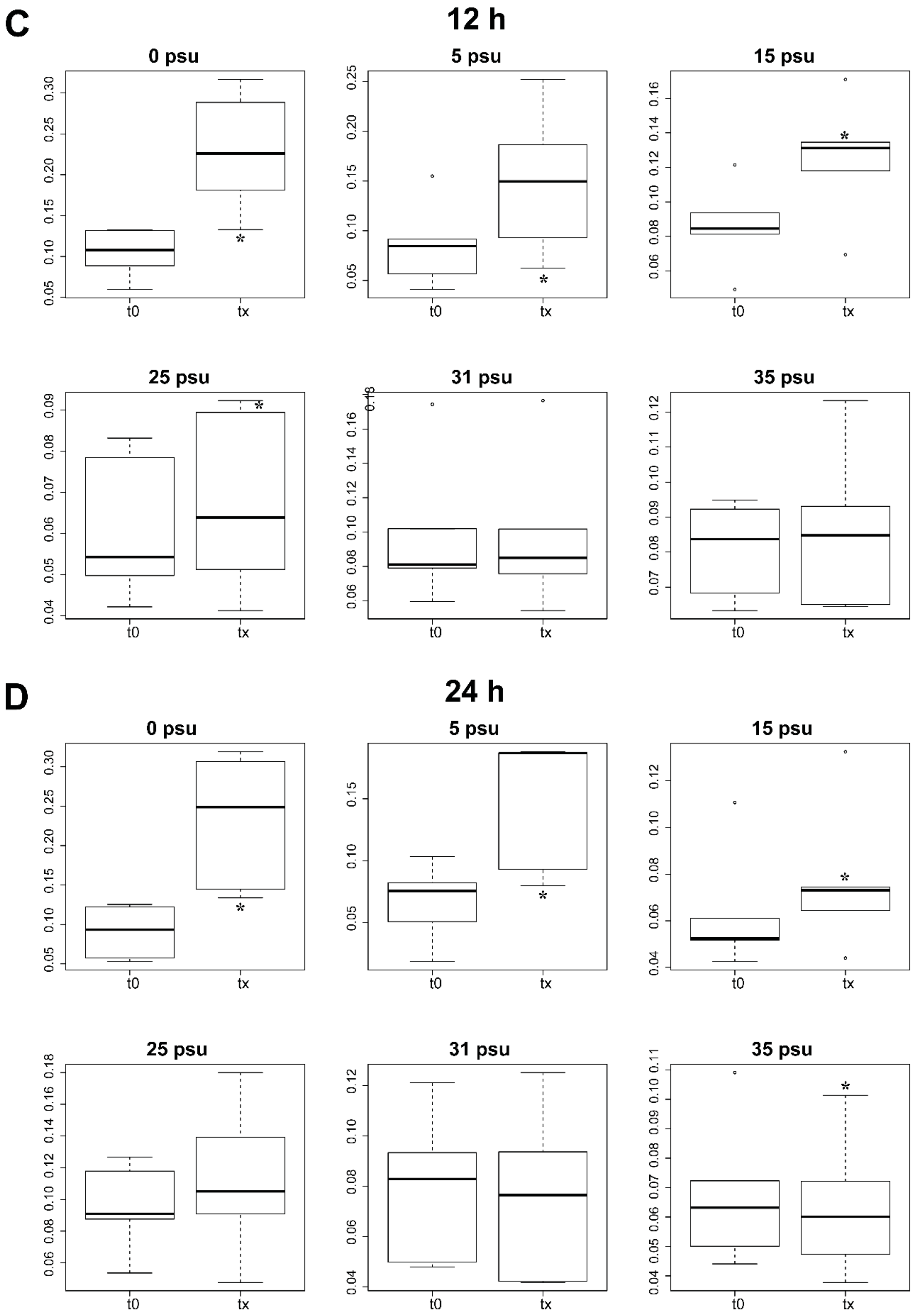

Fig. S1.2. Percentage of variation in body weight of the annelid *Laeonereis culveri* from the time zero (t0) to the second measurement (tx), in each observation time and salinity. Asterisks showing treatment with weights significantly different from time zero.

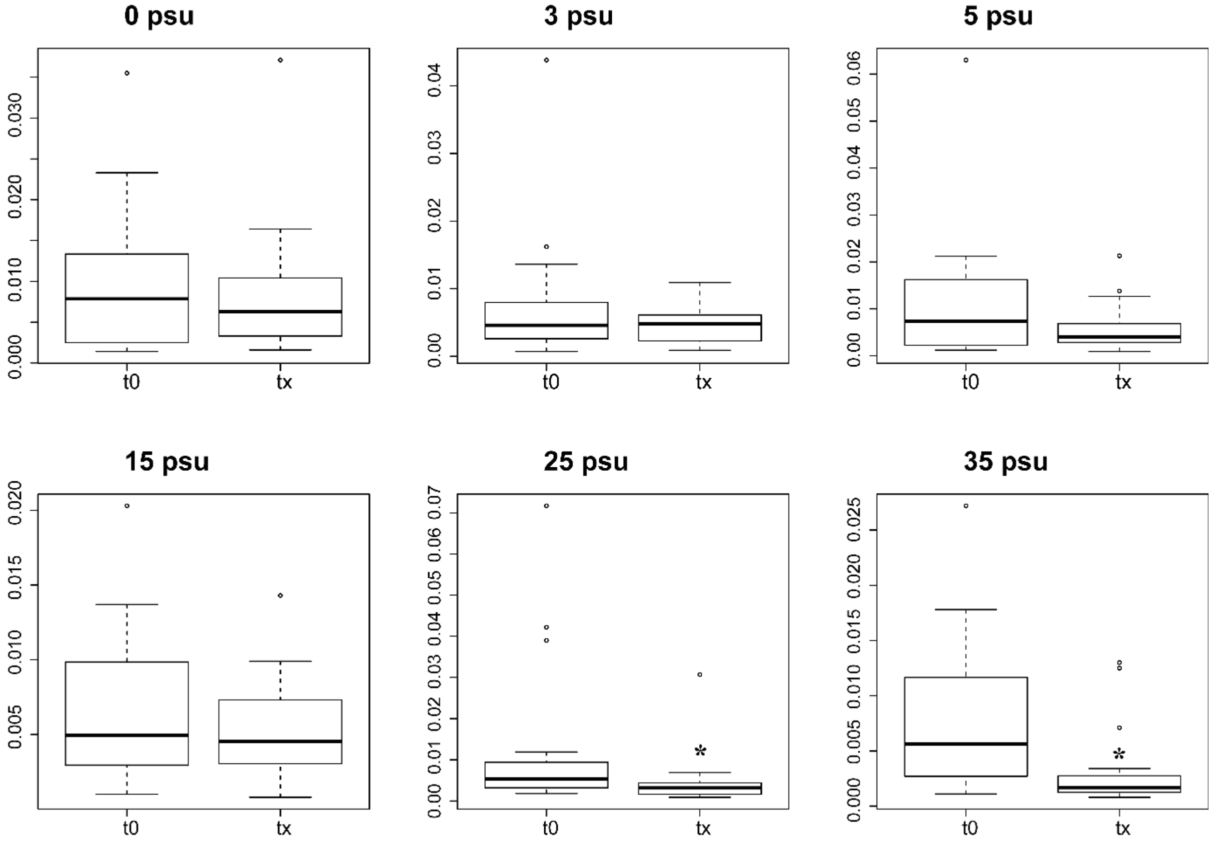

Fig. S1.3. Percentage of variation in body weight of the annelid *Nephtys fluviatilis* from the time zero (t0) to the second measurement (tx), taking into account all the salinities. Asterisks showing treatment with weights significantly different from time zero.

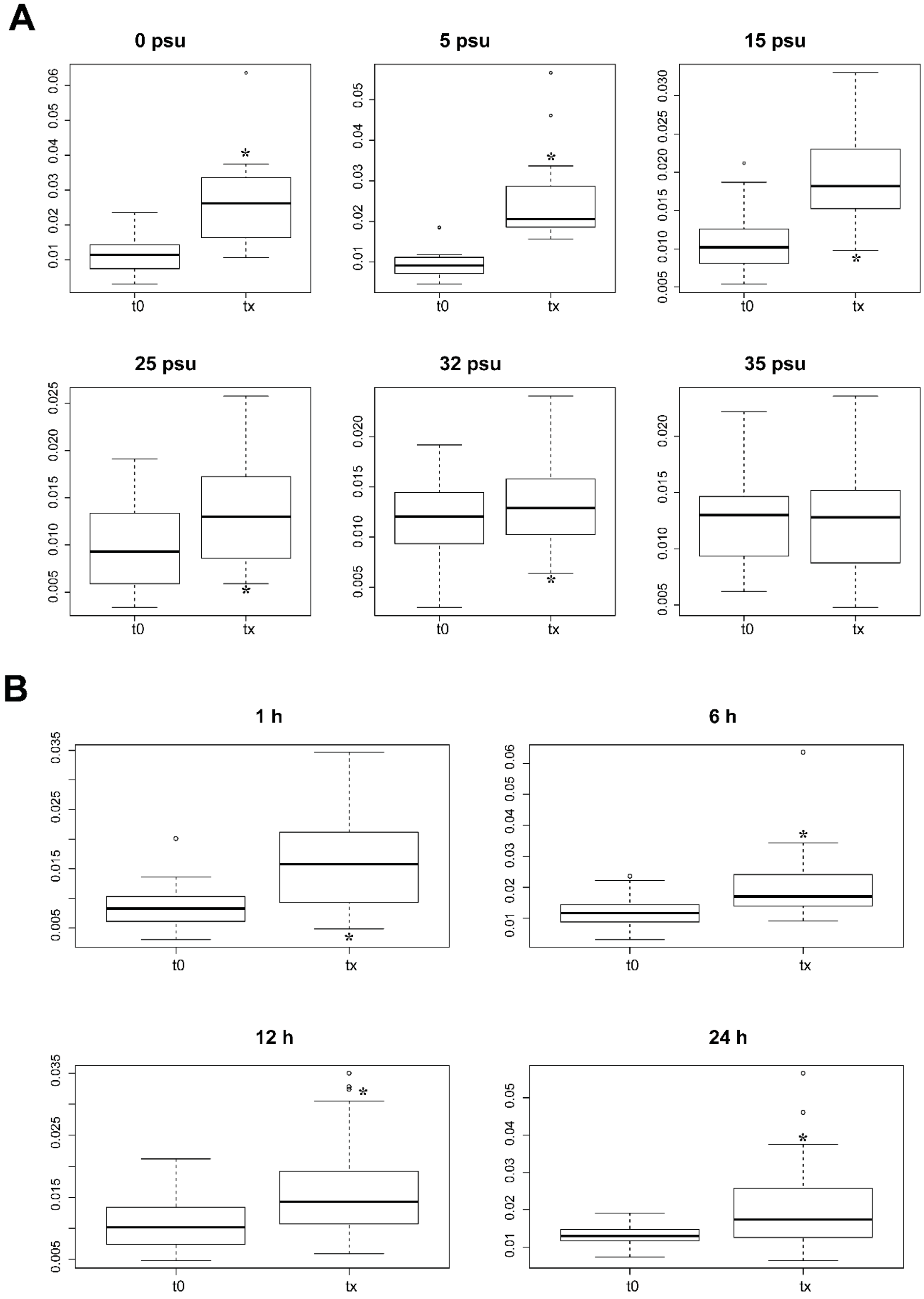

Fig. S1.4. Percentage of variation in body weight of the annelid *Isolda pulchella* from the time zero (t0) to the second measurement (tx), taking into account all the salinities (A) and all the times (B). Asterisks showing treatment with weights significantly different from time zero.

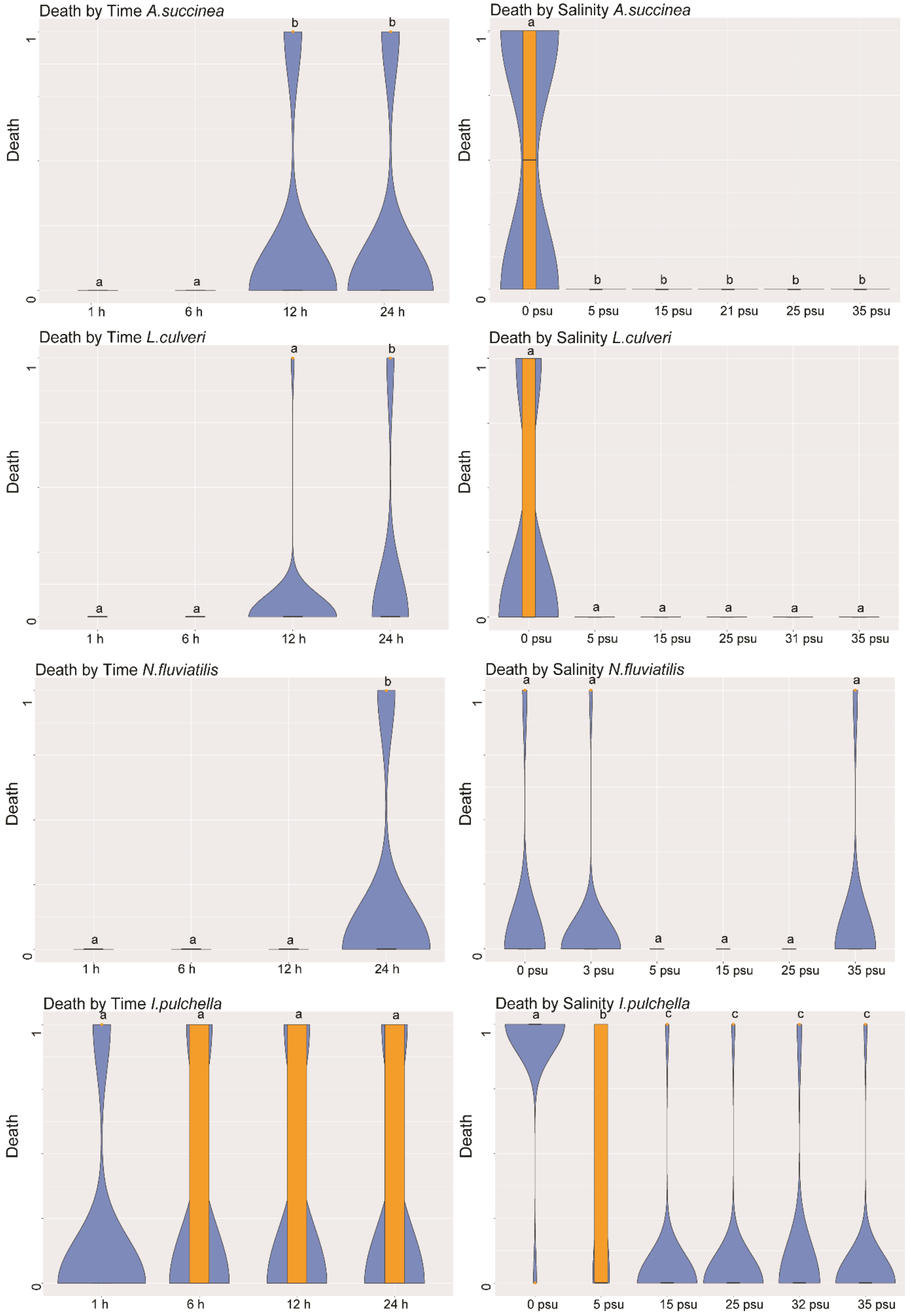

Fig. S2. Violin plots in blue and boxplots in orange referring to effects of salinity and time on the survival of the target species. Letters referring to the groups resulting from the Tukey HSD test.

Table S1. Model selection for each target species. Int = intercept; S = salinity;t = time; S:t = interaction between salinity and time; Df = degree of freedom; logLik = log likelihood; AICc = corrected Akaike Information Criterion; delta = difference between ranked AICc; weight = relative likelihood of the model (AICc Weight). + = factors considered per each model. In bold, best model selected.

	(Int)	**S**	**t**	**S:t**	**Df**	**logLik**	**AICc**	**delta**	**weight**
Alitta succinea									
model1	**24.57**	**+**	**+**	**+**	**25**	**-420.56**	**905.7**	**0**	**1**
model2	65.38	+	+		10	-490.25	1002.6	96.88	0
model3	79.85	+			7	-509.41	1033.9	128.13	0
model4	3.549		+		5	-578.47	1167.5	261.75	0
Laeonereis culveri									
model1	**36.95**	**+**	**+**	**+**	**25**	**-430.95**	**926.3**	**0**	**1**
model2	82.24	+	+		10	-514.91	1051.9	125.57	0
model3	103.6	+			7	-533.51	1082.1	155.72	0
model4	15.58		+		5	-604.74	1220	293.69	0
Nephtys fluviatilis									
model1	**-16.47**	**+**	**+**		**10**	**-632.35**	**1286.7**	**0**	**0.708**
model2	**5.106**	**+**			**7**	**-636.75**	**1288.5**	**1.78**	**0.291**
model3	-39.92		+		5	-644.87	1300.3	13.54	0.001
model4	-8.436	+	+	+	25	-619.61	1303.2	16.46	0
Isolda pulchella									
model1	**155.7**	**+**	**+**		**10**	**-599.22**	**1220.5**	**0**	**1**
model2	154	+	+	+	25	-589.44	1242.9	22.39	0
model3	123.2	+			7	-616.6	1248.2	27.73	0
model4	105.3		+		5	-671.02	1352.6	132.09	0

Chapter II

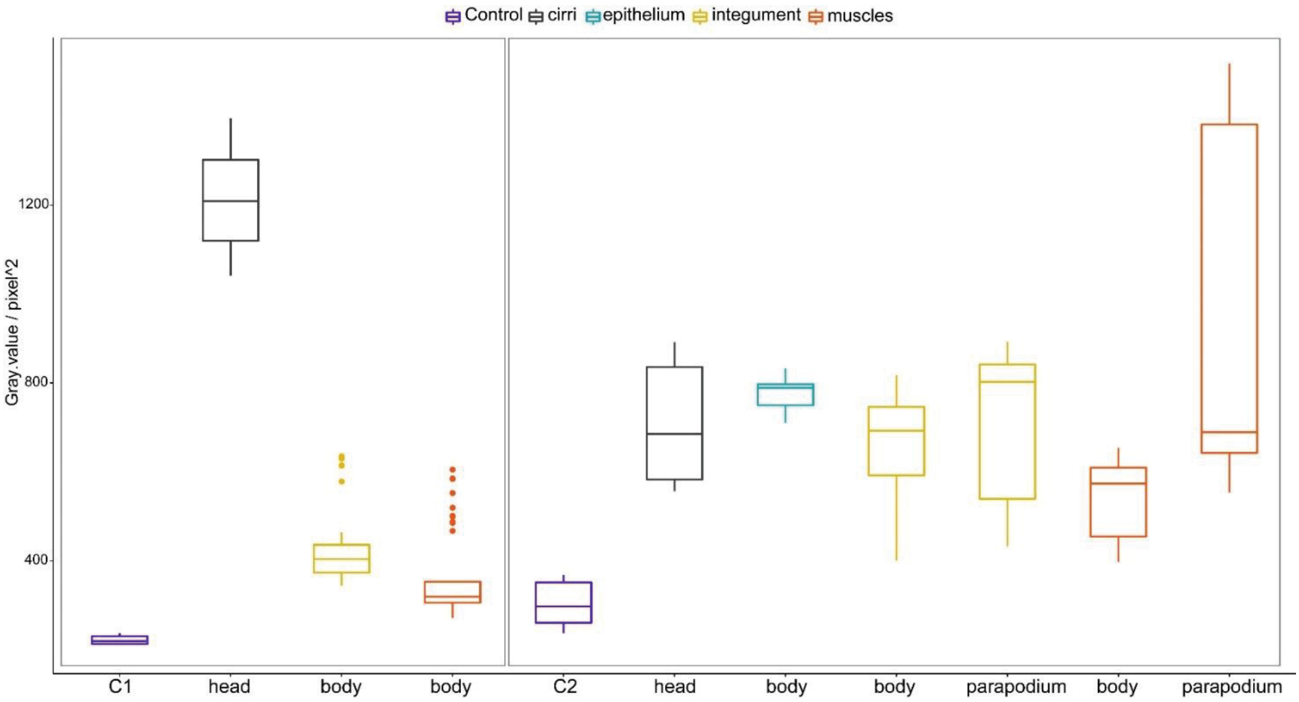

Figure S1. Boxplots of the variation of fluorescence intensity between the negative controls and the treatments in the annelid *Alitta succinea*. Axis x: C= negative control (1, 2, depending on the confocal session), plus the location of the tissues retrieved with the specific signal. Colours refer to the negative control and the tissues: violet= negative control, black= cirri, yellow= integument, orange= muscles, light blue= inner epithelium (digestive). Axis y representing the intensity of the fluorescence converted in gray value in each pixel².

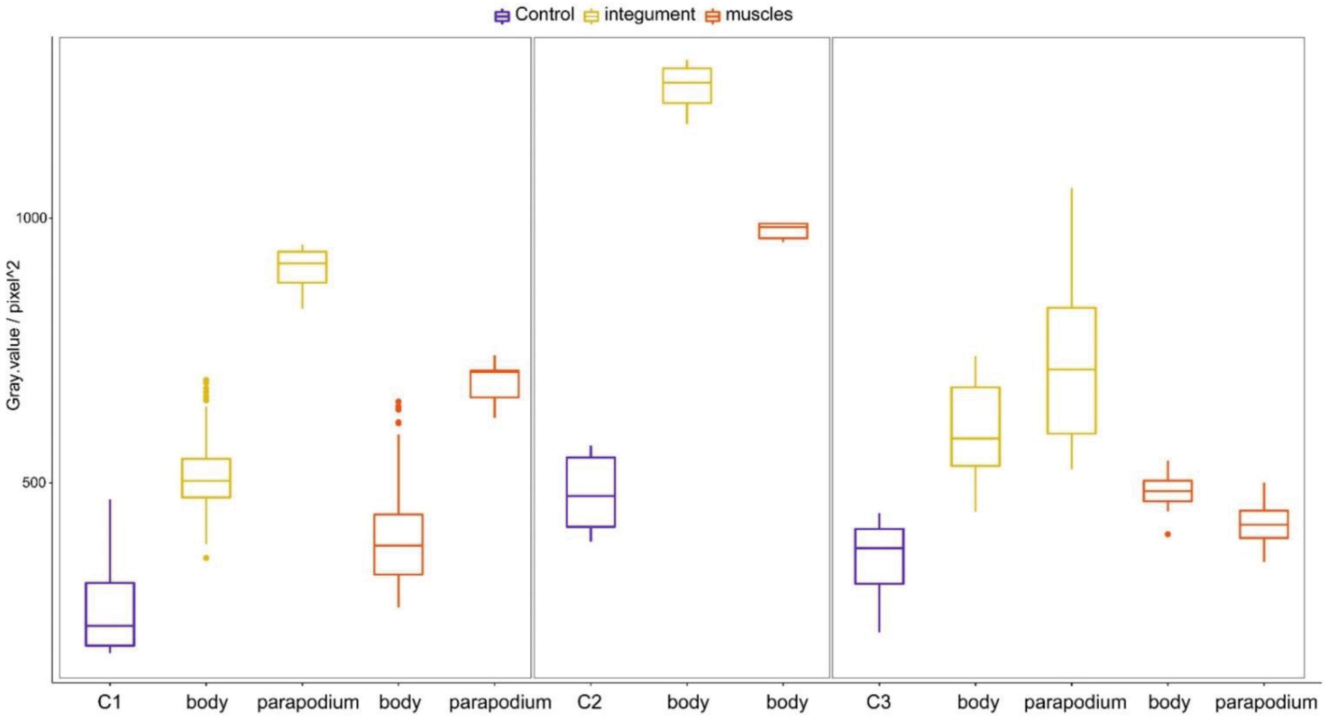

Figure S2. Boxplots of the variation of fluorescence intensity between the negative controls and the treatments in the annelid Laeonereis culveri. Axis x: C= negative control (1-3, depending on the confocal session), plus the location of the tissues retrieved with the specific signal. Colours refer to the negative control and the tissues: violet= negative control, yellow= integument, orange= muscles. Axis y representing the intensity of the fluorescence converted in gray value in each pixel².

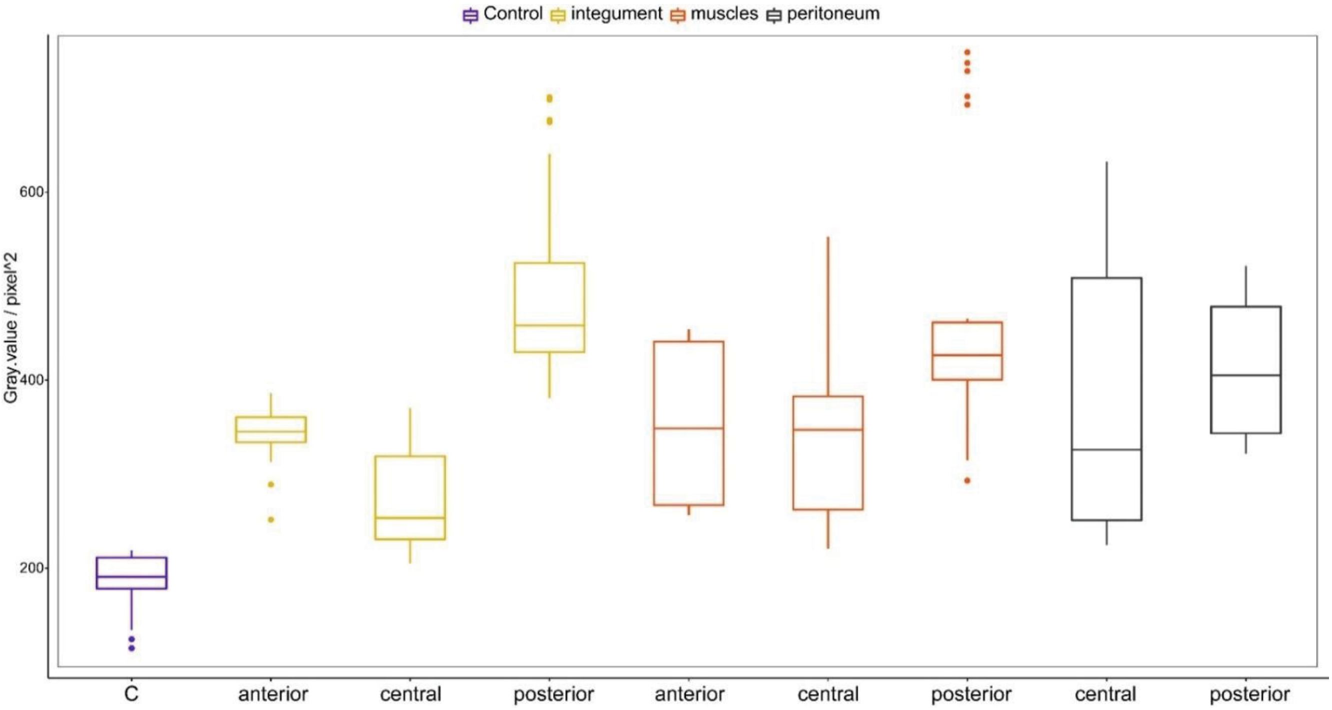

Figure S3. Boxplots of the variation of fluorescence intensity between the negative control and the treatments in the annelid Nephtys fluviatilis. Axis x: C= negative control, plus the location of the tissues retrieved with the specific signal. Colours refer to the negative control and the tissues: violet= negative control, yellow= integument, orange= muscles, black= inner epithelium (peritoneum). Axis y representing the intensity of the fluorescence converted in gray value in each pixel².

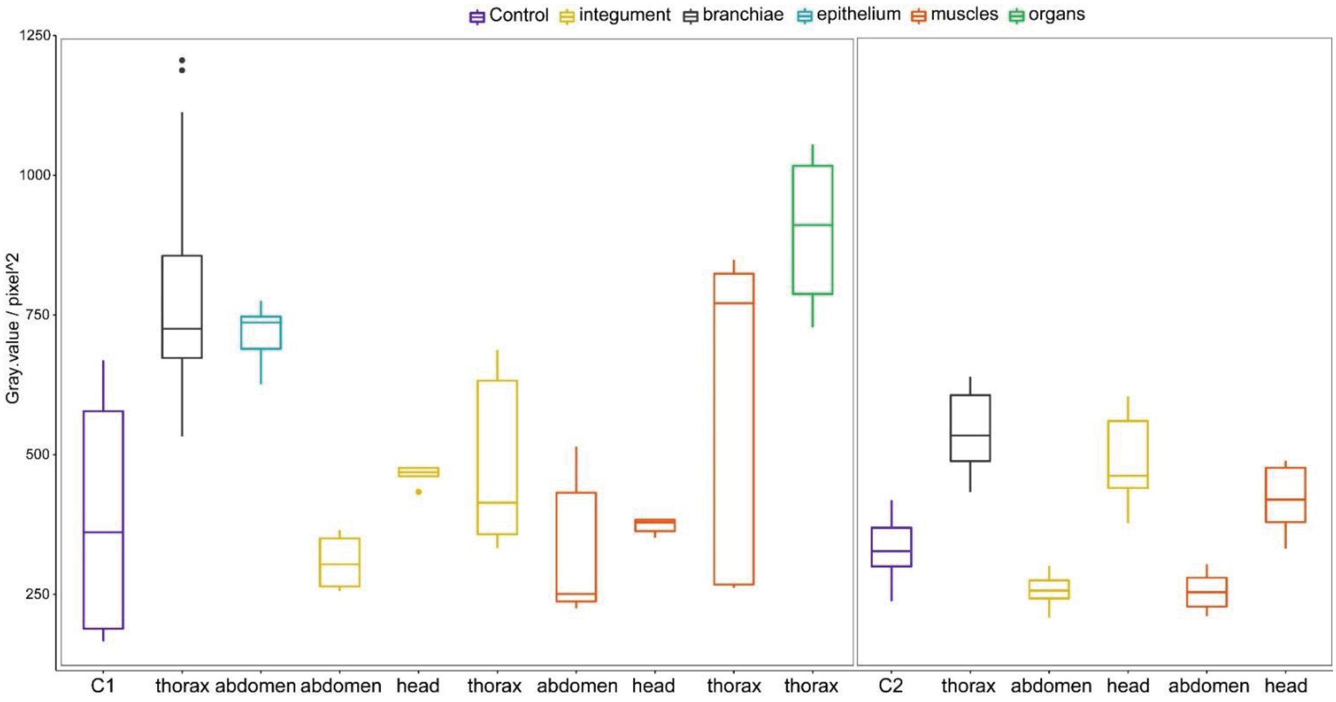

Figure S4. Boxplots of the variation of fluorescence intensity between the negative controls and the treatments in the annelid Isolda pulchella. Axis x: C= negative control (1,2, depending on the confocal session), plus the location of the tissues retrieved with the specific signal. Colours refer to the negative control and the tissues: violet= negative control, black= branchiae, light blue= inner epithelium (digestive), yellow= integument, orange= muscles, green= organs. Axis y representing the intensity of the fluorescence converted in gray value in each pixel².

Table S1. Genbank accession numbers of the NKCC amino-acid sequences used for the phylogenetic analysis.

Taxon		Accession number
Choanoflagellata	*Monosiga brevicollis*_KCCx	XP_001743661.1
Choanoflagellata	*Monosiga brevicollis*_CIP1 isoform1	XP_001742701_1
Porifera	*Amphimedon queenslandica*_N(K)CCx	XP_019858166.1
Placozoa	*Trichoplax adhaerens*_NKCC isoform1	XP_002110612_1
Cnidaria	*Hydra vulgaris*_N(K)CCx	XP_002159353.1
Mollusca	*Lottia gigantea*	XP_009048792.1
Annelida	*Capitella teleta*	ELU01077.1
Annelida	*Helobdella robusta*	XP_009021313.1
Nematoda	*Caeonorhabditis elegans*_NKCC isoform1	NP_001076724_1
Arthropoda	*Drosophila melanogaster*_NKCC isoform1	NP_648572_1
Arthropoda	*Daphnia pulex*_N(K)CCx	EFX74126_1
Chordata	*Ciona intestinalis*_N(K)CCx	XP_002131373_1
Chordata	*Homo sapiens*_NKCC1	NP_001037_1

Table S2. Pairwise test for *A. succinea*. NC= negative control; for the treatments always specified tissue and localization along the body (i.e. head or body). In bold, comparisons with p> 0.05. A and B referring to the confocal sessions, 14/06/2018 and 21/09/2018, respectively.

A	NC	Cirri head	Integument body	Muscle body
Cirri head	**0.000**	-	-	-
Integument body	**0.000**	**0.000**	-	-
Muscle body	**0.000**	**0.000**	**0.000**	-

B	NC	Cirri head	Epithelium body	Integument body	Integument parapodium	Muscle body
Cirri head	**0.000**	-	-	-	-	-
Epithelium body	**0.001**	0.704	-	-	-	-
Integument body	**0.000**	0.473	**0.028**	-	-	-
Integument parapodium	**0.000**	0.935	0.815	0.065	-	-
Muscle body	**0.000**	**0.011**	**0.000**	**0.000**	**0.001**	-
Muscle parapodium	**0.000**	0.320	0.383	0.173	0.629	**0.000**

Table S3. Pairwise comparisons using Wilcoxon rank sum test for the annelid *L. culveri*. NC= negative control; for the treatments always specified tissue and localization along the body. In bold, comparisons with p> 0.05. A, B and C referring to the confocal sessions, 11/05/2018, 08/11/2018 and 22/08/2019, respectively.

A

	NC	Integument body	Muscle body
Integument body	**0.001**	-	-
Muscle body	**0.001**	**0.010**	-

B

	NC	Integument body	Integument parapodium	Muscle body	Muscle parapodium
Integument body	**0.000**	-	-	-	-
Integument parapodium	**0.000**	**0.000**	**-**	**-**	-
Muscle body	**0.000**	**0.000**	**0.000**	**-**	-
Muscle parapodium	**0.000**	**0.001**	**0.010**	**0.000**	-

C

	NC	Integument body	Integument parapodium	Muscle body
Integument body	**0.000**	-	-	-
Integument parapodium	**0.000**	0.055	-	-
Muscle body	**0.000**	**0.007**	**0.000**	-
Muscle parapodium	**0.010**	**0.000**	**0.000**	**0.003**

Table S4. Pairwise comparisons using Wilcoxon rank sum test for the annelid *N. fluviatilis*. NC= negative control; for the treatments always specified tissue and localization along the body. In bold, comparisons with p> 0.05.

	NC	Integument anterior	Integument central	Integument posterior	Muscle anterior	Muscle central	Muscle posterior	Peritoneum central
Integument anterior	**0.000**	-	-	-	-	-	-	-
Integument central	**0.000**	**0.000**	-	-	-	-	-	-
Integument posterior	**0.000**	**0.000**	**0.000**	-	-	-	-	-
Muscle anterior	**0.000**	0.896	**0.007**	**0.002**	-		-	-
Muscle central	**0.000**	0.842	**0.000**	**0.000**	0.842	-	-	-
Muscle posterior	**0.000**	**0.000**	**0.000**	**0.050**	**0.050**	**0.000**	-	-
Peritoneum central	**0.000**	0.420	**0.006**	**0.002**	1.000	0.980	**0.020**	-
Peritoneum posterior	**0.000**	**0.017**	**0.000**	**0.020**	0.070	**0.050**	0.700	0.095

Table S5. Pairwise comparisons using Wilcoxon rank sum test for the annelid *I. pulchella*. NC= negative control; for the treatments always specified tissue and localization along the body. In bold, comparisons with p> 0.05. A and B referring to the confocal sessions, 08/11/2018 and 22/08/2019, respectively.

A	NC	Branchiae thorax	Integument abdomen	Integument head	Muscle abdomen
Branchiae thorax	**0.000**	-	-	-	-
Integument abdomen	**0.004**	**0.000**	-	-	-
Integument head	**0.005**	0.318	**0.002**	-	-
Muscle abdomen	**0.004**	**0.000**	0.979	**0.002**	-
Muscle head	**0.031**	**0.021**	**0.002**	0.467	**0.002**

B	NC	Branchiae thorax	Epithelium abdomen	Integument abdomen	Integument head	Integument thorax	Muscle abdomen	Muscle head	Muscle thorax	Organs thorax
Branchiae thorax	**0.000**	-	-	-	-	-	-	-	-	-
Epithelium abdomen	**0.000**	0.808	-	-	-	-	-	-	-	-
Integument abdomen	0.142	**0.000**	**0.000**	-	-	-	-	-	-	-
Integument head	0.619	**0.000**	**0.014**	**0.000**	-	-	-	-	-	-
Integument thorax	0.185	**0.000**	**0.003**	**0.000**	0.359	-	-	-	-	-
Muscle abdomen	0.302	**0.000**	**0.000**	0.187	0.095	**0.015**	-	-	-	-
Muscle head	0.889	**0.000**	**0.014**	**0.005**	**0.014**	0.255	0.359	-	-	-
Muscle thorax	**0.006**	0.550	0.714	**0.027**	0.359	0.176	**0.000**	0.359	-	-
Organs thorax	**0.000**	0.082	**0.014**	**0.000**	**0.002**	**0.000**	**0.000**	**0.002**	**0.023**	-

Chapter III

Table S1. Table with the species, the acronyms and the accession numbers of the sequences used for the phylogenetic analysis.

Phylum	Species	Short name	Database	Accession number
Porifera	*Amphimedon queenslandica*	Aque_XP_003383384.2	GenBank proteins	XP_003383384.2
Porifera	*Amphimedon queenslandica*	Aque_XP_019851797.1	GenBank proteins	XP_019851797.1
Placozoa	*Trichoplax sp. H2*	Tric_RDD45034.1	GenBank proteins	RDD45034.1
Cnidaria	*Nematostella vectensis*	Nvec_XP_001633187.1	GenBank proteins	XP_001633187.1
Cnidaria	*Nematostella vectensis*	Nvec_XP_001633212.2	GenBank proteins	XP_001633212.2
Cnidaria	*Nematostella vectensis*	Nvec_XP_001622649.2	GenBank proteins	XP_001622649.2
Cnidaria	*Nematostella vectensis*	Nvec_XP_001633227.1	GenBank proteins	XP_001633227.1
Cnidaria	*Nematostella vectensis*	Nvec_XP_032229526.1	GenBank proteins	XP_032229526.1
Xenacoelomorpha	*Isodiametra pulchra*	Ipul_QED21987.1	GenBank proteins	QED21987.1
Xenacoelomorpha	*Isodiametra pulchra*	Ipul_QED21989.1	GenBank proteins	QED21989.1
Xenacoelomorpha	*Isodiametra pulchra*	Ipul_QED21991.1	GenBank proteins	QED21991.1
Platyhelmintes	*Dugesia japonica*	Djap_BAR88202.1	GenBank proteins	BAR88202.1
Platyhelmintes	*Dugesia japonica*	Djap_BAR87948.1	GenBank proteins	BAR87948.1
Syndermata	*Brachionus plicatilis*	Bpli_BAR88202.1	GenBank proteins	RMZ94487.1
Syndermata	*Brachionus plicatilis*	Bpli_RNA05793.1	GenBank proteins	RNA05793.1
Syndermata	*Brachionus plicatilis*	Bpli_RNA41258.1	GenBank proteins	RNA41258.1
Syndermata	*Brachionus plicatilis*	Bpli_RNA34844.1	GenBank proteins	RNA34844.1

Mollusca	*Lottia gigantea*	Lgig_V3ZK38	JGI	V3ZK38
Mollusca	*Lottia gigantea*	Lgig_V4B1U8	JGI	V4B1U8
Mollusca	*Lottia gigantea*	Lgig_V4A5I3	JGI	V4A5I3
Mollusca	*Lottia gigantea*	Lgig_V4A7E8	JGI	V4A7E8
Mollusca	*Lottia gigantea*	Lgig_V3ZP04	JGI	V3ZP04
Mollusca	*Lottia gigantea*	Lgig_V4AZ69	JGI	V4AZ69
Mollusca	*Lottia gigantea*	Lgig_V4AIJ5	JGI	V4AIJ5
Mollusca	*Lottia gigantea*	Lgig_V3ZVR0	JGI	V3ZVR0
Mollusca	*Lottia gigantea*	Lgig_V4CLK0	JGI	V4CLK0
Mollusca	*Lottia gigantea*	Lgig_V3Z755	JGI	V3Z755
Mollusca	*Lottia gigantea*	Lgig_V4BDI9	JGI	V4BDI9
Mollusca	*Lottia gigantea*	Lgig_V3ZVP6	JGI	V3ZVP6
Bryozoa	*Bugula neritina*	Bner_KAF6016817.1	GenBank proteins	KAF6016817.1
Bryozoa	*Bugula neritina*	Bner_KAF6034639.1	GenBank proteins	KAF6034639.1
Brachiopoda	*Lingula anatina*	Lana_XP_013391176.1	GenBank proteins	XP_013391176.1
Brachiopoda	*Lingula anatina*	Lana_XP_013382159.1	GenBank proteins	XP_013382159.1
Brachiopoda	*Lingula anatina*	Lana_XP_013392640.1	GenBank proteins	XP_013392640.1
Brachiopoda	*Lingula anatina*	Lana_XP_013417896.1	GenBank proteins	XP_013417896.1
Brachiopoda	*Lingula anatina*	Lana_XP_013390337.1	GenBank proteins	XP_013390337.1
Brachiopoda	*Lingula anatina*	Lana_XP_013382376.1	GenBank proteins	XP_013382376.1
Brachiopoda	*Lingula anatina*	Lana_XP_013417580.1	GenBank proteins	XP_013417580.1
Brachiopoda	*Lingula anatina*	Lana_XP_013408467.1	GenBank proteins	XP_013408467.1
Annelida	*Alitta succinea*	AsucAQPa	Kocot et al 2016	34078_c0_seq1

Annelida	*Alitta succinea*	AsucAQPb	Kocot et al 2016	51122_c0_seq1
Annelida	*Alitta succinea*	AsucAQPc	Kocot et al 2016	55429_c0_seq1
Annelida	*Alitta succinea*	AsucAQPd	Kocot et al 2016	45933_c0_seq1
Annelida	*Alitta succinea*	AsucAQPe	Kocot et al 2016	387847_c0_seq1 + 296620_c0_seq1
Annelida	*Alitta succinea*	AsucAQPf	Kocot et al 2016	40672_c0_seq1
Annelida	*Alitta succinea*	AsucAQPx	Kocot et al 2016	38822_c0_seq1
Annelida	*Alitta succinea*	AsucAQPb_seq	this study	
Annelida	*Alitta succinea*	AsucAQPc_seq	this study	
Annelida	*Alitta succinea*	AsucAQPf_seq	this study	
Annelida	*Perinereis aibuhitensis*	Paib_71661_c0_seq1	GenBank TSA	71661_c0_seq1
Annelida	*Perinereis aibuhitensis*	Paib_103582_c0_seq2	GenBank TSA	103582_c0_seq2
Annelida	*Perinereis aibuhitensis*	Paib_110021_c0_seq1	GenBank TSA	110021_c0_seq1
Annelida	*Perinereis aibuhitensis*	Paib_52041_c0_seq1	GenBank TSA	52041_c0_seq1
Annelida	*Perinereis aibuhitensis*	Paib_61289_c0_seq1	GenBank TSA	61289_c0_seq1
Annelida	*Perinereis aibuhitensis*	Paib_81940_c0_seq1	GenBank TSA	81940_c0_seq1
Annelida	*Perinereis aibuhitensis*	Paib_108845_c1_seq2	GenBank TSA	108845_c1_seq2
Annelida	*Perinereis aibuhitensis*	Paib_109651_c0_seq1	GenBank TSA	109651_c0_seq1
Annelida	*Perinereis aibuhitensis*	Paib_96014_c0_seq1	GenBank TSA	96014_c0_seq1
Annelida	*Perinereis aibuhitensis*	Paib_108111_c0_seq2	GenBank TSA	108111_c0_seq2
Annelida	*Perinereis aibuhitensis*	Paib_84359_c0_seq1	GenBank TSA	84359_c0_seq1

Annelida	*Platynereis dumerilii*	Pdum_215254_c3	Pdumbase	215254_c3
Annelida	*Platynereis dumerilii*	Pdum_191183_c2_seq1_m.10782	Pdumbase	191183_c2_seq1_m.10782
Annelida	*Platynereis dumerilii*	Pdum_213112_c0	Pdumbase	Pdum_213112_c0
Annelida	*Platynereis dumerilii*	Pdum_8227_c1	Pdumbase	8227_c1
Annelida	*Platynereis dumerilii*	Pdum_118446_c0	Pdumbase	118446_c0
Annelida	*Platynereis dumerilii*	Pdum_218083_c0	Pdumbase	218083_c0
Annelida	*Platynereis dumerilii*	Pdum_215949_c0	Pdumbase	215949_c0
Annelida	*Platynereis dumerilii*	Pdum_213024_c0	Pdumbase	213024_c0
Annelida	*Platynereis dumerilii*	Pdum_117635_c0	Pdumbase	117635_c0
Annelida	*Platynereis dumerilii*	Pdum_211173_c5	Pdumbase	211173_c5
Annelida	*Platynereis dumerilii*	Pdum_211432_c0_seq2	Pdumbase	211432_c0_seq2
Annelida	*Eisenia andrei*	Eand_CAX48970.1	GenBank Proteins	CAX48970.1
Annelida	*Lumbricus rubellus*	Lrub_CAX48992.1	GenBank Proteins	CAX48992.1
Annelida	*Lumbricus rubellus*	Lrub_CAX48991.1	GenBank Proteins	CAX48991.1
Annelida	*Capitella teleta*	Ctel_25505	JGI	25505
Annelida	*Capitella teleta*	Ctel_176137	JGI	176137
Annelida	*Capitella teleta*	Ctel_224285	JGI	224285
Annelida	*Capitella teleta*	Ctel_148029	JGI	148029
Annelida	*Capitella teleta*	Ctel_180401	JGI	180401
Annelida	*Capitella teleta*	Ctel_46197	JGI	46197
Annelida	*Capitella teleta*	Ctel_37043	JGI	37043
Annelida	*Capitella teleta*	Ctel_172599	JGI	172599

Annelida	*Capitella teleta*	Ctel_26353	JGI	26353
Annelida	*Capitella teleta*	Ctel_115384	JGI	115384
Annelida	*Capitella teleta*	Ctel_120819	JGI	120819
Annelida	*Capitella teleta*	Ctel_21078	JGI	21078
Annelida	*Capitella teleta*	Ctel_45698	JGI	45698
Annelida	*Capitella teleta*	Ctel_166556	JGI	166556
Annelida	*Capitella teleta*	Ctel_184073	JGI	184073
Annelida	*Capitella teleta*	Ctel_219373	JGI	219373
Annelida	*Capitella teleta*	Ctel_142373	JGI	142373
Annelida	*Capitella teleta*	Ctel_197140	JGI	197140
Annelida	*Capitella teleta*	Ctel_125390	JGI	125390
Annelida	*Capitella teleta*	Ctel_164915	JGI	164915
Annelida	*Helobdella robusta*	Hrob_95007	JGI	95007
Annelida	*Helobdella robusta*	Hrob_68110	JGI	68110
Annelida	*Helobdella robusta*	Hrob_177042	JGI	177042
Annelida	*Helobdella robusta*	Hrob_185178	JGI	185178
Annelida	*Helobdella robusta*	Hrob_113524	JGI	113524
Annelida	*Helobdella robusta*	Hrob_176474	JGI	176474
Annelida	*Helobdella robusta*	Hrob_154942	JGI	154942
Annelida	*Helobdella robusta*	Hrob_185503	JGI	185503
Annelida	*Helobdella robusta*	Hrob_168509	JGI	168509
Annelida	*Helobdella robusta*	Hrob_185352	JGI	185352
Annelida	*Helobdella robusta*	Hrob_185353	JGI	185353
Annelida	*Helobdella robusta*	Hrob_168508	JGI	168508
Annelida	*Helobdella robusta*	Hrob_95422	JGI	95422
Priapulida	*Priapulus caudatus*	Pcau_XP_014665134.1	GenBank proteins	XP_014665134.1
Priapulida	*Priapulus caudatus*	Pcau_XP_014668506.1	GenBank proteins	XP_014668506.1
Nematoda	*Caenorhabditis elegans*	Cele_NP_001256246.1	GenBank proteins	NP_001256246.1

Nematoda	*Caenorhabditis elegans*	Cele_NP_001370535.1	GenBank proteins	NP_001370535.1
Nematoda	*Caenorhabditis elegans*	Cele_NP_001367603.1	GenBank proteins	NP_001367603.1
Nematoda	*Caenorhabditis elegans*	Cele_NP_001366692.1	GenBank proteins	NP_001366692.1
Nematoda	*Caenorhabditis elegans*	Cele_NP_001359963.1	GenBank proteins	NP_001359963.1
Tardigrada	*Milnesium tardigradus*	Mtar_G5CTG2.1	GenBank proteins	G5CTG2.1
Tardigrada	*Milnesium tardigradus*	Mtar_G5CTG3.2	GenBank proteins	G5CTG3.2
Tardigrada	*Milnesium tardigradus*	Mtar_G5CTG4.1	GenBank proteins	G5CTG4.1
Tardigrada	*Milnesium tardigradus*	Mtar_G5CTG1.2	GenBank proteins	G5CTG1.2
Tardigrada	*Milnesium tardigradus*	Mtar_G5CTG5.1	GenBank proteins	G5CTG5.1
Tardigrada	*Milnesium tardigradus*	Mtar_G5CTG0.1	GenBank proteins	G5CTG0.1
Arthropoda	*Daphnia pulex*	Dpul_EFX88760.1	GenBank proteins	EFX88760.1
Arthropoda	*Daphnia pulex*	Dpul_EFX88757.1	GenBank proteins	EFX88757.1
Arthropoda	*Daphnia pulex*	Dpul_EFX66203.1	GenBank proteins	EFX66203.1
Hemichordata	*Saccoglossus kowalevskii*	Skow_XP_006816957.1	GenBank proteins	XP_006816957.1
Hemichordata	*Saccoglossus kowalevskii*	Skow_XP_002737956.1	GenBank proteins	XP_002737956.1
Hemichordata	*Saccoglossus kowalevskii*	Skow_NP_001158487.1	GenBank proteins	NP_001158487.1
Hemichordata	*Saccoglossus kowalevskii*	Skow_XP_006813480.1	GenBank proteins	XP_006813480.1
Hemichordata	*Saccoglossus kowalevskii*	Skow_XP_006819080.1	GenBank proteins	XP_006819080.1
Echinodermata	*Strongylocentrotus purpuratus*	Spur_XP_001185961.1	GenBank proteins	XP_001185961.1

Echinodermata	*Strongylocentrotus purpuratus*	Spur_XP_799266.2	GenBank proteins	XP_799266.2
Echinodermata	*Strongylocentrotus purpuratus*	Spur_XP_030840396.1	GenBank proteins	XP_030840396.1
Echinodermata	*Strongylocentrotus purpuratus*	Spur_XP_789770.3	GenBank proteins	XP_789770.3
Echinodermata	*Strongylocentrotus purpuratus*	Spur_XP_030833260.1	GenBank proteins	XP_030833260.1
Chordata: CephaloChordata	*Branchiostoma floridae*	Bflo_XP_035679263.1	GenBank proteins	XP_035679263.1
Chordata: CephaloChordata	*Branchiostoma floridae*	Bflo_XP_035683956.1	GenBank proteins	XP_035683956.1
Chordata: CephaloChordata	*Branchiostoma floridae*	Bflo_XP_035695466.1	GenBank proteins	XP_035695466.1
Chordata: CephaloChordata	*Branchiostoma floridae*	Bflo_XP_035695470.1	GenBank proteins	XP_035695470.1
Chordata: CephaloChordata	*Branchiostoma floridae*	Bflo_XP_035668219.1	GenBank proteins	XP_035668219.1
Chordata: Tunicata	*Ciona intestinalis*	Cint_XP_002131566.1	GenBank proteins	XP_002131566.1
Chordata: Tunicata	*Ciona intestinalis*	Cint_XP_002131543.1	GenBank proteins	XP_002131543.1
Chordata: Tunicata	*Ciona intestinalis*	Cint_XP_004226091.2	GenBank proteins	XP_004226091.2
Chordata: Tunicata	*Ciona intestinalis*	Cint_XP_002131906.1	GenBank proteins	XP_002131906.1
Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_H3ADX4	Abascal et al 2014	H3ADX4
Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_H3ADX5	Abascal et al 2014	H3ADX5
Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_H3AEK5	Abascal et al 2014	H3AEK5
Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_H3BF11	Abascal et al 2014	H3BF11
Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_H3BF34	Abascal et al 2014	H3BF34
Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_H3BBE0	Abascal et al 2014	H3BBE0

Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_H3BIC5	Abascal et al 2014	H3BIC5
Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_H3AXY4	Abascal et al 2014	H3AXY4
Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_H3A425	Abascal et al 2014	H3A425
Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_M3XHS6	Abascal et al 2014	M3XHS6
Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_H2ZUR1	Abascal et al 2014	H2ZUR1
Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_H3ACF7	Abascal et al 2014	H3ACF7
Chordata: Vertebrata	*Latimeria chalumnae*	Lcha_M3XLI8	Abascal et al 2014	M3XLI8
Chordata: Vertebrata	*Rattus norvegicus*	Rnor_AQP0	Abascal et al 2014	G3V6E0
Chordata: Vertebrata	*Rattus norvegicus*	Rnor_AQP1	Abascal et al 2014	P29975
Chordata: Vertebrata	*Rattus norvegicus*	Rnor_AQP2	Abascal et al 2014	P34080
Chordata: Vertebrata	*Rattus norvegicus*	Rnor_AQP3	Abascal et al 2014	P47862
Chordata: Vertebrata	*Rattus norvegicus*	Rnor_AQP4	Abascal et al 2014	P47863
Chordata: Vertebrata	*Rattus norvegicus*	Rnor_AQP5_frg	Abascal et al 2014	Q6AYU6
Chordata: Vertebrata	*Rattus norvegicus*	Rnor_AQP6	Abascal et al 2014	Q9WTY0
Chordata: Vertebrata	*Rattus norvegicus*	Rnor_AQP7	Abascal et al 2014	P56403
Chordata: Vertebrata	*Rattus norvegicus*	Rnor_AQP8	Abascal et al 2014	P56405
Chordata: Vertebrata	*Rattus norvegicus*	Rnor_AQP9	Abascal et al 2014	P56627
Chordata: Vertebrata	*Rattus norvegicus*	Rnor_AQP11	Abascal et al 2014	Q8CHM1
Chordata: Vertebrata	*Rattus norvegicus*	Rnor_AQP12A	Abascal et al 2014	D4A9T6

Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP0	Abascal et al 2014	P30301
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP1	Abascal et al 2014	P29972
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP2_1	Abascal et al 2014	P41181
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP3_1	Abascal et al 2014	Q92482
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP4_isoM1x	Abascal et al 2014	NP_001304313.1
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP4_isoX1	Abascal et al 2014	XP_011524244.1
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP5	Abascal et al 2014	P55064
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP6	Abascal et al 2014	Q13520
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP7_1	Abascal et al 2014	Q5T5M1
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP8_isoX1	Abascal et al 2014	XP_011544124.1
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP9	Abascal et al 2014	Q6FGT0
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP10_1	Abascal et al 2014	Q96PS8
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP11	Abascal et al 2014	Q8NBQ7
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP12A	Abascal et al 2014	C9J6F6
Chordata: Vertebrata	*Homo sapiens*	Hsap_AQP12B	Abascal et al 2014	B2RXL4

Supplementary data S2. Alignment of metazoan MIPs sequences used for the phylogenetic analysis.

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>AQP_A
--------MAPSSDGI------R-------T----------PRF-W----------QTVLAESVGTLFLV--MIGCGSFVD---PSNAPPKDNPHAI-----------MPPN---ALRV-------------------
ALSFGLTYGVLVYALRYVS--G--GHLNPAVTVAMLA--ARKISILRMALYLLGQIIGALVGAAL-LRGVT--P----------AS-L---QG-------TL--GATL-PQH------G----I--------TGEKAFM-
VEFLAT-FVFLFVVRATGDCVS--GE-DNPP-A--MRPFVMGIALVAVELYA---V-PISGGGINPARSLGPAA----V-------MG------RM-----E---YH-WV-YWF----
GPILGGVLGALLYEYVFA----S--------------------------------NASLARVKSCLTTNEPPSNKPRQ---------------------------------------
>Paib_71661_c0_seq1
--------MAPSSDGI------R-------T----------SRF-W----------QTVMAESVGTLFLV--MIGCGSFVD---PNSPPSKDDPDAL-----------VPPN---ALRV-------------------
GLSFGLTYGTLVYALRYVS--G--GHLNPAVTIAMLA--ARKISILRALLYVLGQIIGALVGAAL-LRGVT--P----------PN-L---QG-------TL--GATL-PQH------G----I--------TAEKAFM-
VEFFAT-FIFLFVVRATGDTVS--GE-DNPP-A--MRPFVMGLALVAVELYA---V-PISGGGINPARSLGPAA----I-------MG------RM-----E---YH-WV-YWF----
GPILGGVIGAILYEYVFA----S--------------------------------NASLARVKSCLTSNEPPTNKPRQ---------------------------------------
>Ctel_25505
------------------------------------------F-W----------RAVFSEFLGTMLYV--IVGCG---------AWTENS----------------RVPEMTLSVRV-------------------ALAFGLIYAVIIYCVRNVT--D--
AHLNPSITLAMLV--TRRMCFLRAILYIVAQFLGAILGAAL-LYGLT--A----------RE-Y---LA-------QL--GCTI-PGD------E----V--------SDAQGFG-IELFST-FILVFVVFAAYE--K--SK-
REEA-I--TAPFIIGIALAAVSMFA---V-PYTGGSLNTARSFGPAV----I-------KN------VW-----S---HH-WI-YWF----GPLLGGILGGACYEIVYS----T--------------------------------K---------
----------------------------------------------------
>AQP_B
--------AVSSRTDL------K-------T----------KKF-Y----------VAVLAEFLGTMLLV--LASCGSTL------QGPVP-----------------VQST---TVRI-------------------ALTFAFSVGSIVWAIGNVS--
G--GNINPGVTIAFFV--TRRMSLIGFLLYIIAQTIGAITGAAI-LYSLS--P--------D-SY-----KG-------NL--GTPV-LAE------G----V--------SVVQGFF-VEMLLV-FILVLVVFASCD--E--
NR-GDVG-G--SVPLQIGVAIGMCHLWG---V-PLTGAGMNPARSFGPAV----IS------NS------LD-----A---DH-WI-YWI----GPLVGGMLAGIVYEFLFA----V--------------------------------
NATPAKLKGFFTR-NY-DDKEYDA--------------------------------------
>AQPb_seq
--------AVSSRTDL------K-------T----------KKF-Y----------VAVLAEFLGTMLLV--LASCGSTL------QGPDP-----------------VQST---TIRI-------------------ALTFAFSVGSIVWAIGNVS--
G--GNINPGVTIAFFV--TRRMSLIGFLLYIIAQTIGAITGAAI-LYSLT--P--------D-NY-----SR-------NL--GTPQ-LAE------G----V--------SVVQGFF-VEMLLV-FTLVLVVFASCD--E--
NR-GDVG-G--SVPLQIGVAIGMCHLWG---V-PLTGAGMNPARAFGPAV----IS------NS------LD-----A---DH-WI-YWI----GPLVGGMLAGLVYEFLFA----V-------------------------------
-NATPAKLKGFFTR-NY-DDKEYDA--------------------------------------
>Paib_103582_c0_seq2
--------AVSSRQDLRT-----------------------KKF-Y----------VAVFAEYIGTLLLV--LVACGSTL------QGGDD-----------------VQPA---TVRI-------------------SLSFAFSVGSIVWAIGNVS--
G--GNINPGVSIAFFI--TRRLSVVGFFLYIIAQTLGAITGAAL-LYAFV--P-------------EELSSK-------GL--GTPG-LAG-----------V--------SVVQGFF-IEMFLV-FVLVLVVFAACD--D--
LR-GDVG-G--SVPLQIGVAIGMCHLWA---V-PLTGAGMNPARAFGPAV----I-------AN------------RLTSDH-WL-YWI----GPLLGGALAGLMYEFLFA----V-------------------------------
-NATPAKLKGFFTRNYDDKDYDA---------------------------------------
>Pdum_215254_c3
--------GVSSRTDL------K-------T----------KKF-Y----------VAVLAEYIGTLLLV--LVSCGSIV------QGVAD-----------------TQGS---TVRI-------------------ALTFGFSVASVVWAIGNVS--
G--GNINPGVSIAFFV--TRRMSLVSFLFYVIAQVAGAITGAAI-LQALV--P--------S-NY-----NG-------NL--GTPT-FDT------A----T----F---SVVQAFF-VEFLIV-FILVLVVFASCD--E--
RR-VDVG-G--SVPLQIGVAIAMCHLWA---V-PLTGAGMNPARAFGPAV----IS------ND------IR-----G---SH-WV-YWV----GPLLGGMLAGFVYEFLFA----V------------------------------
--NATAAKFRGFFTM-DY-DEKDYDA--------------------------------------
>Eand_COMP57
--------STSSLQDLKT-----------------------RRF-W----------VALVAEFLGTLLLV--LVACGSCASYTTSYSFRNQTDGTEVIK---------TKPLPSDFVQI-------------------
SLAFGLSVATIVWSIAHVS--G--GHINPGVTIGFLV--TRKISLIRAILYTAVQSVGAVLGAVI-LKLVS--P----------PGLND-----------AL--GTTS-PGN------G----V--------SIGQAFT-
IELFIT-FVLVYTVFATCD-----GQRQGFN-G--SGPLAIGLSISMCHLWA---I-PYTGSGMNPARAFGSAL----V-------SG------NL------KADIH-WL-YWA----
GPLLGGALAGVLYDFLFA----T--------------------------------NASLDKLKGFFTSDYDDSQYDS---------------------------------------
>Lrub_COMP65
--------STSSLQDLKT-----------------------RRF-W----------VALLAEFLGTLLLV--LVACGSCAGYTTTYTYRNQTDGSEVVK---------TKPLPSDFVQI-------------------
SLAFGLSVATIVWSIAHVS--G--GHINPGVTIGFLV--TRKISLVRAILYTAVQSVGAVLGAVI-LKLVS--P----------PGLND-----------AL--GTTS-PGN------G----V--------SIGQAFT-
IELFIT-FVLVYTVFATCD-----GQRQGFK-G--SGPLAIGLSISMCHLWA---I-PYTGSGMNPARAFGSAL----V-------AG------KL------EPGIH-WV-YWA----
GPLLGGALAGILYDFLFA----T--------------------------------NATLDKLKGFFTSDYDDSQYDS---------------------------------------
>Hrob_95007
--------ARNSLQDLKS-----------------------VKF-Y----------QALACEFIGTFLLV--LVACGSCGRFVVTET-EVRTNSTNIVT---------SKLIPNDLVQI-------------------
SLCFGLSVATIVWSIAHVS--G--GHINPAVSIAFFV--TRKISFIRFILYAAVQTAGAIVGAYI-LKALT--P----------TGVND-----------SL--GSTL-LGS------G----V--------SKAGGLF-
VELFIT-FVLVFTVFATCD-----SNRQGFA-G--SGPLAIGLSIAMCHLWA---I-PYTGSGMNPARALGSHV----A-------SE------TI------KDHPYVWI-YWI----
GPLIGGMIAGLLYDLAFA----A--------------------------------NAGSSKFAGFFTADYEDENYDS---------------------------------------
>Hrob_68110
--------SSESVYEV------Q-------T----------SGF-W----------LSVAAEFVGTMLLV--IFGCGSCA----------------------------YEAI---PLAI-------------------ALSFGLSVSTVVWAIAHVS--G--
GHINPAVTMGFLA--TRKMSFIKAFMYVVSQMSGAIIGAVI-LNSLY--P----------ES-T---FK-------GL--CTPT-PKT------G----T--------SAIVTFT-VEFLVT-FLLVFVVFATCD--G--QR-
KGFA-G--SGPLAIGLSVTVGHLMG---M-NLTGAGTNPARVFGPAV----I-------SG------TF-----D---RH-WA-YWI----GPMAAGAVAGFIYDFLFA----V-------------------------------
NACTEKLKAFFTTADY-NDADFKK--------------------------------------
>Ctel_176137
--------QCCVPSEL------K-------A----------IRF-Y----------VACLGEVLGTFFLV--LVGCGSCS----------------------------PPGD---VVRI-------------------SLTFTLAIATIVWNVGRVS--G--
GHLNPAVTIGFLV--ARRITVGRAFFYVLAQVVGAILGAVT-LKGLV--AN-----REGWEK-F---RE-------SL--GTST-RAD------G----V--------TEVEVFG-VELLIT-FVLVWTVFATVD--
S--KR-SDTQ-G--SKPLAIGLAIGMCHLWA---V-PFTGAGMNPARVAGPAI----V-------SS------SY-----D---AH-WA-YWA----GPIVGGILAALIYEFIFA----V---------------------------------
NATSSKLRGWATTFTY-DEEEYDN--------------------------------------
>AQP_F
--------------------------------------------M------------------------------------------------------------------------------------------------LI--
ARKVSVARAVLYVLAQCIGAIIGPGL-LHGIP--P----------ED----NRG-------SL--GITA-PAI--------------------HNQQAFG-VEFLIT-FVLVFTVFATCD-----NKRTDIN-G--
SGPLTIGLSVTVCHLFA---I-PFTGSSMNPARSFGPAI----V-------AN------SW-----N---EH-WL-YWC----GPLLGGVLAGLLYDNVFA----A--------------------------------
NASLNKARGYLLASNYDAEKFDE---------------------------------------
>AQPf_seq
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RKVSVARAVLYVFAQCIGAIIGAGL-LHGIT--P----------ED----NRG-------SM--GITA-PTI--------------------HNQQAFG-VEFLIT-FVLVFTVFATCD--N--KR-TDIN-G--
SGPLTIGLSVTVCHLFA---I-PFTGSSMNPARSFGPAI----V-------SN------SW-----N---EH-WL-YWC----GPLLGGVLAGLLYDNVFA----A--------------------------------
NASLNKARGYLLASNYDAEKFDE---------------------------------------
>Pdum_191183_c2_seq1_m.10782
--------MAKSLDDVRS-----------------------PLF-W----------RAVFAELAGTLFLV--LVGCGSCI----------------------------TIKEAPNVVQI-------------------AICFGLSVATMVWCTAHVS--
G--GHINPAVTVAMLI--SRKVSVARAVLYVFAQCIGAIIGAGL-LHGVT-------------PGEQ---RG-------GL--GITAPATI-----------V--------HSQQAFG-VEFLIT-FVLVLTVFATCD-----
NKRTDLN-G--SGPLTIGLSVTMCHLFA---V-PYTGSSMNPARTFGPAV----V-------GN------KW-----D---EH-WV-YWC----GPLLGGVIAGLLYDNVLA----A-------------------------------
--NASMKKARGYLLASNYDAETFNE---------------------------------------
>Paib_110021_c0_seq1
--------MAKSLDDVKS-----------------------PLF-W----------RGVAAELLGTMFLV--FVGCGSCVK---------------------------MGEEGPTVVQI-------------------
ALCFGLIVATMVWCLAHVS--G--GHINPAVTVAMLI--TRKVSVARAVLYVFSQCVGAIIGAGL-LYGVI--P-------------AEKRS-------GM--GFTGPNAD-----------V--------
NPHQAFG-IEFLIT-FVLVFTVFATCD-----SKRTDIN-G--SGPLTIGLSVTVCHLFA---V-PFTGSSMNPARSFGPAL----V-------GK------VW-----E---EH-WV-YWC----
GPLLGGVVAGLLYDNAFA----A--------------------------------NASMKKARGYLLASTYDAENFEE---------------------------------------
>Lgig_V3ZK38
------------------------------------------L-W----------RSLAAETFGTLVIV--LLGCGTWI------TWS-------------------SHPN---LVQI-------------------SLTFGLTVATMVWVFTHIS--G--
GHVNPAVTLAKLV--TRRVSIVRGILYIIAQCLGGVLGAGI-LFGLT--P--------P-HK-----RT-------DL--GATAKLGE------S----V--------TAAQGFG-IELLVS-FVVVMAVFASND--T--
KR-RDLR-G--SQPLTIGLAVMVCHLFA---I-PYTRCGLNPARSFGPSL----V-------MG------IW-----S---NH-WV-FWI----GPLIGGVIAGILYEFVF--------------------------------------------
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>Lgig_V4B1U8
--------MVTSLDDIQS-----------------------VKF-W----------KAVIAEFLGTMLLV--LVACGSCITL--------------------------GKATAPTITQI-------------------SLCFGLSVATIVWNIAHVS--G--
GHINPAVTVGMFA--ARKISLARACFYVLFQLVGSIVGAGL-LMAMT--P-------------VEKHG-------TL--GMTLVSDK-----------I--------NIGQAVG-IEFFIT-FILVFTVFASCD-----
SKRKDLN-G--SIPLTIGLSVTMCHLFA---I-QYTGSSMNTARSFGPAV----I-------MA------VW---------DHHWV-YWL----GPILGGAVAGILYDHVFA----S---------------------------------
NSSLQKVKACLLTSAYDDDKYQA--------------------------------------
>Lrub_COMP64
--------IRDNFEELRS-----------------------WPV-W----------RAMVAEFIGTLMLV--FIGCGACI----GGAWSDL-----------------DDPT---VLGI-------------------
ALAFGLIVATMIWSFGHVS--G--GHVNPAVTFGFLV--ARRITIVRAALYIISQCAGAIVGCGI-LKGLS--P----------HNSNE-----------TF--GLTVVWKQ-----------I--------TPGQGCG-
VEIIIT-FVLVFCVFASVD-----GRRADLN-G--STPLSIGLSVTVCHLFA---V-RYTGSSMNPARTFGPAV----I-------TN------KW-----T---NH-WV-YWV----GPIIGGIIGALLYELVFS----
A---------------------------------SASLRNLKHFFTSPVYGQDEDVD---------------------------------------
>Lana_XP_013391176.1
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--------MATSAEDIRS-----------------------PGL-Y----------RALLAEMLGVLFLV--LVACGACT----------------------------NVKAQQNLITI--------------------SVCFGLSVATLVWCIANVS--
G--GHINPAVTIGFLV--TRKISIVRALFYFVSQLVGGIAGAAI-LMGLT--P----------TEYRA-----------SL--GTTQ-LTQ------G----V--------SVGQGFG-IEFMAT-FVLVFTVFATCD-----
SN-RNDLSG--SGPLAIGLSVAMCHLWA---V-PYTGAGMNTARSFGPAV----I-------FG----GPAW-----D---NH-WV-YWV----GQLLGGVVAALLYDLLFA----V---------------------------
------NSSVDKLKSCGTSRHYNESDTYAV----------------------------------------
>Lana_XP_013382159.1
--------AVRSLQELTS-----------------------LGL-Y----------RSALAEFVGTLFLV--FVACGSV-----------------------------TGRTAPSIVQV--------------------AFTFGLSVATVVWAIAHVS--G-
-GHVNPAVTVAMVT--TRRISVARAVLYVIMQLLGAAAGAGL-LKAVV--P----------YSSINNPRG--------L--GVTTVDGE-----------V--------TAGQALG-IEFLIT-FVLVFTVFATCD-----
GRRSDLS-G--SGPLAIGLSVTMCHLFA---I-QYTGSSMNTARSFGPAL----V-------QG------EW-----S---KH-WI-YWF----GPILGGMAAGLLYDFVFA----A--------------------------------
-DACKNKFKQYLTNWKYDPDTFSE----------------------------------------
>Skow_XP_006816957.1
--------MTSSKDDLRD-----------------------PKF-W----------RAVVGEFLATTLFV--FIGIGSTC------GW--------------------NPPYTPSMVQI--------------------
ALCFGLTIATMVQCFGHVS--G--ANINPAVTCALLV--TRKISFLRAFLYVIAQCIGAVAGAGL-IYGVT--P----------AG----VRG-------GL--GATS-LGT------G----V--------AVEQGFA-
IEYLIT-FELVFTVFATID--P--NR-KDLQ-G--SASLAIGIAVVIGHLFA---I-QFTGASMNSARSFGPAV----I-------MN------FW-----E---DH-WI-YWA----GPILGGITAGVTYEYLFS----
A---------------------------------KTTKDTISSCMLSR-------------------------------------------------
>Hsap_AQP4_isoM1x
--------PLCTRENIMVAFKGVWT----------------QAF-W----------KAVTAEFLAMLIFV--LLSLGSTI------NWGGT-----------------EKPLPVDMVLI--------------------
SLCFGLSIATMVQCFGHIS--G--GHINPAVTVAMVC--TRKISIAKSVFYIAAQCLGAIIGAGI-LYLVT--P----------PS----VVG-------GL--GVTMVHGN-----------L--------TAGHGLL-
VELIIT-FQLVFTIFASCD--S--KR-TDVT-G--SIALAIGFSVAIGHLFA---I-NYTGASMNPARSFGPAV----I-------MG------NW-----E---NH-WI-YWV----GPIIGAVLAGGLYEYVFC----
P---------------------------------DVEFKRRFKEAFSKAAQQTKGSY----------------------------------------
>Hsap_AQP4_isoX1
--------IMVAFKGVWT-----------------------QAF-W----------KAVTAEFLAMLIFV--LLSLGSTI------NWGGT-----------------EKPLPVDMVLI--------------------
SLCFGLSIATMVQCFGHIS--G--GHINPAVTVAMVC--TRKISIAKSVFYIAAQCLGAIIGAGI-LYLVT--P----------PS----VVG-------GL--GVTMVHGN-----------L--------TAGHGLL-
VELIIT-FQLVFTIFASCD--S--KR-TDVT-G--SIALAIGFSVAIGHLFA---I-NYTGASMNPARSFGPAV----I-------MG------NW-----E---NH-WI-YWV----GPIIGAVLAGGLYEYVFC----
P---------------------------------DVEFKRRFKEAFSKAAQQTKGSY----------------------------------------
>Rnor_AQP4
--------PPCSRESIMVAFKGVWT----------------QAF-W----------KAVTAEFLAMLIFV--LLSVGSTI------NWGGS-----------------ENPLPVDMVLI--------------------
SLCFGLSIATMVQCFGHIS--G--GHINPAVTVAMVC--TRKISIAKSVFYITAQCLGAIIGAGI-LYLVT--P----------PS----VVG-------GL--GVTTVHGN-----------L--------TAGHGLL-
VELIIT-FQLVFTIFASCD--S--KR-TDVT-G--SVALAIGFSVAIGHLFA---I-NYTGASMNPARSFGPAV----I-------MG------NW-----E---NH-WI-YWV----GPIIGAVLAGALYEYVFC----
P---------------------------------DVELKRRLKEAFSKAAQQTKGSY----------------------------------------
>Lcha_H3ADX4
--------PLCTKESI------MVAFRGIWT----------QTF-W----------KAVSGEFLAMLIFA--FLSLGSTI------SW-SEN----------------QQ-----LVLM--------------------
SLCFGLSLATMIQCFGHIS--G--THINPAVTISLVC--IKKLSLAKAIFYVAAQCLGAVVGAGL-LYLVT--P----------AD-R---VG-------NL--GATL-VNS------S----L--------SAGQGLL-
IEIIIT-FQLVFNVVASSD--T--KR-NDVK-G--SIALAVGYSVTIGHLFA---I-NYTGASMNPARSFGPAV----I-------MG------IW-----D---NH-WV-YWV----GPLMGGISAAALYEYLYC---
-P---------------------------------DPELKCLLKEVLAKAT-KPSGGKH----------------------------------------
>Lcha_H3ADX5
--------PLCTKESI------MVAFRGIWT----------QTF-W----------KAVSGEFLAMLIFA--FLSLGSTI------SWSENQQ----------------------LVLM--------------------
SLCFGLSLATMIQCFGHIS--G--THINPAVTISLVC--IKKLSLAKAIFYVAAQCLGAVVGAGL-LYLVT-------------PADR---VG-------NL--GATL-VNS------S----L--------SAGQGLL-
IEIIIT-FQLVFNVVASSD--T--KR-NDVK-G--SIALAVGYSVTIGHLFA---I-NYTGASMNPARSFGPAV----I-------MG------IW-----D---NHWWV-YWV----GPLMGGISAAALYEYLYC-
---P---------------------------------DPELKCLLKEVLAKATKPSGGKH-----------------------------------------
>Skow_XP_002737956.1
--------EQTMVGQIRS-----------------------ATF-W----------RAVLAECVAMAIFV--FIGIASTIN---------------------------SPGEDATMVQI--------------------ALGFGISIATMVQCFGHVS--
G--AHINPAVTVAAFC--TRKVNILVTMFYILAQCVGAIVGAAL-LYALL--P------------TSDIRG-------TL--GVTSIAGV--------------------HNWQGLF-IEIILT-FQLVLTIFATID-----
SRRSDLL-G--SASLSIGLSVVIGHLAG---I-RFTGASMNPARSFGPAV----V-------MN------AW-----T---DH-WV-YWV----GPIIGGVLAAFLYEFVFE----P---------------------------------
GSNITRVRNVYSKV--------------------------------------------------
>Lgig_V4A5I3
--------------EL------K-------T----------LNL-W----------RAVFAEFLATMLFV--FLGCAATL------TGT-------------------GTAK---EVKI--------------------AFTFGLAIMAMIQMIGHVS--G--
GHVNPAVTVAMTV--AMKSSVIRAVLYVFAQSGGAIVGAFI-LKGVT--P----------SV-F---HD-------NL--GVND--IA------G----I--------TSAQGFG-IELILT-FVLVIVIFGTTD--G--DR-
PAF--G--SQALLIGLTVTLGHVSS---I-SFTGASMNPARSLGSAV----A-------SN------SW-----D---NH-WI-YWV----GPILGGVLAALVYKLILS----P--------------------------------------------
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>Hrob_177042
--------MERNWKEL------K-------S----------FSL-W----------KAMLAEMVGTSLLV--LFGCGTCI------GHDWQS----------------RDPT---IVQI--------------------
SLTFGLTVATIVHCMEHIS--G--GHINPAVTCAMFI--TRRVTIVRAVLYIAAQLTGSIYGAAI-LKGIT--P----------VV-Y---EG-------DL--GQTS-VDE------T----L----R---ACPDGLT-
VEASIT-FILVLTIFASCE--T--NK-NDRN-G--SAAFSIGFAVAFCHMFA---I-KYTGSSMNPARSFGPAV----I-------GG------NW-----N---DH-YV-FWV----GPVLGAVIAGLSYDFIFA--
--R---------------------------------NAGVEKFIHFFTEEDYNGDSKWS----------------------------------------
>Hrob_185178
--------LTRNVRDL------K-------D----------RVT-W----------QSALAELLGTCILI--VIGTGTCI------GKDWEA----------------NTPT---IVQI--------------------SLTFGLAVATVVRCIGHVS--
G--GHINPAVTCAMLA--TRRVTLSKAIIYIVSQCLGAIIGSAI-VMAIT--P----------AD-Y---MG-------DL--GQTS-VSS------S----I----K---AFPRAVA-VEAFIT-FVLIFTIFASCD--A--NR-
HDLS-G--STPLSIGFAVAFCHMFA---I-KYTGSSMNPARTFGPAV----V-------GG------TW-----E---HH-WV-YWI----GPITGAIFAGLIYEFIFA----S---------------------------------
NATPAKMRKYFSDPDYVGDRIFV----------------------------------------
>Ctel_224285
--------MTSSREDVRS-----------------------PHV-W----------RGVAAEFLGTGLMV--LVGCGACVG---------------------------GKGQAESVVQI--------------------
SLAFGIAVSITMWTVGHVS--G--GHINPAVSFAMLV--SRRISLAKAAMYVLAQCLAATTGAGV-LYGLTPGP----------------IRG-------TL--GATVPHAD-----------I--------TAAQSFT-
IEAILA-FVWVLTLFATSD-----ANRADLSRG---APLATGLSVTMCHLYA---V-RLTGASMNPARSFGPAI----V-------TN------TW-----T---DH-WV-YWF----
GPLLGSALAAILYENTLA--------------------------------------LNPQEGDLL------------------------------------------------------
>Paib_52041_c0_seq1
---------------------------------------------M-------------------------------------------------------------------------------------------------------------------G--------------------------------------------------------------
--------------------------------------------------------------------------------------------SRSLAIGLSICLGHLVA---Y-NYTGASMNPARSIGPAL----V-------MN------IW-----T---DH-WV-YWV----
GPMLGGIIGGFTYEYTHD----S---------------------------------SGKLQTIRRSFRRH-------------------------------------------------
>Pcau_XP_014665134.1
--------MVSVKEEI------R-------N----------FQF-W----------KAVRTEFLITLLLV--FVGCGAAV------RWSEA-----------------SPAN---DIQV--------------------GLAFGLAVATFVQCVGHIS-
-G--GHMNPAVTLGMLV--TRNVSILRAVFYCAVQCLGAIAGSAI-LYGVL--P----------KD-I---RA-------DL--GTTR-VNE------Q----F--------SLAQAFG-VEFMIT-FIYVFTVFANLD--
P--KR-QDMG-S---RSLAIGLSVTLGHLFA---Y-SYTGASMNPARSLGPAL----I-------MN------IW-----E---NH-WL-YWV----GPMMGGIFGGFTYEYTHD----S-----------------------------
----SNAFQYLRRSFRRKWPTAVQRSR----------------------------------------
>Pcau_XP_014668506.1
--------KVYSVREL------T-------N----------FQF-W----------KAVRTELLATLLLA--MTGCGVAL------QCTEA-----------------TPAD---DLKV--------------------
ALAFGLTVATLVQCVGHVS--G--GHMNPAVTVGMLV--TGNISPARAVCYSMAQCTGAIAGTAV-LYGLI--P----------AT-L---RG-------DL--GLTR-VHA------A----V--------
SLAQAFG-IEFIIT-FVYAFAVFANLD--P--KR-KSMG-S---RSLAIGFAALVGNLFG---F-HYTGASMNPARSLGPAV----V-------LN------SW-----E---HH-WI-YWL----
GPVLGGVLGGFTYEYTHD----S---------------------------------KRFPQRLRLSFLTVSASELPRSET----------------------------------------
>Paib_61289_c0_seq1
--------MSLVRN---------------------------SRF-W----------RAVLAEMIGSLFFV--LFTSGSMSTLRGAVCGPVGVNFA-------------TPSGSNPEIDL---
INCVSSNQTLYVVYNFIHSCVTGVSAMTLSWMLYDVS--G--GHISPAVTAAMLT--TRRVSLECAVIYLAAQCVGSLAACGI-LVGLL------------------------------------------------------------
------------------------------------------------------------------G----------SRQVGATV-------------------------------------------------------------------------------------------------------------------------------------
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>Paib_81940_c0_seq1
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-----------------------------------------------------------MS-FFYVFAFFATMD--R--CR-GESS-S--ASLVAFGGAVTMASVLG---V-KSTGASMNPARSLGPAI----I-------EN------VW-----D---
KH-WI-FWF----GPLLGGTVGG---------------------------------------------------------------------------------------------------------------
>Pdum_213112_c0
--------MSLVR-----------------N----------SRF-W----------RAVLAEMIGSLFLV--LFTSGTLS------VW------------
RGAVCGPSDLPDGLTTSSSPVIDTNCLSNQTLFVFNFIQSCMTGVSAMTITWMLFDVS--G--GHLSPAVTAAMLT--TRRISLECAVIYFAAQCAGSLAACGI-LVGLF--SS---------
R----------------QV--GAIV-IPA------E----V--------YSGRAFA-IEFTLT-FFYVFAFFATMD--R--CR-GESS-S--ASLLAFGGAVTLASVLG---I-KTTGASMNPARSLGPAI----V-------EN---
---VW-----D---KH-WI-YWF----GPLLGGSVGGLLFDIVFS----M---------------------------------NASVLRAKAFVNDQLYNADHFDS----------------------------------------
>Hrob_113524
--------MANHCEEIKT-----------------------PAF-W----------KAQFAEFFGTAILV--FIGCGSTLP---------------------------LEGMKRHDLEI--------------------STAFGLAVATSVWIFGHVS--
G--GHINPAVTLGFII--ARRISVLKGFFYMIFQCLGALAGAAV-LYACV------------------GVNG------KSI--GATSIISR-----------M--------GFMEALG-VETIIT-FVLVMTVFASCD-----
VKRRDLG-G--SRPLTIGISVLICHLAA---I-RSTGASMNPARSFGPAV----I-------DN------YW-----Q---NH-WV-YWV----GPMVGGLIAATVYEVIFA---------------------------------------
KDADPRDFCCFTIEREQPAQHKE----------------------------------------
>Hrob_176474
--------MGNMCDEVKT-----------------------AMF-W----------KAQLAEFLGTAILV--FVGCASCL----GDEW--------------------SRVASPRLVEI--------------------
SLAFGLAVATSVWIFGHVS--G--GHINPAVTCAFLI--ARRISILKGLFYILFQCLGAMTGAGF-LYEET--------------------------------------------------------------NCHQKEV--------------------
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>Mtar_G5CTG2.1
--------PRSSIEDLAK-----------------------IQF-W----------KALGAEFIGTAVLV--YIGCGAAVTSTP------------------------DANRDAFVTRV--------------------
SLAFGLTVATMVWAICGVS--G--GHINPAVSLGFLV--TRRISLVRFLLYVAFQCSGAVAGAAL-LYASTFDS---------------VKRG-------GF--GTNSMATE------NGQYLI--------
SPAQGIL-IEAIIT-FVLVFTVFATCD-----AKRSDLK-G--SGPLAIGIAVLISHLVA---I-PLTGTSMNPARSLGPAV----L-------IG------FW-----T---DH-WV-FWV----
GPMLGGAVAGLLYDMAFA----A---------------------------------DASLRKFGECAVADDYDPDADDR----------------------------------------
>Bner_KAF6016817.1
--------LQTTLRDL------K-------D----------LNV-Y----------RATLAEFLGVMFLV--IIGCASPY------GE--------------------RTID---HTRI--------------------SLSFGMSVATIVWVINNVS--G--
GHINPAVTLAMLV--TRKITMVRCILYIGAQMAGAIIGILI-LKGLI--CN-----WQL-AYYT---REQLNITECGL--ALNT-LGK------E----V--------SPGQGFG-VEVLIT-FMLVMTVFATCD-
-Q--SR-TDLH-G--SGPLAIGLAVTIGHLGF---I-EITGASMNPARSFGPAV----V-------LG------DY-----T---NQ-WV-YWV----GPFVGAILAGVVYEFLFA----G-------------------------------
--NAGTTKVVEFFTEVDYNPENEPF----------------------------------------
>Nvec_XP_001633187.1
----------MFLREFLT-----------------------GNF-W----------ASVWAELMATALFV--FLTTGSAI------TW--------------------DPLLPPSIEHI--------------------SLSFGLSIATLAMCTAHIS--G--
GHINPAVTISFMI--VRKVSFLRGAFYVIGQVGGGIAGSAM-LYGLT--P--------------VDKRG-------TL--GATV-PNA------G----V--------STGQAFG-IEFLLT-FLLVLTIFATTD-----AK-
RNHYGY--EVPLAIGLCVTVCHLVG---I-RFTGCGINPARSFGPAV----I-------MN------IW-----T---DH-WV-YWA----GPVAGAILASLLYHFVFR--------------------------------------
ARKELGPN--------------------------------------------------------
>Nvec_XP_001633227.1
--------YCGFTKEL------Y-------S----------RRF-W----------TEILAEFIITSLFV--SIVCGTALQ-----NWS--------------------TPPT---LTHM--------------------ALNSGLAAGTFAMCMWDVS--
S--GLFNPALTIGFLI--TGKKTLLQTIFYIMAQLTGSICGAAA-IYGMA--SQ-----HSVEAS--------------NL--AVNA-RSP------D----V--------SIGQAVG-MEIWAT-FILVLIVFAAGD--S--
DR-QHMRGY--GPPLSIGIVVFINLSLT---I-PISGGSMNPARSFGPAV----V-------MN------SW-----K---DH-WM-YWI----GPIAGSCLASLCYHHVFA----Q----------------------------------
RAINARISSAEEMASKSHQNEGFNC--------------------------------------
>Nvec_XP_001633212.2
--------MPCFQHK---------------------------QLF-I-----------GALAELLASHVFV--FLVCASCV------SWPHF-----------------QAPS---VQHI--------------------ALTAGLTTSTLVMVMSHVT--
G--GQINPAVAVAMVV--TRRVKPVHGLVFVFSQVLGGLLGAAL-LFGLT--P----------SS----IRG-------SL--GMTV-PAP------T----I--------QVGQAVA-METILT-FLLVFGILAATD----
-ER-KALKGY--EKAAAVGFCVFICHMAG---I-PFTGCSMNPARSLGPAV----V-------MD------HW---------RHHWV-YWV----GPFAGSILASLFYGRVFV-----------------------------------
---SGSVAVEQDDTNA--------------------------------------------------
>Nvec_XP_032229526.1
--------ASSSSNDMES-----------------------TGFII----------SASLVEIFGSLLYG--FLGCASTV----------------------------SFDSPRSIESI--------------------ALSYGILYAVLVYILAPFS--G--
GYVNPIVTIALLL--TRKVSVLKAVFYIISQITGGIAGVAL-FSTIL--P------------FENVME-----------VSQF-NGN-----------I--------GPIACFV-MEVVLS-FLFVCTVLVCRE--------
NDLSHF--LVGSASGVTLIACHLAE---F-QLVGWGINPACALGAAV----M-------SN------RP---------VHVWV-YLV----GPVTGSTLAAGFHYASKY--------------------------------------
FPGFVPDDTFLIKGEAINLPEVKRCESG------------------------------------
>Hsap_AQP1
--------MASEFKK--------------------------KLF-W----------RAVVAEFLATTLFV--FISIGSAL----GFKYPVGN----------------NQTAVQDNVKV--------------------
SLAFGLSIATLAQSVGHIS--G--AHLNPAVTLGLLL--SCQISIFRALMYIIAQCVGAIVATAI-LSGIT----------------SSLTGN-------SL--GRND-LAD------G----V--------NSGQGLG-
IEIIGT-LQLVLCVLATTD-----RRRRDLG-G--SAPLAIGLSVALGHLLA---I-DYTGCGINPARSFGSAV----I-------TH------NF-----S---NH-WI-FWV----GPFIGGALAVLIYDFILA----P-
--------------------------------RSSDLTDRVKVWTSGQVEEYDLD----------------------------------------
>Rnor_AQP1
--------MASEIKK--------------------------KLF-W----------RAVVAEFLAMTLFV--FISIGSAL----GFNYPLER----------------NQTLVQDNVKV--------------------SLAFGLSIATLAQSVGHIS-
-G--AHLNPAVTLGLLL--SCQISILRAVMYIIAQCVGAIVASAI-LSGIT----------------SSLLEN-------SL--GRND-LAR------G----V--------NSGQGLG-IEIIGT-LQLVLCVLATTD-----
RRRRDLG-G--SAPLAIGLSVALGHLLA---I-DYTGCGINPARSFGSAV----L-------TR------NF-----S---NH-WI-FWV----GPFIGSALAVLIYDFILA----P---------------------------------
RSSDFTDRMKVWTSGQVEEYDLD----------------------------------------
>Lcha_H3AEK5
-----------MAREF------Q-------Q----------KVF-W----------LAVVAEFLAMTIFV--FIGIGSAI------SFQVVPTT--------------NVTAVQDNVKV--------------------
SLAFGLGITTMAQSVGHVS--G--AHLNPAVTLGLLV--SSQISILKALLYMLAQVLGAIVASAI-LHAVS--P----------N------KH-------VL--GLNQ-LAM------N----L--------SPGQGIV-
FELIIT-FQLVLCVLATTD--K--RR-TDLT-G--SAPLAIGFSVTLGHLIA---ISQHAFVGLQLSTHFSLAR----K-------CR------QF-----S---SL-LI-YWV----GPMIGGLAASLIYDFILA----P-
--------------------------------RSCDLSDRMKVWASGEMEEYQLE----------------------------------------
>Hsap_AQP2_1
-----------MWELRS-----------------------IAF-S----------RAVFAEFLATLLFV--FFGLGSAL------NWPQ------------------ALPS---VLQI--------------------AMAFGLGIGTLVQALGHIS--G--
AHINPAVTVACLV--GCHVSVLRAAFYVAAQLLGAVAGAAL-LHEIT--P----------AD----IRG-------DL--AVNA-LSN------S----T--------TAGQAVT-VELFLT-LQLVLCIFASTD-----ER-
RGENPG--TPALSIGFSVALGHLLG---I-HYTGCSMNPARSLAPAV----V-------TG------KF-----D---DH-WV-FWI----GPLVGAILGSLLYNYVLF----P---------------------------------
PAKSLSERLAVLKG-LEPDTDWEE----------------------------------------
>Nvec_XP_001622649.2
--------LKVGVDEV------F-------T----------GSL-W----------ICVFAEYLGTLLFM--FSVSAASL------RWE-------------------GTPS---TLEI--------------------ALAAGFSMATVTQVFRWVS--
RPLVHANPAVTVASFL--AGDTSLVASFLYVIVQCFGAITGAGL-LHLVC--P----------AY-A---RG-------TL--GATS-LAV------G----T--------TPPQALG-TEIIVT-
FLLVSAILSTLDACP--RD-DNYDRY--DVSAAVGLAVTLGYLVA---L-PLTGAGLNPARSFGPAI----L-------TN------NW-----Q---DH-WV-YWC----GPLVGAVIAGLMYNMLLH----R-
--------------------------------GLKTKRVKHAQSDTE------------------------------------------------
>Rnor_AQP2
-----------MWELRS-----------------------IAF-S----------RAVLAEFLATLLFV--FFGLGSAL------QWAS------------------SPPS---VLQI--------------------AVAFGLGIGILVQALGHVS--G--
AHINPAVTVACLV--GCHVSFLRAAFYVAAQLLGAVAGAAI-LHEIT--P----------VE----IRG-------DL--AVNA-LHN------N----A--------TAGQAVT-VELFLT-MQLVLCIFASTD-----
ER-RGDNLG--SPALSIGFSVTLGHLLG---I-YFTGCSMNPARSLAPAV----V-------TG------KF-----D---DH-WV-FWI----GPLVGAIIGSLLYNYLLF----P---------------------------------
SAKSLQERLAVLKG-LEPDTDWE----------------------------------------
>Hsap_AQP5
----------MKKEVCS-----------------------VAF-L----------KAVFAEFLATLIFV--FFGLGSAL------KWPS------------------ALPT---ILQI--------------------ALAFGLAIGTLAQALGPVS--G--
GHINPAITLALLV--GNQISLLRAFFYVAAQLVGAIAGAGI-LYGVA--P----------LN----ARG-------NL--AVNA-LNN------N----T--------TQGQAMV-VELILT-FQLALCIFASTD-----SR-
RTSPVG--SPALSIGLSVTLGHLVG---I-YFTGCSMNPARSFGPAV----V-------MN------RF-----S--PAH-WV-FWV----GPIVGAVLAAILYFYLLF----P---------------------------------
NSLSLSERVAIIKGTYEPDEDWE----------------------------------------
>Rnor_AQP5_frg
--------KATMKKEVCS-----------------------LAF-F----------KAVFAEFLATLIFV--FFGLGSAL------KWPS------------------ALPT---ILQI--------------------SIAFGLAIGTLAQALGPVS--G--
GHINPAITLALLI--GNQISLLRAVFYVAAQLVGAIAGAGI-LYWLA--P----------LN----ARG-------NL--AVNA-LNN------N----T--------TPGKAMV-VELILT-FQLALCIFSSTD-----SR-
RTSPVG--SPALSIGLSVTLGHLVG---I-YFTGCSMNPARSFGPAV----V-------MN------RF-----S--PSH-WV-FWV----GPIVGAMLAAILYFYLLF----P---------------------------------
SSLSLHDRVAVVKGTYEPEEDWE----------------------------------------
>Hsap_sp_AQP0
-----------MWELRS-----------------------ASF-W----------RAIFAEFFATLFYV--FFGLGSSL------RWAP------------------GPLH---VLQV--------------------AMAFGLALATLVQSVGHIS--
G--AHVNPAVTFAFLV--GSQMSLLRAFCYMAAQLLGAVAGAAV-LYSVT--P----------PA----VRG-------NL--ALNT-LHP------A----V--------SVGQATT-VEIFLT-LQFVLCIFATYD---
--ER-RNGQLG--SVALAVGFSLALGHLFG---M-YYTGAGMNPARSFAPAI----L-------TG------NF-----T---NH-WV-YWV----GPIIGGGLGSLLYDFLLF----P-------------------------------
--RLKSISERLSVLKGAKPDVSNGQ----------------------------------------
>Rnor_AQP0
-----------MWELRS-----------------------ASF-W----------RAIFAEFFATLFYV--FFGLGSSL------RWAP------------------GPLH---VLQV--------------------ALAFGLALATLVQTVGHIS--G-
-AHVNPAVTFAFLV--GSQMSLLRAFCYIAAQLLGAVAGAAV-LYSVT--P----------PA----VRG-------NL--ALNT-LHA------G----V--------SVGQATT-VEIFLT-LQFVLCIFATYD-----
ER-RNGRMG--SVALAVGFSLTLGHLFG---M-YYTGAGMNPARSFAPAI----L-------TR------NF-----S---NH-WV-YWV----GPIIGGGLGSLLYDFLLF----P--------------------------------
-RLKSVSERLSILKG-ARPSDSNGQ---------------------------------------
>Lcha_H3BF11
--------NTNMLWEL------R-------S----------PSF-I----------RAVFAEFLATMVFV--FFGVGSAL------SWSE------------------DPLN---ILQV--------------------SLSFGFTIATMVQGVGHIS--
G--AHLNPAVTLAFLF--GSHISLLRALLYIAAQVLGGMAGAAV-LYGVT--P----------PS-V---RG-------DL--AINK-VQP------G----V--------APGHALV-VEMILT-FQLILCIFATTD--D--
RR-TGCL-G--SPSLSIGLSVTLGHLFG---I-PFTGTSMNPARSFGPAV----V-------VR------KF-----S---YH-WI-FWV----GPIVGAIVAALLYNFILF----P---------------------------------
RKRNFLESIAILKGTLYPEDEDE----------------------------------------
>Lcha_H3BF34
--------MLGELRSL------------------------AF-V----------RAVFAEFSATMVFV--FFGAGSAL------SWST------------------APLN---TLQI--------------------SLAFGLAIATMVQGVGHIS--G-
-AHLNPAVTLAFLF--GSHISMLRALFYITAQVLGGMAGAAV-LYGVT--P----------PS----VRG-------DL--AINN-LHP------E----V--------KSGHAFV-LEAILT-FQLVLCIYATTD--D--
RR-TGCL-G--SQALAIGFSVTLGHLIG---I-PFTGTSMNPARSFGPSV----V-------VG------KF-----P---HHWWV-FWV----GPIIGGVVAGQLYDLVLF----P---------------------------------
RIKKSLERLSVLIGHFPDRDKDE----------------------------------------
>Hsap_AQP6
--------GGRGWASMLA-----------------------CRL-WKAIS------RALFAEFLATGLYV--FFGVGSVM------RWPT------------------ALPS---VLQI--------------------
AITFNLVTAMAVQVTWKAS--G--AHANPAVTLAFLV--GSHISLPRAVAYVAAQLVGATVGAAL-LYGVM--P----------GD----IRE-------TL--GINVVRNS----------V--------
STGQAVA-VELLLT-LQLVLCVFASTD-----SR-QTSG----SPATMIGISVALGHLIG---I-HFTGCSMNPARSFGPAI----I-------IG------KF-----T---VH-WV-FWV----
GPLMGALLASLIYNFVLF----P---------------------------------DTKTLAQRLAILTGTVEVGTGAG----------------------------------------
>Rnor_AQP6
--------MEPGLCNRAYLLV--------------------GGL-WTAIS------KALFAEFLATGLYV--FFGVGSVL------PWPV------------------ALPS---VLQV--------------------
AITFNLATATAVQISWKTS--G--AHANPAVTLAYLV--GSHISLPRAVAYIAAQLAGATVGAAL-LYGVT--P----------GG----VRE-------TL--GVNVVHNS-----------T--------STGQAVA-
VELVLT-LQLVLCVFASMD-----SR-QTLG----SPAAMIGTSVALGHLIG---I-YFTGCSMNPARSFGPAV----I-------VG------KF-----A---VH-WI-FWV----GPLTGAVLASLIYNFILF----
P----------------------------------DTKTVAQRLAILVGTTKVEKVVD----------------------------------------
>Lana_XP_013392640.1
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--------KPIGINEF------R-------N----------PLF-Y----------RAFAAEFIGTLLLV--LVGCGAIT------G--------------------DRSPVVAQLQI--------------------SLVFGFTVGTIVWIFGNSS--G--
GHINPAVTVAMWA--VGNITFVKAVFYIVAQVTGAVGGAAL-LNVLT--P--------ANATDG---GK-------KL--GANL-LSP------G----V--------TVSQGVG-IEIFCT-LVLVFTVFAACD--
G--SR-TDLS-G--SRPLSIGIAVTMAHLYA---H-EYTGCSMNPARSLGPAL----I-------SD------TW-----T---NH-WV-YWV----GPLVGGLIAGFLYEFIFA----E--------------------------------
-DASVKKLKRYLACRCRSLLEEGEESS------------------------------------
>Lcha_H3BBE0
--------LIRRKWTL--------------S------------F-L----------RAILAEFLGTAVFV--FASLASTI------IWNQIPLNIQMQ----------KAHSSDNPVHI--------------------
SLTFGISVAVMSYCMGPIS--G--AHLNPAVTSALLA--GLRISPIKAVSYIFAQVLGAITASGF-LYGLT--P----------GQ-Y---RG-------NL--GINS-LSP------G----V--------TQLQAVG-
IEMAVT-FQLVLCVFASSE-----RK-KDLA-N--CIHLVVGLSVTLGHLVA---I-GYTGCSMNPARSLGPAV----I-------IS------NY-----K---NH-WI-FWM----GPLAGGLLGTILYDFILA----
P--------------------------------RWKSFTDWQKMLKMGLKEESD-----------------------------------------
>Ctel_148029
--------LQCAVAEAKT----------------------LAF-W----------RDALSEFVATFMLM--SVQSALPL------TW--------------------SVSMSSSIVQV--------------------
GLGVGFIVATMAWALGDFG--G--GHMNPAVSLSMAL--SFDISFLRAGVYIAVQTIGAIAGAGF-IYAVT--P----------PS----KRG-------NL--AATE-LGE------G----V--------
DGWQGML-VELWIT-CILVLTIRGSTN----KQRKGNIL----MHTLPIGLAVALGIMSG---F-GHTGGSMNPARSIGPAV----V-------MG------IW-----G---DH-WV-YWV----
GPFLGGTLATLIYVLLLD-------------------------------------KVDKDSTKSSAALAH-----------------------------------------------
>Ctel_180401
--------MTKEIKS-------------------------ATF-W----------RDVIAEFLATFLLM--TVQSAIVM------DWGKD----------------DPAK---SIRV--------------------GLALGFIVATMAWALGDFG--
G--GHINPAVTVAMVF--GGCCTILRGILYVIAQCVGAIAGAGF-IYAVT--P----------AD----FRG-------NL--ALTD-LNE------G----M--------EPWQGYL-VETWVT-CILVLTILGATN-----
ER-RKGNVY--MPTILIGFAVCLGIMSA---F-NHTGGSLNPARSFGPAV----V-------IN------KW-----N---NH-WV-YWA----GPCSGGILASLLYSYMLD-------------------------------------
RVDRGKKEESYDMRG-----------------------------------------------
>Ctel_46197
--------------------------------------------------------------LGTV------------------------------------------------VQV--------------------GLGMGFVVATMAWALGDFG--G--GHINPAVSMAMVV--
RRSITIFRGVMYIIAQSIGAIAGAGF-VYAVT--P----------SN-K---RE-------TL--AVTN-LGP------E----V--------EAWQGFL-VELWAT-FVLVVTILGSTN--A--NR-KGRV-Y--
MPTIFIGFAVTLGIMSA---F-NHTGGSLNPARSFGPAV----V-------MN------LW-----D---NH-WV-YWL----GPIAGGVLAALIYEYVL------------------------------------------------------------
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>Ctel_37043
--------------EL------T-------S----------LAF-W----------RDLLAEFVMTFMLM--SVQAALPL------DWGTNG----------------LL---GGPVQV--------------------GLGVGFLVTAMAWALGDFS-
-G--GHINPAVSIAMMA--CAKISPLRALFYVASQSVGAVAGAGF-VYGMI--P----------SA-S---RG-------HL--SATS-LGP------G----V--------EPHQGFL-IEAWIT-CLLVLTVFGSTN--
K--KR-KGSL-H--MPAVPIGLAVALGIMTGVNAF-GSTGGSMNPARSLGPAV----V-------LS------IW-----D---DH-WV-YWA----GPICGGLLAAMVY----------------------------------------
----------------------------------------------------------------
>Ctel_172599
----------MGREEVCKKGCSLGEARE-------------RSF-W----------RDLAAEFVATFLLV--SVQCALPL------TW--------------------GRNDIGSGIHT--------------------
ALGMTFIVTTTLWSLSEFG--G--IHMNPALSLSMMC--VRRISIFRGLVYMVVQSAGGVAGAAL-IWGLT-------------PEQFRETLA-----------STELNPSM-------------------
TVWQGLG-VEIWLT-FNLILTLHGCTY----TGRKVNIL----MFSVPIGMAVGTGVLSG---F-ASTGASMNPARSLGPAV----M-------MG------KW---------DHHWI-YWV----
GPCLGSVLATFTYYVVFD-------------------------------------KPDKNQKASHGTKPS-----------------------------------------------
>Paib_108845_c1_seq2
--------RMFGVNDL------R-------N----------KVF-W----------RDVGSELFATSLLV--AVQCALPM------QFVDSD----------------DEIDWAILTKI--------------------
ALGMGFVVTCMIETFGEMG--G--AHMNPAVSISLTV--AGNMCILKGFFYIVAQCTGATLGALF-AYSMS--P----------PE-Y---RG-------NL--AVTT-TDS------Q----L--------
PPWKGMM-VELWLT-CMLVLTIWGSTD--S--QR-K-KV-Y--MPSIPIGLAVAMCIMTG---G-LHSGSSLNPARSLGPAI----V-------MN------KW-----D---HH-WV-YWA----
GPIAGGLLATFMYKIVYS----P--------------------------------FDNDEDKFEHVDQVRQHDLLVEAVKAAMNGRTVRRARNEAVCMVDMDIESDYPHDCDSEPIKR
>Lana_XP_013390337.1
--------KFVGTDEVKT----------------------VKF-W----------KSVTAEVLSTAIMM--IVQCSVPL------KWP-------------------GNNDSGTTVQI--------------------
ALGMGFVVMCMIEAFGHIS--G--AQMNPSVSIAMLI--ARKISLLKAIFYIIAQCSGAFLGSGL-IYIIT--P----------AN----ATG-------GL--ATTTVNRD-----------M--------TPAQGMV-
VEMYLT-FMLVFVIFGATD-----SR-KKIT----MPSLVIGMAVAMNICTG---I-NHTGASMNPARSLGPAV----Y-------VN------IW-----T---DH-WV-YWA----GPISGAILATLAYNFGFV--
-----------------------------------LHCTKKEKELEEEKKYAETLPDPTVIERVCGRYCGTLNSG----------------------
>Ctel_26353
-------------SEI------K-------N----------GAF-W----------RDLLAELLATFFLV--AAQCALPL------SYDDTG--------------------SARIGT--------------------ALGMGFVVLSIGWCFGDFS--G--
AHMNPAVTMTLLL--RMKITFLRAFFYWIVQCGGAIGAAFA-IKSLV--T----------ED-MA-ASA-------DL--ALTK-PAA------G----I--------EPWKGML-FEMVLT-AILCFTVHGATN--V--
KR-KGML-F--INTLPIGMALALGILMG---L-PVTGGSLNPARSLGPVVAQYDV-------DG------IW-----D---DH-WI-YWA----GPMLGAIVAAIVYSLL------------------------------------------
----------------------------------------------------------
>Spur_XP_799266.2
--------NESMAKEL------G-------S----------RRF-W----------QAVLAELVGMFFFI--FIGISSTT------AWAPP-----------------IIPS---QVQI--------------------ALAFGLSLATFVHATAHIS--G-
-GHLNPAVSVAFLL--LHRITPLRCLAYSIAQCLGALGAAGM-VYAIT--P----------TA-V---SN-------NV--GPTT-PGA------G----V--------EDWQAFL-MEVCLT-FQLVLVIFSTVD--G--
KR-ASPG-G--SGPLAIGIAVLVAHLGA---I-QYSGASMNPARSLGSAV----V-------GG------VW-----N---AH-WV-YWA----GPLLGGLLGAVTYDYVLD----P--------------------------------
-NVSMGRLRRCPTC-EYGDEDDYTT--------------------------------------
>Skow_NP_001158487.1
--------VVAEVCDIRCCQE-------------------LRF-W----------RAVAAEMLGTFLFL--FILLSSTI------SWND------------------QTPT---ILQI-------------------SFAAGLAIATLVQCFGHIS--
G--GHLNPAVTVAMLF--TGKIGVFKSLFYIIAQCVGAIGGAAL-VFGVT--P----------EE----VRG-------NM--GANV-LNA------Y----V--------TAIQGFG-IEFTLT-FILVFTVFATTD-----
ER-NEIS-G--SKPLAIGIAVIIAHLVG---I-GYTSVGINPARTLGASV----M-------MK------MF-----D---DH-WV-FWA----GPLGGGVAAGWIYVFTFG-------------------------------------
RQFDTEKRETESEKY-----------------------------------------------
>Ctel_115384
--------MEIRSIEL----------------------------W----------RCVIVECLATFLYV--LLGCAATL------RWDPSLYA-------------VHVTSADVIHV--------------------SLAFGFAMCALTQCFGHIS--
G--GHFNPAVTIATTL--TCRVTPLRGALYVGAQCGGGIAGAAL-LYGLT--P----------SA----SRG-------SF--GVTL-LSP------E----L--------NVVQGFA-VELLLT-FLLTLTFFATVD----
-PKRRNHG----NQAMPIGVTVAFVHLVG---Y-RLTGGSCNPARSLGPAF----L-------TN------CW-----D---DH-WI-YWA----GPLGGAVMAGVLHHFIFD----P------------------------------
---SKHRLQAKIPERTE------------------------------------------------
>Lgig_V4A7E8
--------MRVEIRTI-------------------------EF-W----------RAVISECLGTMLYV--LLGCSSTL------SCGT------------------TMDPTNQIVLV--------------------SLCFGFTLATLVQCFGHIS--
G--AHFNPVVSLAMVL--TCKVTPLRGIMYSIAQCGGSVAGAAL-LYGIT--P------------EVCHGHG-------QM--AITS-LMP------G----L--------NIWKAFG-IEAILT-YILVFSVFATID--P-
-NR-RELG----SKPLAIGLAAALCHFAG---V------------SLGFAV------------------------------DH------------DPL-----GVLTHDLA----------------------------------------------------------------------
------------------------------
>Lana_XP_013417896.1
--------FTEVKKELGN----------------------IEF-W----------RAVISECIATLLYV--LLGCASTL------
RWDVYAQNATSDVSSGYVSNAGDAGTTEATSQWTHETLTANSSHDANFYIVRVALCFGLAYTTLQVCFGHIS--G--GHLNPAITIATVV--
TSKISVVRGIGYVLAQCGGSIAGAAL-LYGLS----------------STAVRG-------HM--GVTK-LAA------G----L--------TLEQGLG-MEIVTS-FLLVFTLFSASE-----SSRSDSL----
SSSVAMGAVVTMMHLAT---V-QFTGCGINPARSLGPSF----I-------TN------YW-----E---NH-WI-YWL----GPCLGGIVAGILYKYIFD----P--------------------------------
RKTAPQRSSGVQESQDSDDGTFNYKRCKFRDSNSSKKSPKFSSRSG----------------
>Spur_XP_001185961.1
--------TRWEKDDLGS----------------------ADF-W----------RAVIAECLALTIFI--PIAPGSSV------GW--------------------SGSNTPTNTLI--------------------ALANGLGIATLIQCFSHVS--
G--AHFNPSVTVPLVI--YRQISIVRGLCYVVAQCLGSACGAAI-LKFVT-------------PVDKQTNVG-----------VTLINYDE------G----V--------SLWQGFG-VEFVIT-FHLVLMVFATID---
--SRRTDIQ-G--SSALAIGFTVATGLLYG---I-PYTGASMNPARSFGPAL----V-------AN------YW-----V---DH-WV-YWV----SPIVAGISAASTYKFLFT----D--------------------------------
KAAISRLTSCCCRSKSRDVGRRR---------------------------------------
>Lcha_H3BIC5
---------PLTSQEL------K-------S----------RQF-W----------RCVLAELAGTLILV--SVILGASF------PSQE------------------KAPC---LLQP--------------------ALAAGLSAVSLVHCFGDIS--G-
-AQVNPAVTLAFLC--TRKLDFLRCISYLLAQCLGAMLGSGL-LYLVL--P----------VNST---GG-------HM---VNRINHA------G-------------NAGQALG-MEIFST-FQLVFTIFAVED--H--
RR-REVG-E--PGSLAIGFSLVAGILAA---G-NISSGSLNPARSLGPAI----L-------TG------IW-----E---HH-WV-FWI----GPVLGAVLAGVSYEFFF--------------------------------------------
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>Hrob_154942
--------LPDSHNEI------H-------S----------AAL-V----------KGTIAEFLGTLILV--TIGCGTCLA---K-DWEKD----------------TPTT----VQI--------------------SLAFGLAVAAVGWCFGHVD--
A--GHANPAVTTALFV--SRKISLVRWIFYVFMQCAGAILGADI-LLKLT--P----------SS-V---NG-------TEL-GSPS-INV------A----N--------TAFQGFV-VEAFIT-FVLVLTIFTCCD--K--
NR-KELH-C--IRPIAIGLCVTMCHLFA---L-KYTGSSMNPARALGPHV----V-------AR------EF-----D--PNH-WV-YWV----GPMVGGVLAGCIHEL--------------------------------------------
----------------------------------------------------------
>Hrob_185503
--------ITTNLNDL------K-------S----------SHL-Y----------RSLVCEFIGTFILI--FMGCFSII------TWNRS-----------------EPPS---LVAI--------------------ALCFGIIIAILATAFGGIS--G--
CHINPAVSLGFLV--TRRISLIRFLLYAIVQAAAAIAGAKL-LYELT--P----------EG-P---RR-------EL--QVVVMPLP------G----I--------SDSNAAF-VEFFLT-FLLMFTVYACID--G--GK-
PVAH-G--TAPFIIGLSVTTGVFSA---G-LYSGGCMNPIRALGPAV----V-------QN------YW-----R---HH-WV-YWV----GPMMGAVVAALLYDLLFD----T--------------------------------
AASLRKLKLFFTSLDYDANSFYV---------------------------------------
>Pdum_8227_c1
--------MGTSLDDLKS----------------------PLF-W----------RAVIAELLGTTFLV--LLGAGSCM------EWDV------------------DSDTPPSDLHV--------------------
ALAFGLAVATVVWSVGHLS--G--GHVNPSVTAGFLV--TRRISL-------------------------------------------------------------------------------------------------------------------------------------------
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>Ctel_120819
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--------MFQECRD---------------------------YKF-W----------KAVRCELLCGLVYV--LFGCGSFVY----------------------------VGYSAAQTLVI--------------------AVSFGLSAAILVVCVGGIS--
G--GVCNPALTLGLLV--TRNISVTRATTYFIAQIIGSLCGAAI-LYALS--------------SADHVVSG-------HL--GALS-PHP------Q----M--------TPAQCFG-VEFMAT-LLVTMTTLAAGD--
D-------------SKSFYVGCSIVAAHLFA---L-PYTGCGLNPARCLAPAI----F-------TG------RW-----S---NH-WV-YWI----GPLLGGVIGGFTYEYSKP---------------------------------------
PSSLPQQRVLECDHSAISIETCAT----------------------------------------
>Lgig_V3ZP04
--------MESIFQAIRR-----------------------FSY-W----------KAVKCEFLLTMFYV--LIGCASTI------PTDI------------------HNKQSVNELKV--------------------AFAFGITCLTLMQCFLPIC--
GP-AQMNPAVTIAMLC--TRRVHVITSFIHILAQCVGGLAGAGI-LYGVT--P--------------ARKHG-------HL--GVTFVHND-----------I--------GRGQAFG-IEFITT-FIMTFAYYSGHD--
N-----PGIFGG--FQAAPVGLSIIMGHTLG---W-QFTGASMNPARSLGPAI----V-------CN------EW-----S---DH-WV-YWI----GPVLGAILGGITYEYTTG----G--------------------------------
--GPEIKHLRRTSSLGMMNTETRGS-----------------------------------------
>Ctel_21078
--------MPNSIADLKN------------------------VVF-W----------RDVLVEVLGTGILL--TWITFSFV------TFNPDH----------------YQPN---TTTL--------------------GLFVGFLVFILIETLGPYS--
G--CHMNPAVTLGFFL--NGHLSIARSIFYVIAQCSGGAGGSAL-VYALT--P----------ASKR------------HMFHAITP-SAD-----------V--------SLAQAIG-HECIFT-FLLVFTALFLTL-----
PS-RKSV----NPGFPLGFCVGTSIMCG---G-TYSGSTLNPVVALGPAV----I-------SR------NF-----H---DH-WV-YWV----GPLCGGVVAFLLFKAITV----V----------------------------------
GDKFEKPNNNNNKE--------------------------------------------------
>Ctel_45698
-------------SDIKS------------------------VLF-W----------RDVLVEVLATAILL--IWITFSFV------TFNPEH----------------YQPN---TTTL--------------------GLLVGMVVFILIEALGPYS--G--
CHMNPAVTLGFFL--NGHLSIARAILYTIAQCAGGAGGSAL-VYALT--P----------SSRHHMFHA-------IL-------PHP------E----V--------ALAQAVG-HECIFT-FLLVFTALFLTL-----PN-
RKSV----NPGFPLGFCVGTSIMSA---G-TFSGSTLNPVVALGPAV----I-------SR------NF-----Q---DY-WV-YWV----GPMTGSLIAFLLFKAI-----------------------------------------------------
----------------------------------------------------
>Hrob_168509
----------MSLNDLKK------------------------VSF-W----------TECACELMVSFMLT--TVVMFVLVTNK--------------------------KELYEPNTTHL--------------------GLFVLAFVFMAIETYGPIA---
--CLLNPMAAFSLFL--AGKISLARCVIFTILELCGGLAGTFF-GYLLT--P----------DNRVIPYFD----------------PSH-----HG----M--------NNWQSVT-IEGFFS-FNLIFVVLSVHG-----
TEYPRPLPF--LPNLSIALALAVGLFAA---V--------------------------------------------------------YWL----GPYIGGPLAVLAYKLFAM---------------------------------------GKFYKPREA------------
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>Hrob_185352
--------FKASRRDLAT------------------------LIF-W----------RDVSCEFVVSTFLMTVVILILTTND---------------------------THAYQPSTTHF--------------------GLFAGFFIYSLLETWGPIS----
-CLGHPPAAFCFML--GGKFTVARTVFYTIAETVGCATGAGI-GYALT--P-------------FEKRST-----------FVAFNPAK-----HG----L--------SLAQSVF-VEAVFT-FNLIFCIFSVHG-----
SDYARKFPI--LPNLAIGSAIGTAIMAA---G-TFTGGFMNPLIAFGPAI----V-------SG------DF-----T---NH-WI-YWV----GPYVGGIPAVFLYKFYHW----V----------------------------------
KVRHERLPKRVVPP--------------------------------------------------
>Hrob_185353
--------LQHSKQDLST------------------------LIV-W----------RDVFCELMVTSFLM--IMVTLVLI------TNNVEA----------------YKPG---VTHF--------------------GIFAGFFVYMLLEGYGNIS--
---CVANPMAAFCFYL--AGKFSIAKTILFTAAHVTGCICGSVI-GYELT--P----------AARLA-EPG-------GAFHAFNP-ANH------G----L--------TVTQSVF-VEAILS-FNLIFVVLSLHG--
S--EL-GRPYPI--LPNLAIGIVIGTSIMAA---G-THTGGFMNPLISLGPAF----L-------SH------DF-----K---NH-WI-YWV----GPYIGGPPAVYVYKMFVF----I---------------------------------
KRRNDLMANDVLLESSPRSYISN-----------------------------------------
>Lgig_V4AZ69
--------LMEEIDDL------R-------K----------PSF-W----------KAVVAEFLGCFFLL--IFAVGAGLH---EEGTRGHG------------------------SVHN--------------------ILASGFTIAVLISVFLTVS--
G--AHVNPAVSIGFAV--NRQISFVRFIFYSIAQAGGSVAGTAL-LKAIT--P----------AS-K---IG-------NL--GLVL-PAK------H----V--------TAEQALY-TEIIIT-FFLLFAIFALID--K--DR-
NDVK-G--SIPFMVGLVVCVNIFYG---S-NTSGAAMNPIRAFGPAV----I-------TG------NL-----Q---QH-WI-YWA----GPLIGGAAGAFVYDKIFS----V----------------------------------
ASSNSGIKSCCLGSESYEAVKTS-----------------------------------------
>Lana_XP_013382376.1
--------YKWGFKEV------K-------T----------PAM-W----------KAVAGEFIGTAILL--FTHCQLTI------RWP--------------------RFPEDGNIVQN--------------------ALGTGFTVASIIEGFGHLS-
-R--AVVNPAITLSFVI--ARKVSPTRGLFYVLAQLLGGIAGTGL-SWLVT--P-------------TE-RVG-------NF--GAVF-VHP------D----I--------TLWQGFL-METCLT-FMLAFVNLASID----
-DR-LRPV-N--MPSVAVGLLVCVMVFTG---A-TQTGACMNPAVAMGPHV----I-------LN---QGWMW-----A---QH-WL-YWV----APFTGSAIATVLYMFFFT----W--------------------------
--------GVKSLEFPDGDKEADPYAETRNEL----------------------------------------
>Ipul_QED21987.1
---------AHLKFNI------R-------D----------YKL-W----------RAIVAEFVGTFLLV--FIGCLSVL------PSE--------------------ADDNLDNLVVP--------------------GLCFAAIIFTLIHCIGPTS--G--
CNINPVVTFALVL--LGRCHWAKGLLYLPAQVLGGIAGALL-LWGVS--P----------PD-WC-DAG-------HM--CMTR-VNQ------D----I--------NCFQAVI-IETVAT-GVLVLIVTVISDP-
D--PM-KDFE-G--FPSLAGAMAVLAAVYSA---A-PFTGASLNPARSFGPAL----V-------KM------NF-----E---NH-WV-YWV----GPTLGAFVAVFTYQLLLE----P-----------------------
AIHHEDKSEDTELKLEPTAVFNIERKTPTEDNNM-----------------------------------------
>Ipul_QED21989.1
--------VLHVRIPL------R-------H----------LGL-W----------RAVAAEFIGTFLMV--AMGCLAVF------PSE--------------------VNPDLDNLIVP--------------------GLAFAGIIVTLIHVIGPTS--G-
-CNINPAVTLALVV--IRRCHPLKGILYFVAQILGGIVGSYF-LYLLS--P----------YE-WC-ENA-------HL--CMTR-VKP------V----I--------NCAQAVG-VEVVTT-GVLVLIVMVISDP-D--
PH-KDFE-G--FPSLAGGIAVMTCVYAA---A-PYTGASLNPARSFGPAL----V-------NW------EW-----A---NH-WV-SWV----GPLLGSVCAVGVYKLVLE----Q-----------------------
GIHQNDP---------PPPEYSTERRDNGAEEGF-----------------------------------------
>AQP_C
--------MTDLQRLMNMEDDEPLLTSDDILEEESEETGIIETF-V----------RPASAEFLATAIFV--FIGTTAISSEEL-------------------------SGPGITNLTGI--------------------
AFAHGLTIAMLVMSFGHIS--G--AHINPAVTFGFIL--VKGIPLVKGIVYVFAQLVGSIVGSAM-TRGLLGYASPNMHTN---KTIFEDIGG----------GGHQ-LGP------G----V--------
SVGEGVL-GEVALT-FILVLVILMTAY-----DSNGSNL----LHPLAIGFAVCVDIIAG---A-KVTGASMNPARSFGPAV----I--FSEFNTS------LW-----K---DH-WI-YWL----
GPALGAALAALFLQTYLC---------------------------------------QKE-------------------------------------------------------------
>AQPc_seq
-----------ILEDEHEETGII------------------ETF-V----------RPASAEFLATAIFV--FIGTTATSSEEL-------------------------SGQGVTNLTGI--------------------AIAHGLTIAMLVMSFGHIS--G-
-AHINPAVTFGFIL--VKGIPLVKGIVYVFAQLVGSIVGSAM-TRGLLGYASPNMHTN---KTIFEDISG-----------GGHQ-LGP------G----V--------SVGEGVL-GEIALT-FILVLVILMTAY-
----DSNGSNL----LHPLAIGFAVCVDIIAG---A-KVTGASMNPARSFGPAV----I--LSEFNTS------LW-----K---DH-WI-YWL----GPALGAALAALFYRLIFA------------------------------------
---KRSQRVFKKIN-----------------------------------------------------
>Paib_109651_c0_seq1
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AHINPAVTLGVFI--GRGITWLKAILYIVAQLTGSLVGASL-TRGFLSYKVVGN------TTMYEEILG-----------GGHA-LAK------G----V--------SPAEGVL-GEIVLT-MVLVEVILMSAL----
-DSEGTNV----LHPLAIGFVVFVDIIAA---G-EITGASMNPARSFGPAV----V--LSSLNTT------LW-----K---NH-WV-YWV----GPAAGACIAAIFYRLIFA---------------------------------------
RSNRRLFNNLN-----------------------------------------------------
>Pdum_118446_c0
--------VEESSRNKKF------------------------EEY-V----------QPVAAEFLSTAIFV--FIATASVV---------------------------------QGDIHAI--------------------AVAHGFTIAILVITFGQIS--G--
AHINPAVTLGFII--AGGITWLKAIFYVLAQLIGGIVGAAL-TRGMLSFQVAGN------TTMYEHIKG-----------GGHF-LGP------G----V--------SVAEGVL-GEIALT-TILVIVILMCAY-----
DSNGTNL----LHPLAIGFAIVVDILAA---G-GITGGSMNPARSFGPAV----V--LSSLSTT------LW-----T---NH-WI-YWV----GPAIGASIAGLFYRLIFA---------------------------------------
NNQKRLFK--------------------------------------------------------
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ALAHGLTIALVVASFGHIS--G--AHVNPAVTLGIVI--AGGITLPVAVVYLFAQLLGGMVGAGF-VRLLS--P----------VELFNMTNG-------G---AQTINPSV----------V-------
TWGTAIG-CEIFLT-IVLVFTILMVAV-----DSKTNKT----LAPIIIGVAVVVCILAG---G-WLSGASMNPARTFGPAV------AVTSYNPD------IW---------KYHYI-YWV----
GPFLGACVSGLLYRLVFA---------------------------------------SPDKRVWRR-------------------------------------------------------
>Lgig_V4AIJ5
--------VPKGESNIPLVSIF--------------------ETY-V----------RPCIAEFVGVSFFV--FIGTMVVQ------DSSGLP----------------DKDFRANAVAI--------------------
ALCHGMTIAVLVIALGSIS--G--AHVNPAVTLAIAI--CGEIKPLLAVCYFFSQIVGGMVGAAF-TRAVL--P----------DSMYTQFGG-----------GAHDLATT-----------E--------HVGEAIL-
CEMLLTTVLVLVILLVAVD-----PR-TKTE----LAPLAIGMAVTVDIFAG---I-FVTGASMNPARSFGPAI------AMTVFKNA------IW---------KHHYV-YWI----GPALGSILAALIYKLGLG--
-------------------------------------SASKRL----------------------------------------------------------
>Ctel_166556
--------ARPEPKGMVRVF---------------------LKY-L----------RPPIGEFHAVCLFV--FVGVVAST-----------------------------NVD----TLSV--------------------AVAHGLAIALLVAVYGGIS--G--
GHVNPAVTFGVVI--GGRCEVFIGLLYIIFQLLGGVFGAAL-ARMVL--TVGYTSTGNVTNVDLYEAIG-----------GGVPQLAE------G----V--------TAWNGVF-VEAILT-
FILVLTVLMTAV--D-----QQLS----IAPLCIGFAVLVDIIVG---G-SITGAAMNPARSFGPAL----V-------AG------IW-----T---NE-WI-YWI----GPILGALFAGGSYRVFLA----------------------
-----------------KSEYRILFKDKSE---------------------------------------------------
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--------EPTGLTRL------F------------------LKY-V----------RPPVGEFHAVALFV--FVGVLSST-----------------------------NAD----TLSV--------------------AVAHGLAIALLVAVYGGIS--G--
GHVNPAVTLGVVI--GGGCEVFVGLLYIIFQLLGGVFGAAL-ARMVL--TVGYATTDNVTQVDLYEAIG-----------GGVPQLAD------G----V--------KAWNGVF-VEAILT-
FILVLTVLMTEV--D-----QQLS----VAPLCIGFAVLVDIIVG---G-SITGAAMNPARSFGPAL----V-------AG------IW-----T---NE-WI-YWI----GPILGALFAGGSYRAFLA---------------------
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>Lgig_V3ZVR0
--------SKIFEETVEHGIQKSKMFEEEDHNTNENNVGCYDRF-F----------RPCVVEFMASLLFV--CLGCMSVQNPLG-------------------------ANIPLPSAVAV--------------------
SLCHGFLAAALITIFGNVS--G--GHFNPAVTLGCVI--SRALNPLLGLFYFLSQIVGSIAGAFI-ARAIL--P----------KESYVAIAG-----------GAHI-LTA-----------V--------GPGKGIV-
CEMILT-ALLVFTVLHSFV-----NDRPKDSNL--LGPIAVGFALTASLLAG---I-GITGGSVNPIRSFGPAVALSSIS------TD------AW---------THHYV-YWV----GPICGSIIASLFFILLTA--
-------------------------------------DAGKRCCLQTKK----------------------------------------------------
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--------TGIHFKDMADEKTPFYHASTSTTDRCLRIF---LKY-V----------RPAFAEMTGSFSYV--WVGTLAYA--------------------------------YGGPLWA--------------------
AIAYGVTLTFLVATFAGIS--G--SHINPAITLGVII--AGECELLQGFVYVLAQLVGGIIGAGLAQMSLT----------------AAENNGTTLYDLSNR--GVVRLPNG-----------M--------
GDLIGVV-IVATMT-CLLVLTFIMTAI-----EQ------GTPISSVAIGFAVFGVTMAG-------NGLITNPAQEFGTAV----V-------AN------LW-----S---DQ-WI-FWV----
GPLLGALAAGALYRFIFA----S---------------------------------SDKRLFLKDKYQ---------------------------------------------------
>Skow_XP_006813480.1
--------NTESQRNLASNGGNT------------------NAEVWTPFTVMERYIRPTFAEFFGVMCFV--FIGSMSSV-----VGYPG------------------GGDAAAGLIAV--------------------
ALAHGFAIILFVAGFGNTS--G--GHFNPAITLGIMI--SQEINLIVGVLYIVFQLLGAIVGAAI-VKGVL--T----------DAVYDYING-----------GATLKGDA-----------Y--------SLGEAVG-
CEWILT-MILVSTVLQSAV-----DS-KPSV----LAPMAIGLAVGAGILAG---G-TVSGASMNPARSFGPAV----V-------SG------IW-----T---DH-WI-YWV----GPPIGGITSA------------------
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G--GHFNPAVSLAAML--IGGLNLVMLLPYWVSQLLGGMLGAAL-AKAVS--P----------EERFWNASG-------AA--FVTVQEQG--------------------QVAGALV-AEIILT-
TLLALAVCMGAI--N--EK-TKGP----LAPFSIGFAVTVDILAG---G-PVSGGCMNPARAFGPAV----V-------AN------HW-----N---FH-WI-YWL----GPLLAGLLVGLLIRCFIG--------------
------------------------DGKTRLILKAR----------------------------------------------------
>Rnor_AQP8
--------MADSYHGMSWY----------------------EQY-I----------QPCVVELLGSALFI--FIGCLSVI----------------------------ENSPNTGLLQP--------------------ALAHGLALGLIIATLGNIS--G--
GHFNPAVSLAVTL--VGGLKTMLLIPYWVSQLFGGMIGAAL-AKVVS--P----------EERFWNASG-------AA--FAIVQEQE--------------------QVAEALG-VEIVMT-MLLVLAVCMGAV-
-N--EK-TMGP----LAPFSIGFSVIVDILAG---G-GISGACMNPARAFGPAV----M-------AG------YW-----D---FH-WI-YWL----GPLLAGLFVGLLIRLFIG--------------------------------------
DEKTRLILKSR----------------------------------------------------
>Lcha_H3AXY4
--------MLDGKKESSEKPNEPNLF---------------EKY-V----------QPCVAEMVGVTLIV--SLGCTSVI----------------------------ENVASAGRVQP--------------------
ALAHGLAVGLSVAIYGEIS--G--GHFNPAVSLTAFL--LGALDRIMLLPYCVSQLFGGIIGGSL-AKAMT--T----------HGSYVNASG-----------GAFDVVTS------N----E--------
QIGRAVV-AEIVMT-TLLVITVCMAAI-----NNHSKTP----LAPLCVGFTVAVDILAG---G-DISGACMNPARAFGPAV----V-------SG------HW-----D---YH-WI-YWI----
GPLSGSLVVAAIVRLLLG--------------------------------------DRKLRLILK------------------------------------------------------
>Cele_NP_001256246.1
--------NLKNSSDIVPKMVEDEKD---------------YTI-Y----------SKCAAEFIAVLLFV--YIGSMQAA----------------------------GVFLHDGVLHA--------------------
AFAHGVAIFVLAATFGGVS--G--AHINPAVTFGIAL--VGRISPIHAVCYVVSQLLGSVFGALL-VRISL--P----------YKMYNVISA-----------GATL-CGK------G----Y--------NWQEGLT-
AEIVTT-YILVQTVLLCAV-----DT-DKNR----LAPLAIGFSLIIEILAA---G-AISGASMNPARSFGPNI----MGQVFLKPEHLDAQYMYW---------NYHWI-YYI----
GPIIGAFIAAGVYRMFFA--------------------------------------RDYRVLA--------------------------------------------------------
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--------DASDIVELSKATSVF------------------EKF-I----------QSTFAEFIGTMVYT--FIACMAVT------TMD--------------------------VTSI--------------------ALAEGLGIAFLCSAFLNIS--G--
GLFNPGLTFAMAL--SGGINAVAAIMYFIFQILGALIGAAF-IKAVV--L----------NDSYY-DIKGGCNQYRGL--VPYDKYDA------NRQLDM--------TTGTAVV-IETVLS--
TMIFLVYLMAN--L--DT-KGRQ-S--TGPLAYGFAVLVSIICA---Y-HSSGGSFNPARSFGPAV----I-------SG------YW-----A---EH-YI-YWA----GPALGGLLAGLFYRLILG----D-------
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GNINPAVSIGLLI--ARQISFIRCMLYMLFQVIGALLAAKC-ISIMT-------------YETFNKTHG------------ISH-PSV--------------------PWDKALF-CEIVIS-FILVFTVMRMCV--E--
KSRSEVERG--LAALLIGISIVVGILCG---G-NISGGSMNPARTLGPAI----V-------NN------KL---------NNVWI-YIA----GPLIGGILAGLVQQLLFT----S---------------------------------
ELSKDYLFDYLTNPKFNMEEEYKKYEERK----------------------------------
>Djap_BAR87948.1
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AHINPAVSCGLLV--ARQISLIRYIFYTIFQIIGATAGFGL-ISGVS----SGEVLYKSYYKINFTSSP-------SL--GEAR-IDE-----------VIHRVT---NPYLKII-CELVIT-FVLVFTVMRLCV--
E--KRRSPTEKG--IAPLFIGLSITAGVYCG---M-NISGGSMNPAASLGKYF----F-------KG-----------------GTTIMTHII----GPLLGGALAGVSQQMFFT----S---------------------------------
DLSMSHIKSYLFDSEFEDHSDNY----------------------------------------
>Ctel_142373
--------------------------------------------------------------------------------------------------------------------------------------------SIQVTFILIALILVFMPIS--G--SFFNPVGCFVSYL--
DGRISMAKAILYSIAEISGAATAVLL-LRLRP-------------SSMFTHPLR----------------PAE------S----I--------TPVQGVM-VEAVLT-LFLIFMVMAVTD-----DAVRKVYDM--
NISLVIAFTAGSMIISS---G-SYTGAAMNPLIALGPAV----S-------SA------DF-----S---DH-WV-YWI----GPFIGGTVGYALYAGVRF--------------------------------------
GYHKGHVVASSKCTCF-----------------------------------------------
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-G--AHMNPAISLGFLA--NGSISLVTFASYVIAQLLGATSGAAI-VNMSQ----------------CWKAAG-------DATCGMVT-PDA-----------E--------
SPISAVVALEALFTCFLMLQTLSSAAD-----LRHRFSVPG---MAAVIGATVSFGILIL---G-PVTGAVFNPARYTGPAV----V-------SR------DV---------GLVWV-YWV----
GPMAGSLLAVPLYIRVLR----P---------------------------------EDGTPGE--------------------------------------------------------
>AQP_E
-----------------------------------------VQ--W---------------------------------------------------------------------------------------------------------------------AVMNPVVAVNFMF--
TGRISFLRCIFYIVFDLLAAVSVAYF-VKLVF--P----------EEMVA-----------AI--KVAG-PGE------G----I--------SKIQAMV-IEWVIV-IGHNVAILGTLD-----DDNRSVM----
IPGITIGMATFVFISAG---F-HLTGGILNPFAVFHTAV----I-------TG------DF---------STQWI-YWS----GDFLGGLTALLLYHFSLY--------------------------------------NKGFFK---------------
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>Pdum_218083_c0
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CLMNPVAAVNFMF--TARISFLKCIFYTIFDLLAAVAVAHF-IKVVL--P-------------EE-TVA-------TL--TVAR-PAP------G----M--------SGLQAMV-IEWVLV-IMHQCAIMASID--D--EN-
RTVM----IPGLSIGMATFAAVGAS---Y-RFTGGVLNPFAVFHTAV----L-------TG------DF-----S---TQ-WI-YWT----GDF-----------------------------------------------------------------------------
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F--SVMNPIVGLVFLC--TKRISLFRYIFYIVVDMAGAVAGVAL-IYQCC--P----------PE----VRD-------KF--GPLG-PGK------G----V--------TANQAFG-VEAVLV-MAYMSVICGAMD--
---TDLKKTY----MPGLPIGLTVTIGCLVG---A-PFSGPAMNPIVAFPFAF----L-------SG------NW---------EHHWV-YWA----GDYLGGVLAIALYHIFAY--------------------------------------
DNPKLEIEPCCSCSK------------------------------------------------
>AQP_X
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-------------------------------------------------------------MFFVSVLLGATD-----DKRKTVR----IPNLAIGFCVASAGTIG---G-PF-GVALNPVVSFSLSA----V-------AD------IW---------DK-------
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ALFNPNLVLVLVF--SRRMSIPKGILYIGVEMLMAMGAVTF-IYEIS--P----------ESTKA-----------QF--GPPGPVLD-----------I--------SNNNVWV-CEFIAV-FFFASVLLGAVD--D--
ER-KPVR----IPQLAIGLCVVGAASIG---A-PF-GVTLNPLVSFGFSF----V-------TD------DW---------TRSWV-YWT----AGPVGSIVATILYYFFAY--------------------------------------
DAGKCGNPEEEDEDSD-----------------------------------------------
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--------SVPMLKKVIDPIRGNVE----------------SSYLGSLILFGSDVIVDVVCEFLLTFWLI--VGVVMSNMN---------------------------FPNLRPANLYA--------------------
GNVIGLYVMSLVEGFGHVQ--K--CVMNPNFSLLFVL--QNSMSVAKGIIYIGVQLAATASGAIF-VYNIT--P----------EENRAMIRT----------------PGA----VDG----I--------STAGNWV-
AEAVAV-ASLGVVIIGSGE-----ADKKRTM----MPGLAIGFCVMSTATVG-----APWGVNLNPAVVFAWCV----L-------TN------DW---------GRLWI-YWT----
SGFAGVIVTVLLYNFFVYDGPLV---------------------------------QTNKEALQRFPSMNRTISRAISSDSGVI-----------------------------------
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SEFLAV-SYLVGVTLGTLD--G--DK-KKVM----LPSLAIGFCVMGCNTVG---A-PY-GTNLNPAIVFAFAY----I-------TN------DW---------SRLWV-YWT----
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>Paib_96014_c0_seq1
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MEWICV-TIFMMVILSATD-----RRKKRPM----LPHLAIGFALVSLSLYA---G-PY-GVSLNPISALSIAV----A-------VN------DF---------SYTWI-YFT----
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GIFNPAIGLGFFIASAGQIKIITFLAFLLSELLGGLAGAAL-VRICL-------------TSADY-SNA-------TMTEGVLS-LNL------A----T----A---GIGGGFM-IESILT-FCLAMAVYVTLA--N--
GD-WKQHAG--WGPVAVGAAVCICTLCG---Y-DATGACMNPARALGPAV----IASFYADVNI------AW-----A---YH-YI-YWI----GPGVGAVTAAIIYRVLHF----L---------------------------
------SDQEKQEEDSYQKYEELQDVLKTSESYSS----------------------------------
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---------QNTV----LPSLAVSITVAFGILGT---A-RHTGGFMNPLIPFAFAV----F-------HG------NV---------KNQWI-YWL----GPYIGGTLAVLTYGQMLK----G----------------------------------
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>Cint_XP_002131566.1
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AISDGFVVAILVITIGHIS--G--AHVNFAVTTAVFI--GGGIKLPMVPLYFIAQMLGSLTGAAL-AYICN-------------GSVSFGEFG----------------LGA------G----V--------TVGQGVL-
MEVVLSALLTLAVLLAAVE--------LSSV----NAAFAIGFSILIDILAG---F-NISGACMNPTLSFGPAV----V-------SG------KW-----T---NY-WI-YWV----GPILGAFVSGTVFRLFLG----T-
--------------------------------EARLLFRPDGGDEN--------------------------------------------------
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ANISAILCCEVMIT-GCMVFFTVTMVV-----DK-TYNQ-A--TGPLAIGLTVFQGIIAG---K-WIGAGCLNPSRAFGPAV----V-------LG----GRAW-----N---YH-WV-WWV----
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ANITAIFFCEFITTGFIMFFTLAMVVD--------KTYQQH--TGPFALGMTVFQGVLAG---R-WVGAGCLNPARWFGPAL----V-------TG----GAAW---------NYHWV-LWL----
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IEVVLS-IVLFFTCLSVTD-----HK-----------------------------I-----------RKVDDAV----L---------------------------------YWA----ADYTGGVLAVIMYKILRF---------------------------------------
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G--GHINPAVTLTMCI--LRKCPWKKLLPYWLAQYLGAFAGAAV-VYGVYYDALNAF------DGGVRQVDG--INGTAGI--WATY-PKE-----------F----L---SAESGFG-DQLVGT-
AMLVCLVMAITD-----KRNANIPSG--IQPLCIGLVVVAIGMSF---G-FNCGYAINPARDLGPRV----LTAIAGWGDE------VF---TYRDY---NW--FWV-
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L---STGIAFA-DQVVGT-AVLLLCVMGITD-----DRNSAAPPG--VVPLAVGTAVLGIGASY---G-FNCGYAINPARDMGPRL----FTAMAGWGAD------PF---SFRGT---NW--FWI-
PWLAPHLGAILGVFLYLLLIG--------------------------------------LHHEEEEPEEEKSEV-------------------------------------------------
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NVAWGVAVAMGVYCSAGVS--G--GHINPAVSLAMAI--AKKITMLQCIAYSAAQYFGAIVGSAV-VYGVYYDALNNF------DGGARKTEG--PKATAGI--WSTF-PQE-----------F----
L---STGIGFM-DQVVGT-AILLLCVMGITD-----QKNAGAPPG--VVPLAVGTAVLGIGASF---G-FNCGYALNPARDLGPRL----FTAMAGWGPD------PF---SFRGV---NW--FWI-
PILAPHLGAILGVFLYLLLVG--------------------------------------LHHEEDVVEEEKTGA-------------------------------------------------
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-AHLNPAVTLTMAC--LKKTSWKRVPFYMLAQYLGAFIASVM-VFVVYLDSINNY------DGGIRAVIG--DKSTAGI--WSTYPQSH---------------L---STINGFG-DQIFGT-
ALLLICVLAVTD-----RKNMKPDSG--LVPLSIGLIVVVIGMTF---G-YNCGYAINPARDLGPRI----FTAIAGWGEE------PF---SFRNY---NW--FWV-PVIGSHIGALVGAAIYQLCVG---
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MMLLLPIMAITD-----RRNADVPKF--LVPVLVGLAVFAIGAAY---G-YNCGAAINPARDLSPRI----FTALAGWGTE------VF---SFRNY---NW--FWV-PIVGPHIGAILGAIIYLFLVE-----
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-G--AHINPAVTLAMAT--IGRFDWVKVPLYWLMQFLGSFAACGC-LYGVYYDAIEDF------DGGERQVYG--PNATAAI--WATY-PQD-----------Y----V---TIETGLG-DQIFGT-
MLLLGCIMAIVD-----TRNNKPPHG--MEPLLIGLAVFVIGLAF---G-HNCDYAINPARDLAPRT----FSYWVGYGAE------VW------TPNGINW--WWV-
PVVGPLIGGVCGALIYIVFVE--------------------------------------AHHPDDKDSHSLDQN-------------------------------------------------
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--------RKRLKSALRIK----------------------HPI-L----------KGMLAEFLGTFVLV--VFGDGSVAQVVLSKGAAGT------------------------FLTI--------------------
NIAWGFAVAMGVWVSGGVS--G--GHINPAVTLTLCL--MGREKWRRLIPYWLAQYIGAFVASAC-VYGVYIDALNEF------DGGTRSIRG--TNGTAGI--WSTF-PQE-----------F----
L---TAKVGLA-DQILAT-GLLVCMVLAITD-----KNNMGAPKS--LVPLAVGSVVAVIGMSF---G-YNCGYAINPARDWGPRM----FTALAGWGRG------CWMRIIPEMDFCDTE--VTI-
HTLYTKTPSSLSLWFCQQGVI--------------------------------------LKWALKYDWMYRDSKE-------------------------------------------------
>Hsap_AQP10_1
--------FTQAPAEIMGHLRIR------------------SLL-A----------RQCLAEFLGVFVLM--LLTQGAVAQAVT------------------------SGETKGNFFTM--------------------
FLAGSLAVTIAIYVGGNVS--G--AHLNPAFSLAMCI--VGRLPWVKLPIYILVQLLSAFCASGA-TYVLYHDALQNY------TGGNLTVTG--PKETASI--FATY-PAP-----------Y----L---
SLNNGFL-DQVLGT-GMLIVGLLAILD-----RRNKGVPAG--LEPVVVGMLILALGLSM---G-ANCGIPLNPARDLGPRL----FTYVAGWGPE------VF-------SAGNGW--WWV-
PVVAPLVGATVGTATYQLLVA--------------------------------------LHHPEGPEPAQDLVS-------------------------------------------------
>Hsap_AQP3_1
---------MGRQKELVSRCGEMLHIR--------------YRL-L----------RQALAECLGTLILV--MFGCGSVAQVVL------------------------SRGTHGGFLTI--------------------
NLAFGFAVTLGILIAGQVS--G--AHLNPAVTFAMCF--LAREPWIKLPIYTLAQTLGAFLGAGI-VFGLYYDAIWHF------ADNQLFVSG--PNGTAGI--FATYPSGH---------------L---
DMINGFF-DQFIGT-ASLIVCVLAIVD-----PYNNPVPRG--LEAFTVGLVVLVIGTSM---G-FNSGYAVNPARDFGPRL----FTALAGWGSA------VF-------TTGQHW--WWV-
PIVSPLLGSIAGVFVYQLMIG--------------------------------------CHLEQPPPSNEEENV-------------------------------------------------
>Rnor_AQP3
---------MGRQKELMNRCGEMLHIR--------------YRL-L----------RQALAECLGTLILV--MFGCGSVAQVVL------------------------SRGTHGGFLTI--------------------
NLAFGFAVTLAILVAGQVS--G--AHLNPAVTFAMCF--LAREPWIKLPIYTLAQTLGAFLGAGI-VFGLYYDAIWAF------AGNELVVSG--PNGTAGI--FATYPSGH---------------L---
DMVNGFF-DQFIGT-AALIVCVLAIVD-----PYNNPVPRG--LEAFTVGLVVLVIGTSM---G-FNSGYAVNPARDFGPRL----FTALAGWGSE------VF-------TTGQNW--WWV-
PIVSPLLGSIGGVFVYQLMIG--------------------------------------CHLEQPPPSTEAENV-------------------------------------------------
>Lcha_H3A425
-------------------------------------------------------------------------LFGCGALAQVVL------------------------SNGSHGQFLTV--------------------NLAFGFAATLGVLIAGQISETG--
AHLNPAVTFAVCL--LAREPWIKFPVYSLAQILGGFLASGI-IFAMYFDAIWLY------GNNQLIVMG--PKGTAGI--FATYPSEH---------------L---TLLNGFF-DQLIGT-
AALVLCILAIVD-----PHNNPIPRG--LEAFTIGFVVLNIGLAM---G-FNSGYAVNPARDFGPRL----FTAVAGWGTE------VF-------SAGGHW--WWV-PIVAPLLGAVLGVLVYQLMIG-
--------------------------------------VHFEPETPSTAPENV-------------------------------------------------
>Hsap_AQP9
--------GAEKGKSFKQRLVLK------------------SSL-A----------KETLSEFLGTFILI--VLGCGCVAQAIL------------------------SRGRFGGVITI--------------------
NVGFSMAVAMAIYVAGGVS--G--GHINPAVSLAMCL--FGRMKWFKLPFYVGAQFLGAFVGAAT-VFGIYYDGLMSF------AGGKLLIVG--ENATAHI--FATY-PAP-----------Y----L-
--SLANAFA-DQVVAT-MILLIIVFAIFD-----SRNLGAPRG--LEPIAIGLLIIVIASSL---G-LNSGCAMNPARDLSPRL----FTALAGWGFE------VF-------RAGNNF--WWI-
PVVGPLVGAVIGGLIYVLVIE--------------------------------------IHHPEPDSVFKAEQS-------------------------------------------------
>Rnor_AQP9
--------KDGAKKSLMQRLALK------------------SRI-A----------KETLSEFLGTFIMI--VLGCSSIAQAVL------------------------SRERFGGIITI--------------------NIGFASAVVMALYVTFGIS-
-G--GHINPAVSFAMCA--FGRMEWFKFPFYVGAQFLGAFVGAAT-VFGIYYDGLMAF------AGGKLLVVG--ENATAFI--FATY-PAP-----------F----I---STPGAFV-DQVVST-
MFLLLIVFAMFD-----SRNLGVPRG--LEPVVIGLLIIVLSCSL---G-LNSGCAMNPARDLSPRL----FTALAGWGFE------VF-------TVGNNF--WWI-PVVGPMIGAFLGGLIYILFIQ-----
-----------------------------------MHHSKLDPDMKAEPS-------------------------------------------------
>Lcha_M3XHS6
--------DARDKKSFKDKFVLR------------------NRL-V----------KESLSEFLGTCLLI--VFGCGSIAQSVL------------------------SHESMGGFLTI--------------------
NIAFPMAVTMAIYVGGGVS--G--AHINPAVSFAMCI--VGRLNWLKLPFYIVAQMLGAFVGAGA-VFGIYYEALMTY------TGGVLTVTG--PNATAQI--FATY-PSP-----------F----L---
SIASGLI-DQIIGT-SLLLVCIFGIFD-----NKNNGVPKG--LEPIAVGLCIMVLGLSM---T-LNCGCAINPARDLGPRM----FTAVAGWGTE------VF-------SAGNNW--WWV-
PVVGPMIGAAVGCFIYVMFIE--------------------------------------LHHLDPLDESTGDND-------------------------------------------------
>Hsap_AQP7_1
--------SKMVSWSVIAKIQEILQ----------------RKM-V----------REFLAEFMSTYVMM--VFGLGSVAHMV-------------------------LNKKYGSYLGV--------------------
NLGFGFGVTMGVHVAGRIS--G--AHMNAAVTFANCA--LGRVPWRKFPVYVLGQFLGSFLAAAT-IYSLFYTAILHF------SGGQLMVTG--PVATAGI--FATYLPDH---------------M-
--TLWRGFL-NEAWLT-GMLQLCLFAITD-----QENNPALPG--TEALVIGILVVIIGVSL---G-MNTGYAINPSRDLPPRI----FTFIAGWGKQ------VF-------SNGENW--WWV-
PVVAPLLGAYLGGIIYLVFIG--------------------------------------STIPREPLKLEDSVAYED---------------------------------------------
>Rnor_AQP7
--------AGSVLENIQSVL---------------------QKT-WV---------REFLAEFLSTYVLM--VFGLGSVAHMV-------------------------LGERLGSYLGV--------------------
NLGFGFGVTMGIHVAGGIS--G--AHMNAAVTFTNCA--LGRMAWKKFPIYVLGQFLGSFLAAAT-TYLIFYGAINHY------AGGELLVTG--PKSTANI--FATYLPEH---------------M---
```

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TLWRGFV-DEVFVT-GMLQLCIFAITD-----KLNSPALQG--TEPLMIGILVCVLGVSL---G-MNTGYAINPSRDLPPRF----FTFIAGWGKK------VF-------SAGNNW--WWV-
PVVAPLLGAYLGGIVYLGLIH--------------------------------------AGIPPQGS-------------------------------------------------------
>Lcha_H2ZUR1
--------KVVAKVKTFLRIKN-------------------KTL------------REALAEVLGTFIMM--LFGLGSVAQVII-----------------------GGGKNGEYLSI-------------------
NLSFAMGVTMGIHMAGGVS--G--AHLNTAVSFTMCI--LGKLCWRKLPIYTLAQFFGSFLAAGL-VYFLYYDALHEY------CGGNLTVTG--PKATAEI--FSTY-PAP----------Y----L---
TLSNGFL-DQVVGT-AVLLIGILAIND-----QKNNPALNG--TQGLSVGLLVLVIGMSM---G-MNCGYAINPARDLPPRI----FTAIAGWGLE------VF-------RAGNNW--WWV-
PIVAPLVGSVIGACIYQIFIE---------------------------------------GHHKPEPEGNSSIEF----------------------------------------------
>Cint_XP_002131906.1
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NWAYGFGVTMAVYVTGKVS--G--AHINPAVSVAQCA--FGNLPLYKLPCYIFSQVFGGFVSGAA-VYSIYYEALNAF------DGGQRSVLG--PNGTGGI--FATY-PQD----------Y----
L---SINNGLW-DQVFGT-ALLVGIIFAVTD-----NKNNTIADG--LTPIIIGLLVFILGTSF---G-LNCGYAINPARDFGPRL----FTFAAGWGPG------VF-----TEPNGMSW--WWV-
PIVGPIIGGLTGAILYKLMVG---------------------------------------THLPSGSQSVITDEEDDF-------------------------------------------
>Lgig_V3Z755
------------IKKIALKLRVK-----------------NEL-A----------KDVLAEFFGTFVLC--SFAIGASC------QYVL------------------TRQKLASFLTL--------------------NLANGVGLVFGVYTAGGVS-
-G--ASLNPATTIALCL--HGLMDWYKFPFYTIAETAGFFAAAV-QYRVNIDMLNAY------DGGNRSTYG--PNATAGM--FSTF-PNP----------D----V---STMNCLW-DQIWST-
FLLIVCVFAVID----KKNFMPLDKG--LLPISFGSIVFGIGACW---A-GNCGYPLNPSRDLGPRL----FTAIAGWGIE------PF----SYRN--YNW--FWV-PIVGPCIGASLAYFGYMLFIE--
------------------------------------LHWEAEEGEEPEKVE---------------------------------------------
>Lgig_V4BDI9
------------IKKIALKLRIK-----------------NKL-A----------KDVLAEFFGTFVLC--SFAIGASC------QYVL------------------TRQKLASFLTL--------------------NLANGAGLVFGVYTAGGVS--
G--ASLNPATTIALCL--HGLMDWYKFPFYTIAELAGAFLAAAV-QYGVNYDMLNAY------DGGNRSTEG--PNATAGM--FSTF-PNP----------D----V---STLNCLF-DQIWAT-
CLLIVCVFAVID----KRNFMPIDKG--LLPLSLGTIVFGIGSCW---A-GNCGFPLNPARDLGPRL----FTAIAGWGIE------PF----SHRN--YNW--FWV-PIVGPCIGASLAYFVYMLFIE--
------------------------------------LH-------------------------------------------------------------
>Lgig_V3ZVP6
---------MSIYKKLRVK---------------------NEL-A----------RDILAEFLGTFVLV--SFAIGASCQYVL------------------------TRQTLASFLTL--------------------NFANGLGLVFGIYTAGGIS--
G--ASLNPAATIALCM--HGLIEWYKWPFYTIAELAGAFLAAAV-QYGVNYDMLNAY------DGGNRTTVG--PTATAYV--FSTF-PNP----------D----V---STLNCFF-DQVWGT-
FVLLACTFAIVD-----KR-NFMPIDKGLLPLSLGSIVFAIGTCW---A-GNCGYALNPARDLGPRI----FTAIAGWGIE------PF------SHRNYNW--FWV-PIAGPCVGAILAYFVYMLFIE---
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LTWQGGLADQIFAT-LLLMMGILALTD-----ERNMVGPTGRAYVPLLVGLLVLAIGLAF---G-FNCGYPINPARDFGPRL----FTAMAGWGTQ------VF----SEPRGTYNW--WWI-
PIIGPHVGAIIGALAYNFFIG--------------------------------------YHWPKERDDVQLQSP--------------------------------------------
>Dpul_EFX88760.1
--------FTSALKLLKSSVSAIY-----------------HRLYV----------RAFLAEFISTFAILAFVNGLIAQA---------------------------VLTDGEKGSGV-------------------
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V---SIAGCII-DQLFGS-ALLALCVAALTD-----PKNMRIPLP--LVALYIGFLVLELAIGF---S-LNTGGSPNPARDLSPRI----LTYFAGWGIQ---VFRDW----------TW--FWI-
PVVIPHIGVVIGYLIYFILIE--------------------------------------AHWPTQ-------------------------------------------------------
>Dpul_EFX88757.1
-----MAPKLMKMIRVE-----------------------APL-L----------REIMAEFLGTFILV--LFGDASVAQSKL-----------------------SNEANGDFFSI-------------------
NWGWAVGVMMGVLVAGGVS--G--AHLNPAVTLAMAC--AGRIAIIKVFFYMLAQYLGAFAAAAS-VLGVYSDAIEYYSNTNHNGTLYMNNEA-NDPGLAGI--FSTY-PAP----------
W----L---SIAGGIG-DQILGT-MLLLLCICAITD-----KKNTQIPSA--LVPMYVGFTILGIGVCF---G-ANCGYALNPARDLSPRL----ITLIAGWDQS-----FSW-------N-NYNW--FWI-
PIVGPHIGAILGVFIYIAFVE---------------------------------------AHWPEEGDEVVPITV---------------------------------------------
>Dpul_EFX66203.1
------MRLCCRVHPL----------------------------V----------RAAFAEFIGTFILV--AIGNGSVAQSQL-----------------------TQGEKGNYFTI-------------------NWGWCIGCCLGILVSAKAS--
G--GHLNPAVTMALAV--AKDFPWKRLPVYWFAQYLGALAASGT-VLGVYYEAIIVRKI----DGKFRIHANASEPGSAAI--FANY-PAP----------Y----S---SVVTGLV-EEILCT-
TIFMLVICVVTN-----PKYSKVPSF--LQPFYIGFTLLAVGVAY---G-SNGGYALNPARDLAPRL----VSYFGGFGSR------VF------SFRGYNW--FWI-FLVGPHVGAILGVFIFHFILM--
-------------------------------------ETDKPASVEEKMNSSQLPIVDYEY------------------------------------
>Tric_RDD45034.1
--------TEIHESKMKERLR--------------------PIFRWLN--------PEAMGEFLATFILM--TFGIGSVAQVVL-----------------------GKGNFGDFLSI-------------------
NFGWGLGVTFGCYVAGNVT--G--AHMNPAMTIALAV--YKKFPWRKVPMYITAQMLGSFIASAV-CHAIYIEGINAYDGGVRQTPPHVNATA-------QI--WATY-PQP----------Y----
L---STAAGFF-DQIVGT-GLLALCVFALVD-----DKNWAPPDY--LKPLLIGFVVLNIGDTF---G-LNCGYAINPARDFAPRL----WTLIAGWGTP------TF-------VNGNYW--FWV-
PIVGPILGAIVGGGMYMFFIE--------------------------------------MRWDEIRKSSVAPAN---------------------------------------------
>Hrob_95422
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VWGWGLSVAMACYATMGVS--G--AHINPAVSFAFMS--VGRLSLLGMLMFWLAQYLGAFVGALL-AYALHYEAINLY------DGGKRAITG--QNATAQI--FATY-PQP----------G----
L---GVYTSVI-DQIIGT-MVLLIFVMAISD-----KRNSNIPQG--LKPLVVGALVVFVNYSF---V-YNCMGAINPARDMGPRM----MTLVVGYGVE------VF-------TIYDYF--FWV-
PLVMPHVGAFLGAHIYQLFIG---------------------------------------WHHADDDDDDITITN---------------------------------------------
>Mtar_G5CTG0.1
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-SLVTQIF-DAILGT-GLLVMGIFAIID-----PNNMAVPKG--QIPLYVGFLISSLIFSF---S-YNAGAALNPARDLAPRL----FLWVIGYGAE------AF-----T---ARGHL-
WWLVPVIGPHVGGLLGGVTYQMFIG--------------------------------------AHYQSDRKLKPATIMDEDDDTNAT--------------------------------------
>Mtar_G5CTG1.2
--------MDKEKNDWRSRAARMLYIE--------------SRL-V----------REGLSESLAMFFFMSLLLGCAATAR---------------------------FTGNQNDPMLA-------------------
AFYHGFSIIFAIYVAGGIS--G--GLLNPAITFTIAF--LGRLSWLRCLIYMSAQYFGAFIASAV-VYLIYYDS-----LQNFSGANKVDETG--ANGTAGI--WSTF-PRP----------Y----L---
SLRGAIF-NQIFCT-MLLTIGFLSICD-----FRNSRPDKG--MFPFAVGMLIMTVFLAF---S-YSAGAAMNPARDISPRL----WTLIVGYGDE------VF---SYNNY---KW--FWI-
PWLFPYVGALLGGVIYEIFIG--------------------------------------IHWPKDYA---------------------------------------------------
>Mtar_G5CTF8.1
--------KTGRHPDMVIQFQDDADDQHTSHYEGNW-----RHYFHKKLHIKNRLIRDWLSESLAMFLFMSLLLGGAATAH---------------------------FTGKQDDPMLT-------------------
AVFHGFSAVFGIYVGAGVS--G--GIINPALTFAVAL--LGRVSWRKCLVLVSAQYFGSFIASAV-VYLIYYESLQNYAKTADDNGEFLQKTA-------GI--WSTF-PKP----------Y----L---
SMTGAIF-NQIFCT-MLLSIGFLSISD-----HKNFRPTKG--LFPFAVGLLIMTVFLAF---S-YSAGAAMNPARDLSPRL----WSLIIGYGNE------VF------SHNDYKW--FWI-
PWLFPYVGALFGAVMYQIFVG--------------------------------------VHWPDKQS---------------------------------------------------
>Mtar_G5CTG5.1
--------GITNRKKMDWRGWLRKSTLVR------------SQL-I----------RGCMAEFLAVFVLM--VFIEGSAATAI-----------------------FTNRRQDILFG-------------------
SISSGLGVAMAVYVAGGVS--G--AFLNPAVALAFAV--LGKLSWKNCIFYMISQYLAAFVASCT-MFAYLYEALNNF------DGGERQMFG--PNGTAHI--WSTY-PQP----------F----
L---SPHTAFA-DQVFCT-AILLIVVLAMCD-----SKNWKPHNG--FLPIAIGLLIITISCTL---S-YNAGAAMNPSRDLAPRF----FSYLAGYGTE------PF------GVKGYTW--FFV-
PVLGSHCGAIIGGAIYQLFIG--------------------------------------GQWPDDTSDTNSVSS---------------------------------------------
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--------ELERTEQVRAKIQIK------------------NPL-L----------RNALSEFFGTFLLL--FIGIGIVMQFIL-----------------------SNEKLNTWINI-------------------NLGWGLAIAFTVYTCSKTS--
G--GHFNPAVSIAFLT--LGKLPFKDFLVYCVVQTIGAALGSAA-AFGLYYDQFVKF------AGAYRTILG--PKATAGC--FCSYPALH----------V--------SNTTAFF-DQFAGT-
ALLVLFVCVVID-----KR-NGIPGA--AHPLLFGLVVMMIGTAY---G-MNLGYPINPARDLGPRL----F-SFFIYGSG------VF---------SYHSYYFWI-PVIAPLFGAIFGAWSYTFFVG------
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----GVWGPYI-DQCVGT-GVLAYFLCVVID-----ER-NQIPKI--WHPMFFGFLVMMIGTGF---G-MNIGYPINPARDLGPRL----F-SYFIYGPG------VF---------
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----------MILDKLRAKFHIR------------------KEL-L----------RAVLAEFTGTYLLC--LIGLSVVAQKVL-----------------------PRPEVNEFIGV-------------------NVGFGIAIVFGVAVSAKLS--
G--GHINPAVSFAFLS--VGQITIVQFIAYFVAQFFGAFFGAAT-VYAVYNDAINVF------DGGVRTVGG--PKDTAGI--FASY-PAP----------H----L---GLVNGFV-DQFVAT-
AVFVFLIAHIVD-----KR-NSYPTW--LQPILVGTGFVAIGAAF---G-YNCGYPVNPARDFAPRL----F-------TSIFYGGAVF-------T---KW--FWV-PIVGPFVGAVVGIWLYYFLIG--------
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G--GHLNPAISVAQSI--LGNLPPWKIIPYAIAQVIGAFLGAAV-AYFGHHDDLWKL------DGGIRQVTG--GQATAGL--FTTFPPDH---------------M---SVWGSLL-DQIIGT-
AMLSGLVCLITD-----KR-HQIPTG--VVPVLAGSIMSMVAMTF---G-ANGGFAINPARDFGPRV----FCLCAGYGWE------VF-------SAHGYY--FWI-PIVGALIGSIIGAWIYKIFVG----
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SVWGGFF-DQFIGT-TLFVTGIMSVAD-----KIRDPLPNS--MSAIMVGFLLFVVGNSI---G-YNAGFAVNPARDFGPRF----FTFIAGWGSQ------VF-------SAGDYY--FWI-
PLVAPMFGSLAATIIYLVFIS-------------------------------------NHF--------------------------------------------------------
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NIGFGFGVLMAILLVGTIS--G--AHLNPAVSFSLSL--TGKMTWRKFFIYSLAQTLGAFLGATL-VFLVYLDG-----LKSFGEEMYSLNTA-------GI--FATY-PNE----------V----L---
GVFGGFL-DQVLGT-AVLIMVILAISD-----KKNNDISKE--ISAILIAFLVIVIGCSL---G-FNCGYAINPARDFGPRL----FTFFAGWGSQ------VF----EAGN---GF--FWI-
PIIGPLVGSFIGTLFYLIIVS-------------------------------------NNL--------------------------------------------------------
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LFEANLFNLFL-DQFFST-ALFIITILGVTD-----KRNNDLANE--KTAFMIGISLTVIGTSF---G-YNCGFAVNPARDLAPRI----FTSIAGWGWK------PF-------TVGNYF--FWI-
PVVAPFLGSILGTLLYSLFIS-------------------------------------NHWPDSNNAEDYDDD---------------------------------------------
>Bpli_RNA34844.1
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VSQVGIIADQIIAT-AVYLILILAFTD-----KKNFQFSPE--GTALMAGVALFSICSTL---S-YNSGSPLNPARDFSPRF----FISLVTWNGQ------VY-------SNNNYY--FWT-
PIVCPMIGSAIGTGVYSLVIS-------------------------------------KNLK-------------------------------------------------------
>Spur_XP_789770.3
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-SVPGGVM-DQLVGS-ALLVGGIFAIFD-----KHNIKPPAG--LEPIAVGLLLLVVNIAY---G-YNAGAAVNPARDFSPRL----FTACAGYGKD------IW-----VTPSGDHF--WWI-
PLFVPLVGGPIGGWVYYLTIE-------------------------------------VHHPHKISSRKAKGE---------------------------------------------
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--------MKTWMKKILSVITIR-----------------SDL-G----------RCLMAEFYSTFMLS--VFLRAAIAQD-------------------------LTSGTGSVFNI-------------------
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--SVTTGFF-EQVLST-ALFLAIIGAVLD-----HRNVPPPLN--WAPFFFGLIILALVMTF---G-HNAGAPLNPSIDLSGRL----CLGVMGYGAE------VWVLADGTHDGVH----
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PNPAVFNPNEFTGIT--------SVFEAFL-IETWGT-FILVFVIFSVTH-----PSNAIISNNKGLIPFVIGLTVAILISVY---G-PLTMAGLNPARDFGPRV----IAAIAGWGTI------AI-----
PGPRNGFWL-YIV----GPFIGGVLGGGASDLALY-------------------------------------CMKRFKK-----------------------------------------------------
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ALPAGFGVSISIFISAHFS--D--AHLNPAVSIAFAVVRFHQFHWSRLGPYVLSQFLGSLIAGLT-VFAFYHGAIQSF------EAAHNITRGEPGSQLSGMVFGEYF-
PNPALYNHNEFSSVM--------SVFEAFL-VEAWGT-FILVFVIFSLTH-----PSNSTISNTKVVVPIAIGITVAVIISIY---G-PLTQAGLNPARDFGPRV----IAALAGWGTI------AI-----
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>AQP_D
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GLPGYLIAIFMQAVLHTMTFKD--CRGNPLANVTGYL--HKEQSLKKTILRMICQMIGGIMSYRY-AKTLWRIK----------LNDIHLQRA-------DM--LCSNDLTV-------------------
DPSLGFL-IEFAGTIFETYISFCKLTK-----NP---------ATDMVIKIVISMTNLIL---AVELTGYYVNPANATAQTF--------GCGGGD------PI---------IFILV-YWI----
GPIIATTGAVHIRRKITS-------------------------------------NSKSKNGKIPARNGH---------------------------------------------
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NR-TDLN-G--SASLSIGLSVVIGHLFG---V-KYTGASMNPARSFGPVV----I-------MN------AW-----D---NH-WV-YWV----GPIAGAILAAWLYEFLLD----P--------------------------------
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CEAILT-MTLVMTVIMSAV--------DSASKGKALPPLAIGLAVVTGILSG---G-PFSGASMNPARAFGPAV----V-------AG------VW-----K---DH-YV-WWL----
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DP-SSAEKA--LPPLAIGLAVLVGILAG---G-PFSGASMNPARAFGPAV----A-------AG------VW-----K---NH-YV-WWV----GPIIGGLVAGIIYRMFLA------------------------------------
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L---SIGSGVL-DQVVGT-GLLLCGVLALTD-----SRNNKVTAG--MEPLLVGLLVLAIGTSF---G-FNCGYAINPARDLGPRI----FTAMAGWGIE------VF-------RAGNHW--WWV-
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Supplementary data S3. Alignment of annelid MIPs sequences used for the phylogenetic analysis.

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GSVPLQIGVAIGMCHLWG---VPLTGAGMNPARSFGPAV----I-----SNSLD-ADH---WIYWIGPLVGGMLAGIVYEFLFA----------------------------VNATPAKL------------
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DPSLGFLIEFAGTIFETYISFCKLTKNPA-------TDMVIKIVISMTNLIL--AVELTGYYVNPANATAQTF----GCGGGDPIIFI-------LVYWIGPIIATTGAVHIRRKITS------------------------
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>Ctel_224285
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VSGGHINPAVSFAMLVSRRISLAKAAMYVLAQCLAATTGAGVLYGLTP-----GPIRGTL------------GATVPHADI-TAAQSFTIEAILAFVWVLTLFATSDANRA-DLS-
RGAPLATGLSVTMCHLYA---VRLTGASMNPARSFGPAI----V-----TNTWT--DH---WVYWFGPLLGSALAAILYENTLA-----------------------------LNPQEGDLL---------------------------
--------
>Ctel_148029
-----------------------LQCAVAEAKTLAFWR-----DALSEFVATFMLMSVQSALPLTWSVS-MS----------------SSIVQVGLGV-----------------------------GFIVATMAWALGD---
FGGGHMNPAVSLSMALSFDISFLRAGVYIAVQTIGAIAGAGFIYAVTP-----PSKRGNL------------AATELGEGV-DGWQGMLVELWITCILVLTIRGSTNKQRK-GNI-
LMHTLPIGLAVALGIMSG---FGHTGGSMNPARSIGPAV----V-----MGIWG--DH---WVYWVGPFLGGTLATLIYVLLLD-----------------------------KVDKDSTK--------------------
SSAALAH---------
>Ctel_180401
--------------------------MTKEIKSATFWR-----DVIAEFLATFLLMTVQSAIVMDWGKD-----------------DPAKSIRVGLAL-----------------------------GFIVATMAWALGD---
FGGGHINPAVTVAMVFGGCCTILRGILYVIAQCVGAIAGAGFIYAVTP-----ADFRGNL------------ALTDLNEGM-EPWQGYLVETWVTCILVLTILGATNERRK-GNV-
YMPTILIGFAVCLGIMSA---FNHTGGSLNPARSFGPAV----V-----INKWN--NH---WVYWAGPCSGGILASLLYSYMLD-----------------------------RVDRGKKE--------------------
ESYDMRG---------
>Ctel_46197
-------------------------------------------------LGTV--------------------------------------VQVGLGM-----------------------------GFVVATMAWALGD---
FGGGHINPAVSMAMVVRRSITIFRGVMYIIAQSIGAIAGAGFVYAVTPS----NK-RETL------------AVTNLGPEV-EAWQGFLVELWATFVLVVTILGSTNANRK-GRV-
YMPTIFIGFAVTLGIMSA---FNHTGGSLNPARSFGPAV----V-----MNLWD--NH---WVYWLGPIAGGVLAALIYEYVL--------------------------------------------------------------------------
>Ctel_37043
-----------------------------ELTSLAFWR-----DLLAEFVMTFMLMSVQAALPLDWGTN-GL---------------LGGPVQVGLGV-----------------------------GFLVTAMAWALGD---
FSGGHINPAVSIAMMACAKISPLRALFYVASQSVGAVAGAGFVYGMIPS----AS-RGHL------------SATSLGPGV-EPHQGFLIEAWITCLLVLTVFGSTNKKRK-GSL-
HMPAVPIGLAVALGIMTGVNAFGSTGGSMNPARSLGPAV----V-----LSIWD--DH---WVYWAGPICGGLLAAMVY------------------------------------------------------------------------------
>Ctel_172599
---------------MGREEVCKKGCSLGEARERSFWR-----DLAAEFVATFLLVSVQCALPLTWGRN-----------------DIGSGIHTALGM-----------------------------TFIVTTTLWSLSE---
FGGIHMNPALSLSMMCVRRISIFRGLVYMVVQSAGGVAGAALIWGLTP-----EQFRETL------------ASTELNPSM-TVWQGLGVEIWLTFNLILTLHGCTYTGRK-VNI-
LMFSVPIGMAVGTGVLSG---FASTGASMNPARSLGPAV----M-----MGKWD--HH---WIYWVGPCLGSVLATFTYYVVFD-----------------------------KPDKNQKA--------------------
SHGTKPS---------
>Ctel_26353
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---------------------------SEIKNGAFWR-----DLLAELLATFFLVAAQCALPLSYDDT-----------------GSARIGTALGM----------------------------GFVVLSIGWCFGD---
FSGAHMNPAVTMTLLLRMKITFLRAFFYWIVQCGGAIGAAFAIKSLVTED---MAASADL------------ALTKPAAGI-EPWKGMLFEMVLTAILCFTVHGATNVKRK-GML-
FINTLPIGMALALGILMG---LPVTGGSLNPARSLGPVVAQYDV-----DGIWD--DH---WIYWAGPMLGAIVAAIVYSLL------------------------------------------------------------------------
>Ctel_115384
---------------------------MEIRSIELWR-----CVIVECLATFLYVLLGCAATLRWDPSLYA-----------VHVTSADVIHVSLAF----------------------------GFAMCALTQCFGH---
ISGGHFNPAVTIATTLTCRVTPLRGALYVGAQCGGGIAGAALLYGLTP-----SASRGSF------------GVTLLSPEL-NVVQGFAVELLLTFLLTLTFFATVDPKRR---
NHGNQAMPIGVTVAFVHLVG---YRLTGGSCNPARSLGPAF----L-----TNCWD--DH---WIYWAGPLGGAVMAGVLHHFIFD----------------------------PSKHRLQA-------------------
KIPERTE---------
>Ctel_120819
-------------------------MFQECRDYKFWK-----AVRCELLCGLVYVLFGCGSFVYVGYS-----------------AAQTLVIAVSF----------------------------GLSAAILVVCVGG---
ISGGVCNPALTLGLLVTRNISVTRATTYFIAQIIGSLCGAAILYALSSAD---HVVSGHL------------GALSPHPQM-TPAQCFGVEFMATLLVTMTTLAAGDDSKS---------
FYVGCSIVAAHLFA---LPYTGCGLNPARCLAPAI----F-----TGRWS--NH---WVYWIGPLLGGVIGGFTYEYSKP----------------------------PSSLPQQR----------
VLECDHSAISIETCAT---------
>Ctel_21078
----------------------MPNSIADLKNVVFWR-----DVLVEVLGTGILLTWITFSFVTFNPDHYQ---------------PNTTTLGLFV----------------------------GFLVFILIETLGP---
YSGCHMNPAVTLGFFLNGHLSIARSIFYVIAQCSGGAGGSALVYALTP-----ASKRHMF------------HAITPSADV-SLAQAIGHECIFTFLLVFTALFLTLPSRK-SVN---
PGFPLGFCVGTSIMCG---GTYSGSTLNPVVALGPAV----I-----SRNFH--DH---WVYWVGPLCGGVVAFLLFKAITV----------------------------VGDKFEKP-------------------
NNNNNKE---------
>Ctel_45698
---------------------------SDIKSVLFWR-----DVLVEVLATAILLIWITFSFVTFNPE-HY--------------QPNTTTLGLLV----------------------------GMVVFILIEALGP---
YSGCHMNPAVTLGFFLNGHLSIARAILYTIAQCAGGAGGSALVYALTP-----SSRHHMF------------HAILPHPEV-ALAQAVGHECIFTFLLVFTALFLTLPNRK-SVN---
PGFPLGFCVGTSIMSA---GTFSGSTLNPVVALGPAV----I-----SRNFQ--DY---WVYWVGPMTGSLIAFLLFKAI-----------------------------------------------------------------------
>Ctel_166556
--------------------ARPEPKGMVRVFLKYLR-----PPIGEFHAVCLFVFVGVVASTN----------------------VDTLSVAVAH----------------------------GLAIALLVAVYGG---
ISGGHVNPAVTFGVVIGGRCEVFIGLLYIIFQLLGGVFGAALARMVLTV----GYTSTGNVTNVDLYEAIGGGVPQLAEGV-TAWNGVFVEAILTFILVLTVLMTAVDQQL-SIA----
PLCIGFAVLVDIIVG---GSITGAAMNPARSFGPAL----V-----AGIWT--NE---WIYWIGPILGALFAGGSYRVFLA----------------------------KSEYRILF---------------------KDKSE---------
>Ctel_184073
-----------------------EPTGLTRLFLKYVR-----PPVGEFHAVALFVFVGVLSSTN----------------------ADTLSVAVAH----------------------------GLAIALLVAVYGG---
ISGGHVNPAVTLGVVIGGGCEVFVGLLYIIFQLLGGVFGAALARMVLTVGYATTDNVTQV----DLYEAIGGGVPQLADGV-KAWNGVFVEAILTFILVLTVLMTEVDQQL-----
SVAPLCIGFAVLVDIIVG---GSITGAAMNPARSFGPAL----V-----AGIWT--NE---WIYWIGPILGALFAGGSYRAFLA----------------------------KSEYRILF---------------------KDKSE----
-----
>Ctel_219373
------TGIHFKDMADEKTPFYHASTSTTDRCLRIFLK--YVRPAFAEMTGSFSYVWVGTLAYAY----------------------GGPLWAAIAY----------------------------GVTLTFLVATFAG---
ISGSHINPAITLGVIIAGECELLQGFVYVLAQLVGGIIGAGLAQMSLTAAENNGTTLYDL---------SNRGVVRLPNGM-GDLIGVVIVATMTCLLVLTFIMTAIEQGT-----
PISSVAIGFAVFGVTMAG------NGLITNPAQEFGTAV----V-----ANLWS--DQ---WIFWVGPLLGALAAGALYRFIFA----------------------------SSDKRLFL--------------------KDKYQ---
-------
>Ctel_142373
-------------------------------------------------------------------------------------------SIQV----------------------------TFILIALILVFMP---
ISGSFFNPVGCFVSYLDGRISMAKAILYSIAEISGAATAVLLLR-LRPS----SMFTHPL--------------RPAESI-TPVQGVMVEAVLTLFLIFMVMAVTDDAVR-
KVYDMNISLVIAFTAGSMIISS---GSYTGAAMNPLIALGPAV----S-----SADFS--DH---WVYWIGPFIGGTVGYALYAGVRF----------------------------GYHKGHVV------------------
ASSKCTCF---------
>Ctel_197140
----------------------FGLTLGSLKDLKMWV-----GVLAEFFACFLMMFVIVFLQVNHENS-SY--------------IPSTFQTGMAV----------------------------GVLVMVLVDVFGP---
ISGGFVNPVATFSFFLDGRMPFFQAIFYISAQLSGTVAGCLLLGALIPY-------DMEF-----------MPVIPQGGI-SALRSFVIEVVLSIVLFFTCLSVTDHKIR-KVD------------------------
------------------------------DA---VLYWAADYTGGVLAVIMYKILRF----------------------------GLLYNKNQ-------------------SNEAGDQ---------
>Ctel_125390
----------------------MRIRNQGAR-----------EFCAEFLGTMILIIIGDGSVAQSVLSSET--------------AGAFHSVNWAW----------------------------GLAVAMGVWVSVG---
VSGGHINPAVTLTMCILRKCPWKKLLPYWLAQYLGAFAGAAVVYGVYYDAL--NAFDGGVRQVDGINGTAGIWATYPKEFL-SAESGFGDQLVGTAMLVCLVMAITDKRNA-
NIPSGIQPLCIGLVVVAIGMSF---GFNCGYAINPARDLGPRVLTAIAGWGDEVFTYR--DYNWFWVPLAGPHVGAMVGAALYEWLVG----------------------------LHFPDEEE---------
-------------YMFNN---------
>Ctel_164915
--------------------------RKRLKSALRIKHPILKGMLAEFLGTFVLVVFGDGSVAQVVLSKGA--------------AGTFLTINIAW----------------------------GFAVAMGVWVSGG---
VSGGHINPAVTLTLCLMGREKWRRLIPYWLAQYIGAFVASACVYGVYIDAL--NEFDGGTRSIRGTNGTAGIWSTFPQEFL-TAKVGLADQILATGLLVCMVLAITDKNNM-
GAPKSLVPLAVGSVVAVIGMSF---GYNCGYAINPARDWGPRM----F---TALAGWG-RGC---WMRII-PEMDFCDTEVTIHTLYT----------------------------KTPSSLSL---
WFCQQGVILKWALKYDWMYRDSKE---------
>Hrob_95007
----------------------ARNSLQDLKSVKFYQ-----ALACEFIGTFLLVLVACGSCGRFVVT-ETEVRTNSTNIVTSKLIPNDLVQISLCF----------------------------GLSVATIVWSIAH---
VSGGHINPAVSIAFFVTRKISFIRFILYAAVQTAGAIVGAYILKALTP-----TGVNDSL------------GSTLLGSGV-SKAGGLFVELFITFVLVFTVFATCDSNRQ-GFA-
GSGPLAIGLSIAMCHLWA---IPYTGSGMNPARALGSHV----A-----SETIK--DHPYVWIYWIGPLIGGMIAGLLYDLAFA----------------------------ANAGSSKF------------
AGFFTADYEDENYDS---------
>Hrob_68110
----------------------SSESVYEVQTSGFWL-----SVAAEFVGTMLLVIFGCGSCAYEAIP---------------------LAIALSF----------------------------GLSVSTVVWAIAH---
VSGGHINPAVTMGFLATRKMSFIKAFMYVVSQMSGAIIGAVILNSLYP-----ESTFKGL------------CTPTPKTGT-SAIVTFTVEFLVTFLLVFVVFATCDGQRK-GFA-
GSGPLAIGLSVTVGHLMG---MNLTGAGTNPARVFGPAV----I-----SGTFD--RH---WAYWIGPMAAGAVAGFIYDFLFA----------------------------VNACTEKL-----------
KAFFTTADYNDADFKK---------
>Hrob_177042
----------------------MERNWKELKSFSLWK-----AMLAEMVGTSLLVLFGCGTCIGHDWQSRD---------------PTIVQISLTF----------------------------GLTVATIVHCMEH---
ISGGHINPAVTCAMFITRRVTIVRAVLYIAAQLTGSIYGAAILKGITP-----VVYEGDL------------GQTSVDETLRACPDGLTVEASITFILVLTIFASCETNKN-DRN-
GSAAFSIGFAVAFCHMFA---IKYTGSSMNPARSFGPAV----I-----GGNWN--DH---YVFWVGPVLGAVIAGLSYDFIFA----------------------------RNAGVEKF-----------
IHFFTEEDYNGDSKWS---------
>Hrob_185178
----------------------LTRNVRDLKDRVTWQ-----SALAELLGTCILIVIGTGTCIGKDWEANT---------------PTIVQISLTF----------------------------GLAVATVVRCIGH---
VSGGHINPAVTCAMLATRRVTLSKAIIYIVSQCLGAIIGSAIVMAITP-----ADYMGDL------------GQTSVSSSIKAFPRAVAVEAFITFVLIFTIFASCDANRH-DLS-
GSTPLSIGFAVAFCHMFA---IKYTGSSMNPARTFGPAV----V-----GGTWE--HH---WVYWIGPITGAIFAGLIYEFIFA----------------------------SNATPAKM-----------
RKYFSDPDYVGDRIFV---------
>Hrob_113524
----------------------MANHCEEIKTPAFWK-----AQFAEFFGTAILVFIGCGSTLPLEGM-----------------KRHDLEISTAF----------------------------GLAVATSVWIFGH---
VSGGHINPAVTLGFIIARRISVLKGFFYMIFQCLGALAGAAVLYACV------GVNGKSI------------GATSIISRM-GFMEALGVETIITFVLVMTVFASCDVKRR-DLG-
GSRPLTIGISVLICHLAA---IRSTGASMNPARSFGPAV----I-----DNYWQ--NH---WVYWVGPMVGGLIAATVYEVIFA----------------------------KDADPRDF------------
CCFTIEREQPAQHKE---------
>Hrob_176474
----------------------MGNMCDEVKTAMFWK-----AQLAEFLGTAILVFVGCASCLGDEWSRVA--------------SPRLVEISLAF----------------------------GLAVATSVWIFGH---
VSGGHINPAVTCAFLIARRISILKGLFYILFQCLGAMTGAGFLYEETN-----------------------------------------------------------------------------CHQKE-----------------------V---------------
--------------------------------------------------------------------------------------------
>Hrob_154942
----------------------LPDSHNEIHSAALVK-----GTIAEFLGTLILVTIGCGTCLAKDWEKDT---------------PTTVQISLAF----------------------------GLAVAAVGWCFGH---
VDAGHANPAVTTALFVSRKISLVRWIFYVFMQCAGAILGADILLKLTPS----SVNGTEL------------GSPSINVAN-TAFQGFVVEAFITFVLVLTIFTCCDKNRK-ELH-
CIRPIAIGLCVTMCHLFA---LKYTGSSMNPARALGPHV----V-----AREFD-PNH---WVYWVGPMVGGVLAGCIHEL------------------------------------------------------------------------
>Hrob_185503
----------------------ITTNLNDLKSSHLYR-----SLVCEFIGTFILIFMGCFSIITWNRS-EP---------------PSLVAIALCF----------------------------GIIIAILATAFGG---
ISGCHINPAVSLGFLVTRRISLIRFLLYAIVQAAAAIAGAKLLYELTPE----GPRRELQ------------VVVMPLPGI-SDSNAAFVEFFLTFLLMFTVYACIDGGKP-VAH-
GTAPFIIGLSVTTGVFSA---GLYSGGCMNPIRALGPAV----V-----QNYWR--HH---WVYWVGPMMGAVVAALLYDLLFD----------------------------TAASLRKL-----------
KLFFTSLDYDANSFYV---------
>Hrob_168509
------------------------MSLNDLKKVSFWT-----ECACELMVSFMLTTVVMFVLVTNKKE-LY--------------EPNTTHLGLFV----------------------------LAFVFMAIETYGP---I-
ACLLNPMAAFSLFLAGKISLARCVIFTILELCGGLAGTFFGYLLTP------DNRVIP------------YFDPSHHGM-NNWQSVTIEGFFSFNLIFVVLSVHGTEYP-
RPLPFLPNLSIALALAVGLFAA------------------------------------------VYWLGPYIGGPLAVLAYKLFAM----------------------------GKFYK----------------------PREA------------
>Hrob_185352
----------------------FKASRRDLATLIFWR-----DVSCEFVVSTFLMTVVILILTTNDTHAYQ---------------PSTTHFGLFA----------------------------GFFIYSLLETWGP---IS-
CLGHPPAAFCFMLGGKFTVARTVFYTIAEVGCATGAGIGYALTPF----EKRSTFV------------AFNPAKHGL-SLAQSVFVEAVFTFNLIFCIFSVHGSDYA-
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RKFPILPNLAIGSAIGTAIMAA---GTFTGGFMNPLIAFGPAI----V-----SGDFT--NH---WIYWVGPYVGGIPAVFLYKFYHW----------------------------VKVRHERL--------------------
PKRVVPP---------
>Hrob_185353
-----------------------LQHSKQDLSTLIVWR-----DVFCELMVTSFLMIMVTLVLITNNVE-AY---------------KPGVTHFGIFA----------------------------GFFVYMLLEGYGN---IS-
CVANPMAAFCFYLAGKFSIAKTILFTAAHVTGCICGSVIGYELTPAARLAEPGGAFH------------AFNPANHGL-TVTQSVFVEAILSFNLIFVVLSLHGSELG-
RPYPILPNLAIGIVIGTSIMAA---GTHTGGFMNPLISLGPAF----L-----SHDFK--NH---WIYWVGPYIGGPPAVYVYKMFVF----------------------------IKRRNDLM-----------
ANDVLLESSPRSYISN---------
>Hrob_168508
-----------------------FEWSLDDLARPSFWT-----ECACEMLVTFILLSTMTLVASVSEQD-AN---------------PASTVHVCFYL----------------------------ARFMYVAYEGYGA---I-
GCCVSPVVTFSILLAREISLAKILLA-------------------------EDSELP------------MVDPGRSGL-KIWQATTVEGLYSQNTV------------------LPSLAVSITVAFGILGT---
ARHTGGFMNPLIPFAFAV----F-----HGNVK--NQ---WIYWLGPYIGGTLAVLTYGQMLK----------------------------GRKYKPKF-----------K--------QTQTTQN---------
>Hrob_95422
---------------------PVHRLEKLGHIKNIYVK-----YFLAEFLGTCLLMLGGCSSSAQFVLSKGT----------------SSSDGPVWGW----------------------------GLSVAMACYATMG---
VSGAHINPAVSFAFMSVGRLSLLGMLMFWLAQYLGAFVGALLAYALHYEAI--NLYDGGKRAITGQNATAQIFATYPQPGL-GVYTSVIDQIIGTMVLLIFVMAISDKRNS-
NIPQGLKPLVVGALVVFVNYSF---VYNCMGAINPARDMGPRMMTLVVGYG--VEVFTIYDY-FFWVPLVMPHVGAFLGAHIYQLFIG----------------------------WHHADDDD---------
-----------DDITITN---------
>Eand_COMP57
-----------------------STSSLQDLKTRRFWV-----ALVAEFLGTLLLVLVACGSCASYTTSYSFRNQTDGTEVIKTKPLPSDFVQISLAF----------------------------GLSVATIVWSIAH---
VSGGHINPGVTIGFLVTRKISLIRAILYTAVQSVGAVLGAVILKLVSP-----PGLNDAL------------GTTSPGNGV-SIGQAFTIELFITFVLVYTVFATCDGQRQ-GFN-
GSGPLAIGLSISMCHLWA---IPYTGSGMNPARAFGSAL----V-----SGNLKADIH---WLYWAGPLLGGALAGVLYDFLFA----------------------------TNASLDKL-----------
KGFFTSDYDDSQYDS---------
>Lrub_COMP65
-----------------------STSSLQDLKTRRFWV-----ALLAEFLGTLLLVLVACGSCAGYTTTYTYRNQTDGSEVVKTKPLPSDFVQISLAF----------------------------GLSVATIVWSIAH---
VSGGHINPGVTIGFLVTRKISLVRAILYTAVQSVGAVLGAVILKLVSP-----PGLNDAL------------GTTSPGNGV-SIGQAFTIELFITFVLVYTVFATCDGQRQ-GFK-
GSGPLAIGLSISMCHLWA---IPYTGSGMNPARAFGSAL----V-----AGKLEPGIH---WVYWAGPLLGGALAGILYDFLFA----------------------------TNATLDKL-----------
KGFFTSDYDDSQYDS---------
>Lrub_COMP64
-----------------------IRDNFEELRSWPVWR-----AMVAEFIGTLMLVFIGCGACIGGAWSDLD---------------DPTVLGIALAF----------------------------GLIVATMIWSFGH---
VSGGHVNPAVTFGFLVARRITIVRAALYIISQCAGAIVGCGILKGLSP-----HNSNETF------------GLTVVWKQI-TPGQGCGVEIIITFVLVFCVFASVDGRRA-DLN-
GSTPLSIGLSVTVCHLFA---VRYTGSSMNPARTFGPAV----I-----TNKWT--NH---WVYWVGPIIGGIIGALLYELVFS----------------------------ASASLRNL-----------
KHFFTSPVYGQDEDVD---------
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Chapter IV

Table S1. Table with the process ID, the species, the amplified marker, the estuary and the geographical coordinates of the annelid specimens used in this work.

Process ID	Species	Amplified marker	Estuary	Latitude	Longitude
ESPBR001-21	*Alitta* sp. A	COI	PEC	-25.449	-48.689
ESPBR002-21	*Alitta* sp. A	COI	PEC	-25.243	-48.415
ESPBR003-21	*Alitta* sp. A	COI	PEC	-25.435	-48.705
ESPBR004-21	*Alitta* sp. A	COI	PEC	-25.243	-48.415
ESPBR005-21	*Alitta* sp. A	COI	PEC	-25.449	-48.689
ESPBR006-21	*Alitta* sp. A	COI	PEC	-25.496	-48.498
ESPBR007-21	*Alitta succinea*	COI	PEC	-25.297	-48.331
ESPBR008-21	*Alitta succinea*	COI	PEC	-25.51	-48.468
ESPBR009-21	*Alitta succinea*	COI	PEC	-25.552	-48.395
ESPBR010-21	*Alitta succinea*	COI	PEC	-25.51	-48.468
ESPBR011-21	*Alitta succinea*	COI	PEC	-25.297	-48.331
ESPBR012-21	Alitta sp. A	COI	PEC	-25.435	-48.705
ESPBR013-21	*Nereis* sp.	COI	PEC	-25.548	-48.434
ESPBR014-21	*Nereis* sp.	COI	PEC	-25.548	-48.434
ESPBR015-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.844	-48.582
ESPBR016-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.844	-48.582
ESPBR017-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.86	-48.64
ESPBR018-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.859	-48.64
ESPBR019-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.868	-48.708
ESPBR020-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.859	-48.64
ESPBR021-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.868	-48.708
ESPBR022-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.854	-48.571
ESPBR023-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.873	-48.606
ESPBR024-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.86	-48.64
ESPBR025-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.86	-48.597
ESPBR026-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.864	-48.646
ESPBR027-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.854	-48.571
ESPBR028-21	*Alitta* sp. A	COI	Guaratuba Bay	-25.86	-48.597
ESPBR029-21	*Pseudonereis* sp.	COI	Guaratuba Bay	-25.873	-48.606
ESPBR030-21	*Alitta* sp. A	COI	Babitonga Bay	-26.264	-48.653
ESPBR031-21	*Alitta* sp. A	COI	Babitonga Bay	-26.275	-48.715
ESPBR032-21	*Alitta* sp. A	COI	Babitonga Bay	-26.249	-48.701
ESPBR033-21	*Alitta* sp. A	COI	Babitonga Bay	-26.3	-48.798
ESPBR034-21	*Alitta* sp. A	COI	Babitonga Bay	-26.275	-48.715

ESPBR035-21	*Alitta* sp. A	COI	Babitonga Bay	-26.264	-48.653
ESPBR036-21	*Alitta* sp. A	COI	Babitonga Bay	-26.307	-48.687
ESPBR037-21	*Alitta succinea*	COI	Babitonga Bay	-26.235	-48.641
ESPBR038-21	*Alitta succinea*	COI	Babitonga Bay	-26.235	-48.641
ESPBR039-21	*Alitta succinea*	COI	Babitonga Bay	-26.249	-48.701
ESPBR040-21	*Alitta succinea*	COI	Babitonga Bay	-26.296	-48.697
ESPBR041-21	*Pseudonereis* sp.	COI	Babitonga Bay	-26.185	-48.613
ESPBR042-21	*Isolda pulchella*	COI	Babitonga Bay	-26.213	-48.592
ESPBR043-21	*Isolda pulchella*	COI	PEC	-25.557	-48.31
ESPBR044-21	*Isolda pulchella*	COI	PEC	-25.552	-48.395
ESPBR045-21	*Isolda pulchella*	COI	PEC	-25.557	-48.31
ESPBR046-21	*Isolda pulchella*	COI	PEC	-25.337	-48.394
ESPBR047-21	*Isolda pulchella*	COI	PEC	-25.553	-48.437
ESPBR048-21	*Isolda pulchella*	COI	PEC	-25.552	-48.395
ESPBR049-21	*Isolda pulchella*	COI	PEC	-25.365	-48.424
ESPBR050-21	*Isolda pulchella*	COI	PEC	-25.553	-48.437
ESPBR051-21	*Isolda pulchella*	COI	PEC	-25.409	-48.717
ESPBR052-21	*Isolda pulchella*	COI	PEC	-25.51	-48.468
ESPBR053-21	*Isolda pulchella*	COI	PEC	-25.365	-48.424
ESPBR054-21	*Isolda pulchella*	COI	PEC	-25.557	-48.31
ESPBR055-21	*Isolda pulchella*	COI	PEC	-25.409	-48.717
ESPBR056-21	*Isolda pulchella*	COI	PEC	-25.365	-48.424
ESPBR057-21	*Isolda pulchella*	COI	PEC	-25.337	-48.394
ESPBR058-21	*Isolda pulchella*	COI	Babitonga Bay	-26.184	-48.623
ESPBR059-21	*Isolda pulchella*	COI	Babitonga Bay	-26.213	-48.592
ESPBR060-21	*Isolda pulchella*	COI	Babitonga Bay	-26.213	-48.592
ESPBR061-21	*Isolda pulchella*	COI	Babitonga Bay	-26.184	-48.623
ESPBR062-21	*Isolda pulchella*	COI	Babitonga Bay	-26.315	-48.731
ESPBR063-21	*Isolda pulchella*	COI	Babitonga Bay	-26.338	-48.685
ESPBR064-21	*Isolda pulchella*	COI	Babitonga Bay	-26.315	-48.731
ESPBR065-21	*Isolda pulchella*	COI	Babitonga Bay	-26.315	-48.731
ESPBR066-21	*Isolda pulchella*	COI	Babitonga Bay	-26.184	-48.623
ESPBR067-21	*Isolda pulchella*	COI	Babitonga Bay	-26.184	-48.623
ESPBR068-21	*Isolda pulchella*	COI	Babitonga Bay	-26.184	-48.623
ESPBR069-21	*Isolda pulchella*	COI	Babitonga Bay	-26.315	-48.731
ESPBR070-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.862	-48.589
ESPBR071-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.862	-48.589
ESPBR072-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.86	-48.597
ESPBR073-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.86	-48.64
ESPBR074-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.86	-48.597
ESPBR075-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.859	-48.64
ESPBR076-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.859	-48.64

ESPBR077-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.859	-48.64
ESPBR078-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.86	-48.64
ESPBR079-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.864	-48.661
ESPBR080-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.86	-48.64
ESPBR081-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.86	-48.597
ESPBR082-21	*Isolda pulchella*	COI	Guaratuba Bay	-25.862	-48.589
ESPBR083-21	*Laeonereis acuta*	COI	Babitonga Bay	-26.213	-48.592
ESPBR084-21	*Laeonereis acuta*	COI	Babitonga Bay	-26.213	-48.592
ESPBR085-21	*Laeonereis acuta*	COI	Babitonga Bay	-26.264	-48.653
ESPBR086-21	*Laeonereis acuta*	COI	Babitonga Bay	-26.264	-48.653
ESPBR087-21	*Laeonereis acuta*	COI	Babitonga Bay	-26.282	-48.675
ESPBR088-21	*Laeonereis acuta*	COI	Babitonga Bay	-26.338	-48.685
ESPBR089-21	*Laeonereis acuta*	COI	Babitonga Bay	-26.338	-48.685
ESPBR090-21	*Laeonereis acuta*	COI	Babitonga Bay	-26.264	-48.653
ESPBR091-21	*Laeonereis pandoensis*	COI	Babitonga Bay	-26.296	-48.697
ESPBR092-21	*Laeonereis acuta*	COI	PEC	-25.246	-48.416
ESPBR093-21	*Laeonereis acuta*	COI	PEC	-25.446	-48.691
ESPBR094-21	*Laeonereis acuta*	COI	PEC	-25.553	-48.437
ESPBR095-21	*Laeonereis acuta*	COI	PEC	-25.514	-48.491
ESPBR096-21	*Laeonereis acuta*	COI	PEC	-25.449	-48.689
ESPBR097-21	*Laeonereis acuta*	COI	PEC	-25.514	-48.491
ESPBR098-21	*Laeonereis acuta*	COI	PEC	-25.553	-48.437
ESPBR099-21	*Laeonereis acuta*	COI	PEC	-25.562	-48.36
ESPBR100-21	*Laeonereis acuta*	COI	PEC	-25.514	-48.491
ESPBR101-21	*Laeonereis pandoensis*	COI	PEC	-25.243	-48.415
ESPBR102-21	*Laeonereis pandoensis*	COI	PEC	-25.243	-48.415
ESPBR103-21	*Laeonereis pandoensis*	COI	PEC	-25.243	-48.415
ESPBR104-21	*Laeonereis pandoensis*	COI	PEC	-25.246	-48.416
ESPBR105-21	*Laeonereis pandoensis*	COI	Guaratuba Bay	-25.868	-48.708
ESPBR106-21	*Laeonereis pandoensis*	COI	Guaratuba Bay	-25.86	-48.64
ESPBR107-21	*Laeonereis pandoensis*	COI	Guaratuba Bay	-25.86	-48.597
ESPBR108-21	*Laeonereis pandoensis*	COI	Guaratuba Bay	-25.86	-48.64
ESPBR109-21	*Laeonereis pandoensis*	COI	Guaratuba Bay	-25.868	-48.708
ESPBR110-21	*Laeonereis pandoensis*	COI	Guaratuba Bay	-25.864	-48.661
ESPBR111-21	*Laeonereis pandoensis*	COI	Guaratuba Bay	-25.86	-48.64
ESPBR112-21	*Laeonereis pandoensis*	COI	Guaratuba Bay	-25.862	-48.589
ESPBR113-21	*Laeonereis pandoensis*	COI	Guaratuba Bay	-25.866	-48.712

ESPBR114-21	*Laeonereis pandoensis*	COI	Guaratuba Bay	-25.868	-48.708
ESPBR115-21	*Laeonereis pandoensis*	COI	Guaratuba Bay	-25.86	-48.64
ESPBR116-21	*Laeonereis pandoensis*	COI	Guaratuba Bay	-25.864	-48.661
ESPBR117-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.862	-48.589
ESPBR118-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.112	-48.788
ESPBR119-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.112	-48.788
ESPBR120-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.112	-48.788
ESPBR121-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.3	-48.798
ESPBR122-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.112	-48.788
ESPBR123-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.112	-48.788
ESPBR124-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.112	-48.788
ESPBR125-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.3	-48.798
ESPBR126-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.3	-48.798
ESPBR127-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.3	-48.798
ESPBR128-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.112	-48.788
ESPBR129-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.868	-48.708
ESPBR130-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.3	-48.798
ESPBR131-21	*Nephtys fluviatilis*	COI	PEC	-25.421	-48.707
ESPBR132-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.3	-48.798
ESPBR133-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.3	-48.798
ESPBR134-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.864	-48.661
ESPBR135-21	*Nephtys fluviatilis*	COI	PEC	-25.243	-48.415
ESPBR136-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.862	-48.589
ESPBR137-21	*Nephtys fluviatilis*	COI	Babitonga Bay	-26.112	-48.788
ESPBR138-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.868	-48.708
ESPBR139-21	*Nephtys fluviatilis*	COI	PEC	-25.243	-48.415
ESPBR140-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.862	-48.589
ESPBR141-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.86	-48.64
ESPBR142-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.864	-48.661
ESPBR143-21	*Nephtys fluviatilis*	COI	PEC	-25.446	-48.691
ESPBR144-21	*Nephtys fluviatilis*	COI	PEC	-25.421	-48.707
ESPBR145-21	*Nephtys fluviatilis*	COI	PEC	-25.246	-48.416
ESPBR146-21	*Nephtys fluviatilis*	COI	PEC	-25.446	-48.691
ESPBR147-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.859	-48.64
ESPBR148-21	*Nephtys fluviatilis*	COI	PEC	-25.243	-48.415
ESPBR149-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.868	-48.708
ESPBR150-21	*Nephtys fluviatilis*	COI	PEC	-25.421	-48.707
ESPBR151-21	*Nephtys fluviatilis*	COI	PEC	-25.246	-48.416
ESPBR152-21	*Nephtys fluviatilis*	COI	PEC	-25.246	-48.416

ESPBR153-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.868	-48.708
ESPBR154-21	*Nephtys fluviatilis*	COI	PEC	-25.446	-48.691
ESPBR155-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.86	-48.64
ESPBR156-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.862	-48.589
ESPBR157-21	*Nephtys fluviatilis*	COI	PEC	-25.243	-48.415
ESPBR158-21	*Nephtys fluviatilis*	COI	PEC	-25.243	-48.415
ESPBR159-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.868	-48.708
ESPBR160-21	*Nephtys fluviatilis*	COI	Guaratuba Bay	-25.862	-48.589

Table S2. Genetic distances among the target species and for each species.

Label	**n° specimens**	**Taxa**	**Comparisons**	**Min Dist (%)**	**Mean Dist (%)**	**Max Dist (%)**	**SE Dist (%)**
Within Species	156	4	2993	0.00	3.88	20.93	0.00
Within Genus	0	0	0	0.00	0.00	0.00	0.00
Within Family	71	1	1258	22.75	26.97	28.57	0.00

Alitta succinea

Label	**n° specimens**	**Taxa**	**Comparisons**	**Min Dist (%)**	**Mean Dist (%)**	**Max Dist (%)**	**SE Dist (%)**
Within Species	37	1	666	0.00	7.91	21.07	0.01

Laeonereis culveri

Label	**n° specimens**	**Taxa**	**Comparisons**	**Min Dist (%)**	**Mean Dist (%)**	**Max Dist (%)**	**SE Dist (%)**
Within Species	34	1	561	0.00	9.66	19.23	0.02

Nephtys fluviatilis

Label	**n° specimens**	**Taxa**	**Comparisons**	**Min Dist (%)**	**Mean Dist (%)**	**Max Dist (%)**	**SE Dist (%)**
Within Species	44	1	946	0.00	0.72	2.08	0.00

Isolda pulchella

Label	**n° specimens**	**Taxa**	**Comparisons**	**Min Dist (%)**	**Mean Dist (%)**	**Max Dist (%)**	**SE Dist (%)**
Within Species	41	1	820	0.00	0.33	1.04	0.00

Table S3. Diagnostic characters between the MOTUs of *Alitta succinea*.

Group name (n° sequences)	n° diagnostic characters	n° diagnostic or partial characters	n° partial characters	n° partial or uninformative characters	n° invalid characters
Alitta n. sp. A (28)	95	0	11	0	0
Alitta succinea (9)	91	0	7	0	0

Table S4. Fst results for each species among the estuaries. PEC= Paranaguá Estuarine Complex; GB= Guaratuba Bay; BB= Babitonga Bay.

Alitta sp. A

	PEC	**GB**	**BB**
PEC		-	-
GB	-		-
BB	-	-	

Alitta succinea

	PEC	**GB**
PEC		-
GB	-	

Laeonereis pandoensis

	PEC	**GB**	**BB**
PEC		-	-
GB	-		-
BB	-	-	

Laeonereis acuta

	PEC	**BB**
PEC		+
BB	+	

Nephtys fluviatilis

	PEC	**GB**	**BB**
PEC		-	+
GB	-		+
BB	+	+	

Isolda pulchella

	PEC	**GB**	**BB**
PEC		-	-
GB	-		-
BB	-	-	

Table S5. Correlations between genetic diversity of each species and geographical distances.

Species	Record Count	n° BINs	Linear Regression Rsq	Linreg Slope	Gen Dist Max	Geo Mst Sum	Geo Dist Max	Mantel Rsq	Mantel Pvalue
Alitta sp. A	28	1	0.001	-0.00021	0.846	171.373	123.667	0.001	0.54
Alitta succinea	9	1	0.016	0.00291	2.538	127.372	117.004	0.016	0.28
Laeonereis pandoensis	17	1	0.005	0.0004	0.855	131.099	120.479	0.005	0.35
Laeonereis acuta	17	1	0.125	0.00188	0.855	159.651	124.411	0.125	0.01
Nephtys fluviatilis	44	1	0.106	0.00408	2.051	146.24	123.667	0.106	0.01
Isolda pulchella	41	1	0	-0.00002	1.036	174.156	115.085	0	0.54