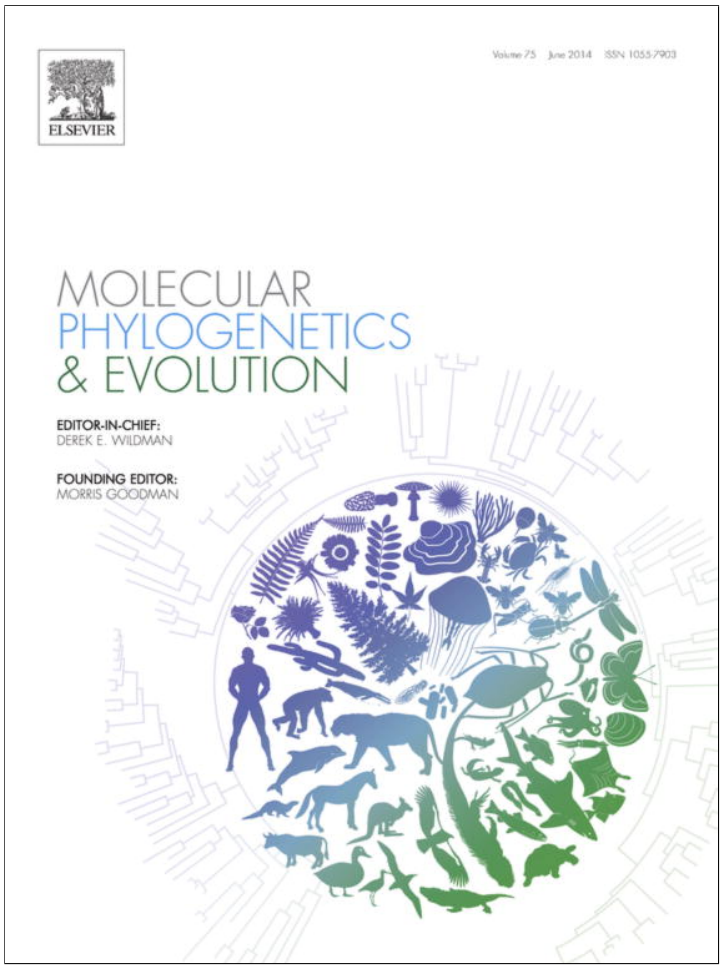

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Short Communication

Molecular data do not provide unambiguous support for the monophyly of flatfishes (Pleuronectiformes): A reply to Betancur-R and Ortí

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ABSTRACT

Betancur-R and Ortí (2014) offer a criticism of our recent examination of the monophyly of extant flatfishes (Pleuronectiformes; Campbell et al., 2013). We welcome this opportunity to examine and respond to the main issues presented in Betancur-R and Ortí (2014). Briefly, this debate centers on the question of whether or not analyses of the available evidence support a stable and confident conclusion regarding a sister group relationship between the two recognized pleuronectiform suborders: Psettodoidei (four species) and Pleuronectoidei (>700 species). In Campbell et al. (2013), we reported results based on sequences from six nuclear genes compatible with monophyly of Pleuronectoidei but not with that of Pleuronectiformes. In our analyses, the most closely related percomorph family to the Pleuronectoidei was resolved to be the Centropomidae. In Campbell et al. (2013), we also provided a critical review of the morphological evidence in favor flatfish monophyly showing that this evidence requires a careful re-examination where it concerns psettodoids. Here we present our perspective on the issues raised in Betancur-R and Ortí (2014).

Introduction

In the following discussion, we use the notations of Betancur-R and Ortí (2014, hereafter BO) to refer to Betancur-R et al. (2013, hereafter BET) and to Campbell et al. (2013, hereafter CAM).

Issue 1: Phylogenetic analyses from concatenated data sets are sensitive to conflicting gene tree histories

BO claims that concatenation of unlinked loci can circumvent the effects of differences in individual gene histories. However, concatenated analyses are affected by these differences (Kubatko and Degnan, 2007; McVay and Carstens, 2013). Most analyses reported in BET (15/19, BET Table 3) do not support pleuronectiform monophyly. These include all those analyses conducted by BET using species tree methods (STAR) (Liu et al., 2009), which are designed to account for incomplete lineage sorting. Additional results of individual gene trees support pleuronectiform monophyly in no more of 4/18 cases across six additional analyses presented in BET. Given the sensitivity of the pleuronectoid plus psettodoid clade to varying samples (taxonomic and molecular) and analyses frameworks, we consider the molecular evidence equivocal concerning pleuronectiform monophyly. Overall, the results to date do not provide consistent or unambiguous support for monophyly.

To examine if incomplete lineage sorting is likely to influence inference of basal pleuronectiform relationships, and if the current molecular data available can resolve the question of pleuronectiform monophyly we conducted a triplet analysis (Cranston, 2010; Pamilo and Nei, 1988). Using RAxML version 7.3.0 (Stamatakis, 2006), we generated gene trees for rooted three taxon cases to test pleuronectiform monophyly with the BET and CAM datasets (Table 1). Unambiguous support for neither pleuronectiform monophyly nor non-monophyly is evident in this analysis. However, it suggests a high degree of incomplete lineage sorting or inability to infer an accurate gene tree through the use of a homogeneous model among other constraints. The incongruence among gene tree analyses indicates that a coalescent uncertainty is present and must be addressed using a species tree method (McVay and Carstens, 2013). Analyses that attempt to incorporate

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Table 1
Triplet analysis of rooted four taxon cases from the BET and CAM datasets. In all triplet comparisons we used representatives of the genera *Eopsetta* (Pleuronectoidei), *Psettodes* (Psettodoidei), and rooted with *Channa*. (Channidae). Three different carangimorph (clade L) representatives were included in the three triplet analyses, *Toxotes*, *Sphyraena*, and *Centropomus*. From BET the taxa used from the Pleuronectiformes were *Eopsetta jordani* and *Psettodes erumei*. The three different carangimorph fish rotated through the analysis were *Toxotes jaculatrix*. *Sphyraena putnamae*, and *Centropomus undecimalis*. The sequences were rooted at *Channa striata* and are drawn for the no missing dataset of BET plus the SH3PX3 locus from the full BET dataset for a 19 loci. Four additional loci, EGR1, EGR2B, EGR3, and MLL (for 23 total) are drawn for the CAM dataset using *Eopsetta jordani* and *Psettodes erumei* to represent the Pleuronectiformes. *Toxotes jaculatrix*, *Sphyraena argentea*, and *Centropomus undecimalis* were used in the three different analyses to represent Carangimorpha. *Channa maculata* is used to root the gene trees from CAM, except for MLL where *Channa striata* (GenBank Accession: AY362241.1) was aligned to the existing sequences using MUSCLE v3.8.31 (Edgar, 2004a, 2004b). In all analyses completely undetermined sites were removed. Gene trees were inferred under a General Time Reversible (GTR) with four category gamma distribution (Γ) in RAxML HPC-PTHREADS v7.3.0 (Stamatakis, 2006). We report which pairs of taxa are found to be most closely related to each other across the rooted gene trees. Only three possibilities are present: ((A,B),C), ((A,C),B), and ((B,C),A). The correct relationship should occur at some frequency greater than 1/3, the other two possibilities should occur at some frequency that is less than 1/3 (Cranston, 2010; Pamilo and Nei, 1988).

Toxotes as non-pleuronectiform		*Centropomus* as non-pleuronectiform		*Sphyraena* as non-pleuronectiform	
Pair	Occurrence	Pair	Occurrence	Pair	Occurrence
BET dataset					
Eopsetta and Psettodes	9	Eopsetta and Psettodes	7	Eopsetta and Psettodes	8
Psettodes and Toxotes	8	Psettodes and Centropomus	6	Psettodes and Sphyraena	7
Eopsetta and Toxotes	2	Eopsetta and Centropomus	6	Eopsetta and Sphyraena	4
CAM dataset (using EGR1, EGR2B, EGR3, MLL)					
Eopsetta and Psettodes	1	Eopsetta and Psettodes	1	Eopsetta and Psettodes	2
Psettodes and Toxotes	3	Psettodes and Centropomus	2	Psettodes and Sphyraena	1
Eopsetta and Toxotes	0	Eopsetta and Centropomus	1	Eopsetta and Sphyraena	1
Total proportions					
Proportion Supporting Psettodes + Eopsetta	0.435	Proportion Supporting Psettodes + Eopsetta	0.348	Proportion Supporting Psettodes + Eopsetta	0.435
Proportion Supporting Psettodes + Toxotes	0.478	Proportion Supporting Psettodes + Centropomus	0.348	Proportion Supporting Psettodes + Sphyraena	0.348
Proportion Supporting Eopsetta + Toxotes	0.087	Proportion Supporting Eopsetta + Centropomus	0.304	Proportion Supporting Eopsetta + Sphyraena	0.217

the effects of incomplete lineage sorting using a non-homogeneous model result in a non-monophyletic Pleuronectiformes and a monophyletic Pleuronectoidei from the BET dataset, which are the same conclusions presented in CAM.

Issue 2: Higher bootstrap values in the analyses are not necessarily indicative of phylogenetic signal

A confounding factor in this debate is the interpretation of bootstrap values from datasets of differing lengths. In BO, bootstrap values were used to gauge the phylogenetic signal in the data. A monophyletic Pleuronectiformes was resolved with 65% bootstrap support, which led the authors to conclude that a "stronger phylogenetic signal" was retained in a longer (23 genes) dataset. However, the authors ignored the fact that in the analyses reported in CAM, a group incompatible with pleuronectiform monophyly (i.e., pleuronectoids + centropomids) was resolved with much higher bootstrap support (73% from ML analysis with $1_N2_N3_{RY}$ and 87% from ML analysis with 1_N2_N no missing data; see Table 1 in CAM).

Further, it is known that the bootstrap index can be a poor measure of "phylogenetic signal" in concatenated analyses because it may take on high values even in the face of conflict or systematic error (Chen et al., 2003; Felsenstein, 1978; Hillis and Bull, 1993; Huelsenbeck, 1997; Salichos and Rokas, 2013). One must keep in mind that these indicators assess the strength of the signal used to order the data hierarchically (Swofford et al., 1996). That "signal" can originate either from common ancestry or non-phylogenetic sources like convergent constrainsts in the nucleotide substitution process. Therefore, the numerical bootstrap value does not necessarily measure the reliability of a phylogenetic inference (Chen et al., 2003). In CAM, concomitant with numerical or statistic nodal supports, the question of pleuronectiform monophyly was evaluated with an analytical strategy involving alternative coding schemes, inference methods and models of molecular evolution designed to assess the influence of phylogenetic artifacts. Non-stationarity in nucleotide evolution seems to be a problem affecting the monophyly of the Pleuronectoidei in past studies due to compositional biases in some lineages (e.g. Bothidae). In the case of *Psettodes*, this taxon does not appear to be distinctly biased in base composition, thus we deemed non-stationarity an unlikely factor in producing a non-monophyletic Pleuronectiformes. Among all the different phylogenetic methods employed and data treatments employed by CAM, no treatment of the data yielded support of a monophyletic Pleuronectiformes in the optimal consensus topology.

The bootstrap support values in the analyses presented by BET and BO derive from long concatenated datasets. Thus, the value of comparing bootstrap indices presented in CAM, BET and BO is compromised by differences in the lengths of the different datasets. Additionally, an increase in bootstrap support in concatenated analyses for the support of pleuronectiform monophyly from below 50 in BET to 65 in BO should be expected as an artifact, and highlights the need to move away from using bootstrap support as a sole indicator of support for concatenated analyses of large datasets (Salichos and Rokas, 2013). The potential effects of increased proportions of missing data on the larger datasets are discussed in more detail below.

Issue 3. The CAM dataset contains fewer sites than those examined in BET/BO but it is not necessarily more prone to error than those used in BET/BO

This point touches on important and open questions in molecular phylogenetics: How is phylogenetic information distributed across the genome and how variable is that information from

region to region? This aspect of BO's criticism is based on the differences the number of sites and loci between CAM and BET (BO Table 1). While generally increasing the number of sites can increase the accuracy of inference by reducing stochastic errors (Chen and Mayden, 2010). However, number of sites is not the essential consideration in phylogenetic inference. Rather, quality and quantity of phylogenetic signal captured by the data are the essential characteristics that increased character sampling attempts to address.

The gene markers chosen in particular for the CAM dataset RAG1, Rhodopsin, EGR1, EGR2B, EGR3, and MLL have been shown to be phylogenetically informative for the investigation of the deep evolutionary relationships for several teleost groups (e.g., Elopomorpha, Ostariophysi, Cypriniformes, Esociformes, Acathomorpha, etc.; Chen, 2001; Chen et al., 2003, 2007, 2008, 2013; Chen and Mayden, 2010; Chen et al., 2014; Dettaï and Lecointre, 2005; López et al., 2004; Mayden and Chen, 2010). In addition, this character sampling design (six nuclear genes) is as good as most of those used in the other papers recently published in highly respected journals in the research field of molecular systematics.

The CAM sequence alignment consists of ninety taxa with only 7.6% gaps and missing data with a total of 5664 total characters 3034 variable sites (2525 parsimony informative) from nuclear gene loci with moderate substitution rates. The complete BET alignment of twenty loci contains 29.1% gaps and missing data, 19,461 total characters and 11,501 variable sites (9812 parsimony informative). The BET alignment includes the highly variable 16S mitochondrial rRNA locus, which inflates the number of variable sites and can be expected to increase homoplasy in the dataset as the product of multiple substitution hits. In CAM there are 33.7 variable sites per taxon with few missing data compared to BET with 53.7 variable sites per taxon and almost 4 times as much missing data. It has been shown that missing data can result in topological and branch length errors, and can lead to strong support of false bipartitions which otherwise would have weak support (Lemmon et al., 2009) except when the following conditions are met: (1) missing data are not randomly distributed among taxa; (2) overall branch lengths are not long and/or characters do not evolve rapidly; (3) sufficient informative data are analyzed with appropriate methods such as Bayesian and likelihood analyses in which realistic sequence evolution models are implemented (Wiens and Morrill, 2011). Significantly, an analysis of the BET matrix excluding sites with missing data produces the same conclusions as those reported in CAM (i.e. paraphyletic pleuronectiforms and monophyletic pleuronectoids). Monophyly of Pleuronectiformes is found in BO and BET only when missing data sites are included highlighting the possible influence of missing data in the conclusions of BET and BO. As molecular datasets grow exponentially larger, it will be interesting to examine how and why missing data affects the stability of inferred pleuronectiform relationships.

Issue 4: Pleuronectiform monophyly has not been conclusively demonstrated morphologically

We presented a discussion of the issues surrounding the morphological evidence for pleuronectiform monophyly in CAM but review them here for added emphasis. As previously noted the conditions of the three putative pleuronectiform synapomorphies (Chapleau, 1993) in psettodoids raise questions about the value of these traits to support pleuronectiform monophyly. First, the presence of a *recessus orbitalis* in psettoids has yet to be conclusively established (Hensley, 1997). It was only assumed to be present in *Psettodes* by Chapleau (1993). However, it is important to note that *Psettodes* does not exhibit the skin folds around the eyes and eye protrusion ability associated with a *recessus orbitalis* (Chabanaud, 1937). Second, the condition of the insertion of the dorsal fin is distinct in *Psettodes* from all other flatfishes and it is posterior to the eye (Nelson, 2006). Finally, the extent of cranial asymmetry, is different in *Psettodes*, where eye migration is arrested at the dorsal midline (Friedman, 2008). In summary, there is no overwhelming morphological evidence in support of pleuronectiform monophyly. Therefore, tests of that hypothesis using independent sources of evidence contribute to establishing the degree of confidence that can be associated with the group. Using the compelling shared body plan of flatfishes to differentially weigh conflicting results invalidates the value of conducting these studies. Alternatively, highlighting ambiguous and contradictory results may point to interesting aspects of the evolution of this group either at the paleontological or molecular levels.

Issue 5: The CAM study presents our findings from extensive analyses of a relevant dataset. Confidence in molecular phylogenetic research does not rest on selective reporting of results that match expectations

In CAM, we presented results of an extensive set of analyses of a large collection of DNA sequences from a representative set of pleuronectiform taxa and potential outgroups to examine the evolutionary history of the Pleuronectiformes. As mentioned above, filtering those results using previously proposed hypothesis would negate the value of conducting repeated tests. Further, while producing a stable classification of extant species is an important goal of phylogenetics, it is not the only or even the most important goal. Perhaps of greater significance are inferences concerning the timing and dynamics of diversification of different lineages, which in turn can be used to identify regularities and peculiarities in the process of evolution. In this respect, it is essential to highlight and understand the history of ambiguously supported groups. These results can then guide further studies that will clarify why these species that share a unique body plan do not carry a strong and unambiguous signal of shared history in their genomes. We strongly disagree with the proposition that reporting results that are in conflict with currently accepted hypotheses or classifications, whether or not they concern emblematic groups, weakens the field.

Issue 6: Taxon sampling and its effect on phylogenetic inference may be one of the factors that lead to different conclusions in the CAM, BET and BO analyses, but the use of "less" taxa in CAM is not necessarily more prone to error than a "dense" taxon sampling in BET/BO for the question of monophyletic pleuronectiforms

Dense taxonomic sampling can reduce effects of systematic biases, such as long-branch attraction, on phylogenetic inference (Hillis, 1998; Zwickl and Hillis, 2002). In practice, phylogenetic analyses examine only a subset of ingroup diversity due to practical limitations and the fact that only a subset of lineages in an ingroup survived to be sampled in molecular studies (Chen and Mayden, 2010). In CAM, our taxon sampling was designed to allow an examination of the relationship of pleuronectiforms to the other percomorph fishes. Instead of sampling a diverse set of pleuronectiform taxa, which would be important in the study of the pleuronectiform intra-relationships, our emphasis was inclusion of major pleuronectiform lineages and the representative taxa from the proposed pleuronectiform allies from the Carangimorpha (or clade L). We made our best efforts in sequencing our targeted loci from these samples to avoid the issue of missing data. Our taxonomic sampling is specifically relevant given our study objectives. We think that a simple comparison of the number of taxa used in a dataset (see Table 1 in BO) is not a useful indicator of the relative merits of

different sampling schemes as they relate to particular phylogenetic questions. Importantly, if long-branch attraction is influencing inference of basal pleuronectiform relationships then all conceivable molecular datasets will be prone to its effects given the lack of extant or extinct stem taxa that may "break" the psettodoid branch for example. Finally, as molecular data continue to accumulate, it will soon be possible and interesting to study the effects of taxonomic composition on inference of pleuronectiform phylogeny using either taxon jackknifing or simulation approaches.

Issue 7: Rejecting pleuronectiform monophyly and the value of topology tests

Topology tests that BO claims to be essential for phylogenetic hypothesis testing are subject to limitations (e.g. Goldman et al., 2000) and a suite of issues are raised by their use. It is generally accepted that such the tests present only the most conservative way to assess different hypotheses. Indeed, a correct hypothesis may not be able to be statistically rejected while it still makes the observed data the most likely. Moreover, to perform topology tests, a strongly justified null hypothesis should first be established. As we discussed above, our perspective is that morphological evidence of sufficient clarity and weight to warrant the assumption of pleuronectiform monophyly has not been presented. That evidence may exist but the work of assembling the relevant observations and analyzing them to identify synapomorphic traits encompassing the psettodoids is not available in the scientific literature. In CAM we describe seventeen different phylogenetic analyses using four different analytical frameworks. All of these yielded topologies with a monophyletic Pleuronectoidei and a non-monophyletic Pleuronectiformes. We derive confidence in our conclusions from the congruence between these results.

Issue 8. We conducted an independent analysis of the pleuronectiform question using a different dataset

This is an important point to consider. CAM represents a parallel examination of flatfish phylogenetic relationships. Our position is that the use of an independent non-overlapping source of evidence is a strength of our contribution and not a weakness. Taxonomic groupings that find consistent and unambiguous support in independent datasets merit higher confidence than those whose support is low and highly sensitive to analyses configuration. Phylogenetic patterns can be highly heterogeneous across genomes (Yoder et al., 2013). An approach to phylogenetic inference that relies solely on continued concatenation of all available evidence will obscure that heterogeneity and, in our opinion, unjustifiably inflate confidence on the resulting phylogenetic arrangements.

Concluding remarks

As in other fields of study, it is important to take into account the degree of conflict and uncertainty associated with a result. Our contribution in CAM and the arguments outlined above show that evidence for flatfish monophyly is not broadly, cleanly or strongly preserved in living flatfish genomes. Under no definition of unequivocal known to us can it be said that DNA sequences produce unequivocal support for a monophyletic pleuronectiforms. The absence of a clear and strong molecular signal for flatfish monophyly that emerges from the studies published to date will help guide future examinations of pleuronectiform evolutionary history and molecular evolution. We find it exciting to be working at the infancy or at least the early youth of molecular evolution research. The imminent widespread availability of genome-scale datasets will allow for in depth and extensive investigations of how heterogeneity in the evolutionary dynamics of lineages and of genomic compartments led to the puzzling array of patterns evident in extant genomes (Chen and Mayden, 2010). To maximally benefit from these advances, it will be important to not prejudice our conclusions or omit reports of conflicting findings based on expectations generated from not firmly established assumptions. While BO's reference to Carl Sagan's well-worn statement regarding burdens of proof seems to place our findings concerning a particular arrangement of fish species on par with claims for the supernatural, we believe that a measured perspective, more in line with Hume's dictum: "*A wise man, therefore, proportions his belief to the evidence*", promotes richer opportunities to gain knowledge from the study of molecular phylogenetics and lead us to conclude that the evidence for flatfish monophyly is not unequivocal.

It should be noted that the graphical abstract presented by Betancur-R and Ortí (2014) manages to misrepresent the debate. They use two pleuronectoids to represent the pleuronectoid and the psettodoid lineages. Our graphical abstract includes appropriate representations of these two suborders. Additionally, prior to review our response was written to a manuscript entitled "Molecular data provide unambiguous support for the monophyly of flatfishes (Carangimorphariae: Pleuronectiformes)" which was lacking methodologically. We are pleased by the results of peer review in shaping the criticism manuscript and creating a much more civil atmosphere for dialogue.

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