Evolution of sexual dimorphism and Rensch's rule in the beetle genus *Limnebius* (Hydraenidae) - is sexual selection opportunistic?

Andrey Rudoy [1] , **Ignacio Ribera** Corresp. [1]

[1] Institute of Evolutionary Biology (CSIC-Universitat Pompeu Fabra), Barcelona, Spain

Corresponding Author: Ignacio Ribera
Email address: ignacio.ribera@ibe.upf-csic.es

Body size sexual dimorphism (SSD) is widespread among animals, with larger females usually attributed to an optimization of resources in reproduction and larger males to sexual selection. A general pattern in the evolution of SSD is Rensch's rule, which states that SSD increases with body size in species with larger males but decreases when females are larger. We studied the evolution of SSD in the genus *Limnebius* (Coleoptera, Hydraenidae), measuring SSD and male genital size and complexity of ca. 80% of its 150 species and reconstructing its evolution in a molecular phylogeny with 71 species. We found strong support for a higher evolutionary lability of male body size, which had an overall positive allometry with respect to females and higher evolutionary rates measured over the individual branches of the phylogeny. Increases in SSD were associated to increases in body size, but there were some exceptions with an increase associated to changes in only one sex. Male secondary sexual characters appeared several times independently, generally on species that had already increased their size. There was an overall significant correlation between SSD and male genital size and complexity, although some lineages with complex genitalia had low SSD, and some small species with complex genitalia had no SSD. Our results suggest that the origin of the higher evolutionary variance of male body size may be due to lack of constraints rather than to sexual selection, that may start to act in species with already larger males due to random variation.

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Author for correspondence: Ignacio Ribera (e-mail address: ignacio.ribera@ibe.upf-csic.es)

Running title: Evolution of sexual dimorphism in *Limnebius*

Abstract

Body size sexual dimorphism (SSD) is widespread among animals, with larger females usually attributed to an optimization of resources in reproduction and larger males to sexual selection. A general pattern in the evolution of SSD is Rensch's rule, which states that SSD increases with body size in species with larger males but decreases when females are larger. We studied the evolution of SSD in the genus *Limnebius* (Coleoptera, Hydraenidae), measuring SSD and male genital size and complexity of ca. 80% of its 150 species and reconstructing its evolution in a molecular phylogeny with 71 species. We found strong support for a higher evolutionary lability of male body size, which had an overall positive allometry with respect to females and higher evolutionary rates measured over the individual branches of the phylogeny. Increases in SSD were associated to increases in body size, but there were some exceptions with an increase associated to changes in only one sex. Male secondary sexual characters appeared several times independently, generally on species that had already increased their size. There was an overall significant correlation between SSD and male genital size and complexity, although some lineages with complex genitalia had low SSD, and some small species with complex genitalia had no SSD. Our results suggest that the origin of the higher evolutionary variance of male body size may be due to lack of constraints rather than to sexual selection, that may start to act in species with already larger males due to random variation.

Keywords: body size, genital complexity, Rensch's rule, sexual selection, sexual size dimorphism.

INTRODUCTION

Sexual size dimorphism (SSD) is highly variable among animal species, from minuscule males with comparatively giant females to males much larger than females (Darwin, 1871; Hedrick & Temeles, 1989; Fairbairn, 1997; Vollrath, 1998). In most insect species females are larger than males (Darwin, 1871; Arak, 1988; Shine, 1988; Fairbairn, 1997), a fact usually explained because the energetic investment in the progeny is larger in females than in males, which mostly provide just genetic information. For this reason, population fertility depends more on females than on males, so females should be as big and males as small as possible, to minimise resources spend on their maintenance (Darwin, 1871; Thornhill & Alcock 1983; but see Shine, 1988 for some alternative views).

Although some species have reached this "optimum" state of minimised males, in the absence of selection both sexes will tend to have the same size, given the strong genetic correlation between sexes for most traits (Lande, 1980). However, rapid changes in SSD can occur even when selection pressure is small (Reeve & Fairbairn, 2001), which begs the question not why sexual dimorphism exist, but why are there so many species in which males are about the same size or bigger than females. One reason could be ecological (Slatkin, 1984; Shine, 1989; Musterud, 2000): when sexes have widely different sizes, they may not be able to share the same ecological niche. This may be an advantage in avoiding intraspecific competition, but there is little evidence that SSD may have originated primarily through ecological divergence in any group (Fairbairn, 1997). There could also be social reasons, if males contribute to raising the progeny either by protecting females or providing resources, thus equalizing the investment of the two sexes. An alternative explanation is sexual selection: larger males may have an advantage, either because they can gain better access to females (male-male competition), or because females prefer them (female choice). In the first case, differences may affect the size of the body or other structures used for male-male competition; in the second there may be other characters involved, especially genital characters when there is cryptic female choice (Eberhard, 1985; Kuijper et al., 2012).

Despite the large body of work on sexual dimorphism, there is still a lack of understanding of its long-term evolution in diverse lineages, particularly among invertebrates (Fairbairn, 1997). There are a number of unresolved questions on the origin and evolution of sexual dimorphism that can be addressed with a phylogenetic reconstruction in speciose lineages

with a diversity of male genital and body sizes. One of the few recognised general trends in the evolution of SSD is the so-called Rensch's rule (Abouheif & Fairbairn, 1997; Fairbairn, 1997; Székely et al., 2004). Rensch (1950) observed that sexual differences increased with body size in species where males were larger, but decreased in species where females were larger. This implies that male body size varies more over evolutionary time than female body size, irrespective of which sex is larger (Fairbairn, 1997). Fairbairn & Preziosi (1994) hypothesized that sexual selection for large male size may be the primary force driving Rensch's rule, a hypothesis supported by the observation of Rensch's rule in sexually selected characters other than size, such as male pigmentation (Santos & Machado, 2016). An alternative possibility is that males have a larger evolutionary plasticity, somehow equivalent to a larger intraspecific phenotypic plasticity (Fairbairn, 2005; Gómez-Mestre & Jovani, 2013).

Data supporting Rensch's rule is mostly intraspecific or from closely related small species groups (Abouheif & Fairbairn, 1997; Fairbairn, 1997; Székely et al. 2000; Kraushaar & Blanckenhorn, 2002), with only few global studies of diverse lineages, and mostly among vertebrates (e.g. Lindenfors et al., 2003; Székely et al., 2004). In two reviews of Rensch's rule support in some groups of arthropods, Blanckenhorn et al. (2007a,b) found strong support in some groups (some Diptera and Hemiptera Gerridae), which show negative allometry in plots of females vs. males; while in others there was isometry (e.g. some beetles and hymenopterans) or only weak tendencies (butterflies and spiders). Rensch's rule was mostly supported in groups with males larger than females, something unusual in ectotherms (contrary to mammals and birds, Fairbairn, 1997), and there was little evidence to support its prevalence at the intraspecific level (see also Martin et al., 2016).

In this work we reconstruct the macroevolutionary patterns of SSD evolution in a diverse lineage of insects with the aim to investigate the origin and evolution of body size differences, and to determine the underlying causes of Rensch's rule over long evolutionary periods. We particularly focus on the relationship between SSD and evolutionary changes in body size of males and females, and whether SSD is linked to size variation in both sexes or can appear through changes in one sex only (Fairbain, 1997). We also study the correlation of SSD with other characters of the male genitalia, such as size and complexity.

As a study group we use a diverse and ancient lineage of beetles, the genus *Limnebius* (family Hydraenidae). *Limnebius* includes ca. 150 species with an almost cosmopolitan

distribution, all of them aquatic, living in all types of continental waters with the only exception of saline habitats (Perkins, 1980; Jäch, 1993; Hansen, 1998; Rudoy et al., 2016). In a recent work, *Limnebius* was shown to be divided in two sister lineages with an estimated Oligocene origin, the subgenera *Bilimneus* and *Limnebius* s.str., with ca. 60 and 90 described species respectively (Rudoy et al., 2016). The two subgenera differ in a number of traits, including variation in body size and in the size and complexity of the male genitalia, much larger in *Limnebius* s.str. (Rudoy et al., 2016; Rudoy & Ribera, 2016). They also differ in sexual dimorphism and the presence of secondary sexual characters (SSC). In *Bilimneus* females are slightly larger than males, which do not have strongly developed SSC; on the contrary, within *Limnebius* s.str. there are a wide range of different situations, including males much larger than females and with well developed SSC (Jäch, 1993; Rudoy & Ribera, 2016), providing thus a suitable system for the study of the origin and evolution of SSD.

MATERIAL AND METHODS

Taxon sampling

We obtained morphological data of the males of 120 and the females of 86 of the ca. 150 described species of *Limnebius*, among them four undescribed species (Table S1). Females were identified mostly by association with males, as there are few characters that could identify them unequivocally (Perkins, 1980; Jäch, 1993), but in some species this was not possible as several species of similar size could coexist in the same locality. In some cases very few specimens of some of the sexes could be studied (Table S1). In some species with a low number of specimens (e.g. *L. paranuristanus*, *L. angustulus* or *L. fontinalis*; see Table S1 for the taxonomic classification of the genus) the SSD was very similar to that of the most closely related species with enough data, but in other cases (e.g. *L. canariensis*) the few specimens that could be obtained differ in SSD from related species, but there is the possibility that the measured specimens were not representative.

Morphometric measurements

We measured body length of adults (males, *lm* and females, *lf*) as the sum of the individual maximum lengths of pronotum and elytra, as the different position of the articulation between the two could alter the total length when measured together. Similarly, the head was not

134 measured, as in many specimens it was partly concealed below the pronotum. Measures were
135 obtained with stereoscope microscopes equipped with an ocular micrometer.
136 Measures of the genitalia were obtained from Rudoy & Ribera (2016). Briefly, male
137 genitalia (aedeagi) were dissected and mounted on transparent labels with dimethyl hydantoin
138 formaldehyde (DMHF). For size measurements we used as a single value the average of each
139 measure in all studied specimens of the same species (Table S1). For shape characterisation a
140 single specimen was used as species show in general a very constant shape of the aedeagus, with
141 very low intraspecific variability as compared with the marked differences between species
142 (Jäch, 1993; Rudoy et al., 2016). We measured the maximum length of the male genitalia (*lg*)
143 orientated in ventral view according to the foramen. We did not include setae or apical
144 membranous structures but included appendages when they were longer than the median lobe (as
145 in e.g. some species of the *L. nitidus* group, Rudoy et al., 2016). Measurements were directly
146 obtained from the digital images using ImageJ v.1.49 (National Institutes of Health, US,
147 http://imagej.nih.gov/ij/) (Fig. S1).
148 We used two different measures to characterise the complexity of the aedeagus, following
149 Ribera & Rudoy (2016): (1) Perimeter (*per*) of the aedeagus in ventral view, including the
150 median lobe and the main appendages. We obtained an outline of the genitalia from digital
151 images using ImageJ. The total perimeter was the sum of the values of the different parts of the
152 genitalia (median lobe and left parameter, plus main appendages if present, see Rudoy et al.,
153 2016). We standardised the values by dividing the perimeter by the length of the aedeagus, to
154 obtain a measure of complexity by unit of length (Fig. S1; Table S1).
155 (2) Fractal dimension (*fd*). We estimated the fractal dimension of the outline of the
156 aedeagus in ventral view on images of standard size (2100x2100 pixels, 2000 pixels from base to
157 apex of the aedeagus) with the software Fractal Dimension Estimator (http://www.fractal-
158 lab.org/index.html). This software estimates the Minkowski fractal dimension of bidimensional
159 images using the box-counting method (Falconer, 1990). The software converts the image to
160 binary data, selects the scaling window of the box, and counts how many boxes are necessary to
161 cover the image. The absolute value of the slope of a log-log graph of the scale with the number
162 of boxes is the fractal dimension of the image (Fig. S1; Table S1).
163
164 **Phylogenetic analyses**

For our analyses we use two phylogenetic reconstructions, one with the species for which there were molecular data available and a second one with all species for which there were morphological data, added to the molecular phylogeny according to their morphological similarities.

The molecular phylogeny was based on that obtained in Rudoy et al. (2016) and Rudoy & Ribera (2016), including 71 species of *Limnebius* (Table S2). Taxon sampling was denser for the Palaearctic lineages in subgenus *Limnebius* s.str., including the full range of body sizes and structural variation of the aedeagus. We used as outgroup and to root the tree the genus *Laeliaena*, considered to be sister to *Limnebius* based on multiple morphological synapomorphies (Hansen, 1991; Jäch, 1995; Perkins, 1997; Beutel et al., 2003).

The phylogeny was constructed with Bayesian methods in BEAST 1.8 (Drummond et al., 2012) using a combined data matrix with three partitions, (1) mitochondrial protein coding genes (two *cox1* fragments plus *nad1*); (2) mitochondrial ribosomal genes (*rrnL* plus *trnL*) and (3) nuclear ribosomal genes (*SSU* plus *LSU*) (Table S2; Rudoy & Ribera, 2016), with a Yule speciation process as the tree prior and an uncorrelated relaxed clock.

Trees were calibrated with the rates estimated in Cieslak et al. (2014) for family Leiodidae, within the same superfamily Staphylinoidea (Beutel & Leschen, 2005) and the same gene combination based on the tectonic separation of the Sardinian plate 33 Ma. It must be noted that for our objectives only relative rates are needed. An absolute calibration would only be necessary to obtain absolute estimates of character change, which is not our main objective and does not affect our conclusions.

We reconstructed the ancestral values of the morphological variables using the values of the terminals (extant species) in BEAST 1.8. We implemented a Brownian movement model of evolution (BM), a null model of homogeneous evolution in which variation accumulates proportionally with time, with incremental changes drawn from a random distribution with zero mean and finite constant variance (Hunt & Raboski, 2012; Adams, 2014). The reconstruction of ancestral values using a BM model of evolution is biased toward average or intermediate values (Pagel, 1999; Finarelly & Goswami, 2013), which may result in an underestimation of the rates of evolution of some characters. Due to these limitations our reconstruction needs to be understood as the simplest null model explaining the evolutionary change in the studied characters.

We reconstructed the origin and secondary lost of some SSC with MESQUITE v.3 (Midfort et al., 2011) using parsimony. Secondary male sexual characters in the genus *Limnebius* affect mostly the tibiae and the last abdominal sternites (Jäch, 1993). In many species of *Limnebius* (but mostly in *Limnebius* s.str.) males have slightly curved and apically wider tibiae, especially in the anterior and medial legs, which also have suction setae (Jäch, 1993). However, these characters are difficult to quantify precisely and more observations are needed to establish their prevalence. The hind tibiae of males are also modified in some species of *Limnebius* s.str (Fig. S2). There are three different types of SSC in the abdominal sternites of males: (1) a more or less developed medial protuberance; (2) two parallel tuffs of setae; and (3) a medial impression delimited by ridges (Jäch, 1993; Fig. S2). All of them occur mostly in large species.

We studied the evolution of the morphological characters trough the full evolutionary path of species (i.e. from root to tips) and in the individual branches, using phylogenetic ancestor-descendant comparisons (PAD; Baker et al., 2015; Rudoy & Ribera, 2016). We measured three values for each of the individual branches (including terminals): (1) amount of phenotypic change, equal to the arithmetic difference between the final and initial values of the branch; (2) absolute amount of phenotypic change, equal to the absolute value of the amount of phenotypic change; (3) phenotypic change measured in darwins (Haldane, 1949), computed as the absolute value of the natural logarithm of the ratio between the final and initial values divided by the length of the branch in million years (Myr) (Table S3). The use of the natural logarithm standardises the change so it is proportional and directly comparable among species with different sizes (Haldane, 1949; Gingerich, 2009). To qualitatively characterise phenotypic change in the individual branches, we coded as positive or negative the increase or decrease of body size in each sex, as well as the SSD measured as the absolute difference between male and female body size. An estimated change lower than 5% in body size of males or females was considered within experimental error (i.e. "without change"). For SSD, we considered as "without change" branches with a change lower than 5% of the total range of observed differences. As individual branches are in principle independent from each other we analysed these variables with standard statistical procedures (see e.g. Baker et al., 2015).

We estimated the phylogenetic signal of the morphological variables in the whole tree using the *K* metric (Blomberg et al., 2003), which tests whether the topology and branch lengths of a given tree better fits a set of tip data compared with the fit obtained when the data have been

randomly permuted. The higher the *K* statistic, the more phylogenetic signal in a trait. *K* values of 1 correspond to a BM model, which implies some degree of phylogenetic signal. *K* values closer to zero correspond to a random or convergent pattern of evolution, while *K* values greater than 1 indicate strong phylogenetic signal. We used the R package 'Picante' (Kembel et al., 2010) to compute *K* and the significance test. We also measured the correlation between some variables across the whole tree with a regression of phylogenetic independent contrasts with the PDAP package in MESQUITE v.3. We use a type II regression with reduced major axis (RMA) to relate the independent contrasts obtained in PDAP of log10 male and female size (Fairbairn, 1997; Blanckenhorn et al., 2007b) using the package PAST v.3 (Hammer et al., 2001).

To test for the possible effect of the incomplete taxon sampling, in some analyses we also used a phylogeny including species with only morphological data. We used the tree provided in Rudoy et al. (2016), in which species without molecular data were placed mostly according to the similarities of the male aedeagus (Fig. S3). When relationships were uncertain, a polytomy was formed with all the species sharing a similar structure of the aedeagus, and whenever necessary for the analyses these polytomies were randomly resolved in MESQUITE v.3. For comparison, some of the correlations were also repeated using the species values directly, without phylogenetic correction.

RESULTS

Overall interspecific allometry in SSD

Both for the whole genus *Limnebius* and subgenus *Limnebius* s.str. the slope of the regression between the size of females and the size of males was significantly larger than one (Fig. 1; Table 1), i.e. there was a positive allometry in the size of males with respect to females. Although the estimate of the slope of the regression for subgenus *Bilimneus* was also larger than one, the 95% confidence interval could not reject isometry between both sexes (Table 1). Results were similar for both phylogenies (only with species with molecular data and with all species), or when data were compared without phylogenetic correction (Table 1).

The phylogenetic signal *K* of the SSD, as measured with the ratio male/female body size (rSSD), was lower than one, suggesting lack of phylogenetic signal ($K = 0.56$; $p < 0.001$). The *K* values for body size of males and females were, on the contrary, clearly larger than one, suggesting a strong phylogenetic signal (1.35 and 1.65 respectively, both $p < 0.01$).

In the whole genus *Limnebius*, rSSD was significantly correlated with male and female body size (*lm* and *lf*), but correlation values were much larger for males than for females (Table 2). When the two subgenera were considered separately, the correlation with *lf* was not significant in subgenus *Limnebius* s.str. when using the phylogeny with all species included, and never in *Bilimneus* (Table 2).

The correlations of rSSD with the measures of aedeagus size and complexity were also highly significant, although weaker when using the phylogenetic tree with all species than when using the phylogeny with only species with molecular data, or when using raw data without phylogenetic correction (Table 3). Correlations were also weaker within the species of *Bilimneus*, and significant only for the perimeter.

Evolution of SSD

According to our reconstruction, the ancestral condition of *Limnebius* was a SSD close to a 1:1 ratio; Fig. 2, Table S3). The general evolution in *Bilimneus* was to a slight decrease in size, with females larger than males, while in *Limnebius* s.str. the general trend was an increase in size (although some lineages maintained the ancestral small size), with males larger than females (Fig. 2; Table S3). This increase in size and SSD was continuous for the extant species with the largest SSD.

Of all 130 individual branches of the phylogenetic molecular tree, only in 32 female body size had a faster evolutionary rate than male body size, as measured in darwins (average difference of male minus female darwins = 0.006, std = 0.017; Fig. 3). Many of the branches in which females evolved faster than males were in the *L. nitidus* subgroup, with an uncertain basal topology but reconstructed as having an overall decrease in SSD (Figs 2, 4F and S5). Differences were similar when measured with absolute phenotypic change, with only 33 branches out of 130 in which female body size changed more than male body size (average difference of male minus female absolute body size = 0.029 mm, std = 0.045) (Figs 4D and S5; Table S3).

The reconstructed changes in SSD in the individual branches of the phylogenetic tree were clearly associated to changes in body size. In almost 50% of the branches (63 out of 130) SSD increased when the body size of males and females also increased, and decreased when body sizes decreased (Table 4; Fig. 4B,E). There were, however, a number of possible alternative situations, the most common of them that SSD increased when body size of both

sexes decreased (Fig. 4A,C; Table 4). In all individual branches in which this happened females were larger than males, so the increase in SSD was due to a relative larger reduction in male size (Table 4). The inverse situation, i.e. a decrease in SSD when body size of both sexes increased, was much less frequent (Fig. 4C,F; Table 4). But again, in the single branch with a SSD decrease larger than 5% when both sexes increased in size females were larger than males, so the reduction in SSD was due to a relative larger increase of the male body size (Tables 4 and S3). When only change above 5% was considered, the most common situation was no change in either males or females (Table 4), but the general pattern did not change.

There were branches in which there was a significant change in SSD with an increase in body size of only one of the sexes. When males were the sex that changed there was generally an increase in SSD (17 out of 24 branches, Table 4). When females were the only sex to significantly increase their size, SSD increased when female size decreased (3 branches), or decreased when female body size increased (also 3 branches, Table 4). There was only one case in which SSD decreased when only female body size decreased (4 for the males), an none in which SSD increased only due to an increase in female body size (against 10 branches for males, Table 4). In all these cases it can be considered that changes in SSD were not associated with an overall size increase. Overall, there were 24 branches in which SSD significantly changed only due to male change, while change was only due to females in seven branches (Table 4; $p<0.005$ of equal probabilities assuming a binomial distribution). For all studied species, the reconstructed average size change along the evolutionary path was larger for males than females.

Differences in the rate of phenotypic evolution of male and female body size in the individual branches, as measured in darwins, were positively correlated to the change in males, but negatively (albeit not significantly) with that of females (Table 5). When measured in absolute phenotypic change, differences between male and female body size were positively correlated with the change of both sexes, but with a stronger correlation and a steeper slope for males (Table 5).

Evolution of secondary sexual characters

According to our reconstruction, modifications in the hind tibiae appeared three times independently in the phylogeny (Fig. 5). Within the *L. nitidus* group, species in the *L. nitiduloides* subgroup have a row of setae (Figs 4E and S3), and two species, *L. truncatellus* and

L. mesatlanticus, have the distal part of the male hind tibia strongly widened (Fig. S2; Jäch, 1993). In addition to these, in three closely related species within the *L. parvulus* group males have wider hind tibiae (*L. doderoi*, *L. furcatus* and *L. gridellii*, Fig. 5).

According to our reconstruction, the protuberance in the male abdomen appeared independently in the *L. gracilipes* group (in the clade excluded *L. cordobanus*, Rudoy et al., 2016) (Figs 4D and S3) and in the *L. nitidus* group. Within the later, it was secondarily lost in the *L. nitidus* subgroup, with the exception of *L. kocheri* (Fig. 4F,S3). The other modifications of the abdomen of males occur in two of the subgroups of the *L. parvulus* group (Fig. 5). The two species of the *L. setifer* subgroup have a medial impression, and the species of the *L. parvulus* subgroup a tuff of setae, with the exception of *L. glabriventris*, very close to *L. parvulus*, which likely lost it secondarily (Jäch, 1993). There was no molecular data for the species of the *L. setifer* subgroup (Fig. S3), so it remains uncertain whether there may have been a single origin for the secondary modifications of the abdomen, which subsequently diverged in the two subgroups, or they appeared independently.

DISCUSSION

Rensch's rule

Our results confirm the general validity of Rensch's rule in the genus *Limnebius*, that is, that body size of males is evolutionary more labile than that of females (Rensch, 1950; Fairbairn, 1997). There are several lines of evidence supporting this conclusion: (1) the correlation between male (y-axis) and female (x-axis) body size had a slope larger than one (i.e. a positive allometry) both for the genus and the subgenera, although for *Bilimneus* (with small species and females larger than males) when considered separately the regression was not significant. (2) The ratio male/female body size (rSSD) was correlated mostly with male body size, while the correlation with female body size was lower and in some cases not significant, indicating that males drive the evolution of SSD. And (3) when the evolution of SSD was reconstructed in the individual branches of the phylogeny evolutionary rates of male body size were generally higher than in females, and when females had higher rates there was a secondary reduction in SSD. When the change in SSD was measured in darwins (a compound measure including rate) it was also correlated to absolute changes in male, but not female body size, for which the correlations were negative but not significant. Results were very similar when regressions were obtained using raw

data or phylogenetic independent contrasts, and for the later, when using the molecular phylogeny (with a subset of the species) or the estimated phylogeny with all species.

The most commonly accepted cause for Rensch's rule is the continued action of directional sexual selection on the body size of males (Abouheif & Fairbairn, 1997; Székely et al., 2004). The increase in male body size results in an increase in SSD when males are larger, but a decrease in SSD if females are larger. This model assumes a correlated evolution between male and female body size, so that when males increase in size females also increase, although at a lower rate (i.e. the correlation is <1) (Maynard Smith, 1977; Fairbairn & Preziosi, 1994). Our results are in general agreement with this model, as changes in SSD were most frequently associated to changes in body size of both sexes (although larger in males). However, there were a variety of particular cases, the most common being an increase in SSD while both sexes decrease in size. The contrary situation, with an increase in body size of both sexes leading to a decrease in SSD, was more infrequent, being found only in one branch within *Bilimneus*, the subgenus with females generally larger than males. Both cases contradict the association of SSD with an overall increase in size, but still show larger changes in males than in females, in agreement with Rensch's rule.

There are two other possible cases in which SSD is not associated with changes in body size of both sexes. One is isometry, defined in our case as a change larger than 5% in body size of both sexes in parallel, but with a change in SSD of less than 5% (i.e., considered to be not significant). The reconstructed branches with isometric change in the *Limnebius* phylogeny occurred in lineages with small species with low SSD. The second case is an increase in SSD due only to an increase in male body size, with no change (or a change lower than 5%) in the females. This situation was more frequent in the phylogeny, suggesting that in some circumstances there may be a decoupling of the evolution of the male and female body size. It has been suggested that when body size is subjected to other selective forces females should approach their optimal size independently of the size of males, which may be mostly driven by sexual selection (Lande, 1980). There were also cases in which changes in SSD were associated to changes in only one sex, challenging the assumption that selection on body size of one sex will always drive the evolution of the other due to their overall genetic correlation. Our results are in agreement with simulation studies showing that in species with large populations genetic

correlations between the sexes do not pose strong constraints to the evolution of SSD (Reeve & Fairbairn, 2001).

In general, although the overall evolution of SSD in *Limnebius* seems to conform to a standard model with sexual selection favouring an increase in male body size with female body size also increasing due to genetic correlation, there was considerable variation, and some lineages show deviating patterns. The high evolutionary lability of SSD was confirmed by its low phylogenetic signal as measured with the K statistic, lower than for male and -especially- female body size.

Relationship between SSD and aedeagus size and complexity

We found a general positive correlation between SSD and size and complexity of male genitalia, which would suggest that genital characters are also subjected to sexual selection, in parallel to male body size. However, in a previous study Rudoy & Ribera (2016) did not found clear evidence for the presence of directional selection in the evolution of the complexity and size of the male genitalia in the genus *Limnebius*. Although the most complex genitalia are always present in the larger species, small species may also have complex genitalia. The size of the male genitalia was also evolutionary very labile, with no clear trends and a large variance, specially in *Limnebius* s.str., with the larger species and the more complex genitalia (Rudoy & Ribera, 2016). Similarly, there are lineages with uniformly complex genitalia, but with a wide variation in SSD (as in e.g. the species of the *Limnebius punctatus* subgroup, Table S1), contrary to hypotheses linking Rensch's rule with the evolution of genital characters (Bonduriansky & Day, 2003). In other insects (e.g. water striders) a positive correlated evolution between non-intromittent genitalia and sexual size dimorphism has been reported, but there was no correlation between the shape of intromittent genital traits and sexual size dimorphism (Arnqvist & Rowe, 2002).

Secondary sexual characters

Secondary sexual characters (SSC) appeared several times independently in *Limnebius*, but generally in large species with high SSD. Male secondary sexual traits are often linked to directional sexual selection (Petrie, 1988; Wilkinson, 1993; Simmons & Tomkins, 1996; Wilkinson & Taper, 1999; Simmons, 2013; Santos & Machado, 2016), which will be supported by their association with species with high SSD. It is also interesting to note that the only loss of

SSC affecting a relatively diverse lineage (within the *Limnebius nitidus* subgroup) occurred in a lineage that also secondarily reduced the SSD and the complexity of the male genitalia in some of the species, although other still have relatively complex aedeagus (Rudoy et al., 2016). Due to the uncertainty in the topology of the *L. nitidus* subgroup (Rudoy et al., 2016) it is not possible to assess if the presence of protuberance in *L. kocheri* is homologous to that of the other species of the group (i.e., it is sister to the rest of the species within the subgroup) or if it acquired the character independently, although the different conformation (short and acute in *L. kocheri*, long, oblique and medially impressed in other species) suggest the later possibility (Jäch, 1993). This would also agree with an alternative topology grouping in a monophyletic clade all linages within the *L. nitidus* group with SSC in the abdomen, in which case the absence of SSC in the *L. nitidus* subgroup (with the exception of *L. kocheri*) could be ancestral and not secondary (Rudoy et al., 2016).

The presence of SSC seems to be more linked to the complexity of the genitalia, both for the characters present in the terminal segments of the abdomen and the extreme modifications of the legs. According to our reconstruction, the modification of the posterior legs appeared after the development of SSC in the abdomen, and they are also not linked to SSD. Thus, they are conserved in species with a secondarily rSSD close to 1, as for example in some species of the *L. nitiduloides* group, which has a strong variation in SSD despite having uniformly complex genitalia (Rudoy & Ribera, 2016). As already noted, the relationship between SSC and complex genitalia is, however, not reciprocal, as there are groups with a complex genitalia but without SSC (as in e.g. the *L. nitidus* complex or the *L. punctatus* subgroup, Table S1).

Concluding remarks

Our results demonstrate that the evolution of SSD dimorphism in the genus *Limnebius* was largely driven by changes in males, thus providing strong support for the prevalence of Rensch's rule. However, the increase in SSD was not always linked to an overall size increase in both sexes, and was not always associated to the presence of male secondary sexual characters (SSC), contrary to the expectations under the hypothesis of sexual selection as the primary cause of Rensch's rule. Although most species with SSC had a strong SSD, with males larger than females, SSC are evolutionary more derived, appearing generally when species had already increased their size. In Rudoy & Ribera (2016) it was shown that differences in the evolution of

the male genitalia between *Bilimneus* and *Limnebius* s.str. were largely due to an increase in the variance of the change in the later, in which males are generally larger than females and which includes the larger species and the species with the stronger SSD and the more complex genitalia. This raises the possibility that the primary driver for the evolution of male body size is simply their larger evolutionary variance, maybe related to the lack of constraints associated with egg development and reproduction acting on females. In *Limnebius* s.str. sexual selection, with the subsequent development of SSC, may have been triggered in lineages that already had larger males and complex genitalias, reinforcing these pre-existing traits. The stronger constraints in the variability of males in subgenus *Bilimneus* remains to be explained, but it may be related to unknown differences in mating behaviour or other traits related to reproduction.

ACKNOWLEDGEMENTS

We are grateful to M.A. Jäch (NMW, Wien) and P. Perkins (MCZ, Harvard) for allowing us to study the collections of their institutions and for supporting this work in many ways. A.R. thanks M.A. Jäch for his help during his stay in the NMW. We also thank the comments and advice of F. García-González (EBD, Seville) and A. Cordero-Rivera (University of Vigo). This work was partly funded by a JAE PhD studentship (CSIC) to A.R., the Spanish Ministerio de Economía y Competitividad (projects CGL2010-15755 and CGL2013-48950-C2-1-P), a Salvador de Madariaga grant in the Phyletisches Museum in Jena (PRX14/00583) to I.R. and the "Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement de la Generalitat de Catalunya" (project SGR1532).

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598 **Figures**

599

600 **Fig. 1.** Regression between male (y-axis) and female (x-axis) body size. A, phylogenetic
601 independent contrasts of species in the molecular phylogeny (Figs 2 and 5); B, phylogenetic
602 independent contrasts of the estimated phylogeny with all species (Fig. S3); C, regression with
603 the raw data (without phylogenetic correction). Dotted lines, isometric relationship (slope = 1).
604 See Table 1 for the numerical values of the regressions.

605

606 **Fig. 2.** Evolution of the sexual dimorphism (rSSD) in the phylogeny of *Limnebius*, reconstructed
607 in BEAST using a Brownian model of evolution. Numbers in nodes, reconstructed value of rSSD
608 with 95% confidence interval in square brackets (see Fig. S4 for node support values).

609

610 **Fig. 3.** Histogram of the differences in darwins of the phenotypic change in males and females in
611 the individual branches of the phylogeny (see Table S3 for the values of the individual branches).

612

613 **Fig. 4.** Evolutionary trajectories of SSD in selected species of *Limnebius*. In the y-axis,
614 reconstructed values of male (filled circles) and female (white circle) body sizes (mm); in the x-
615 axis, nodes in the reconstructed evolution of the species (Figs 2 and 5). Distances in the x-axis
616 are proportional to time (Ma, note that the scale is reversed, i.e. the root of the Limnebiini tree is
617 dated with time 0). Filled arrows, apparition of abdominal secondary sexual characters (SSC);
618 empty arrows, apparition of SSC in the posterior tibiae; crosses, secondary lost of SSC, all
619 according to the reconstruction in Fig. 5. The trajectory represented in F (*L. nitidus* complex) is
620 the same for all the species in this complex, due to the short terminal branches and the uncertain
621 relationships among the species.

622

623 **Fig. 5.** Evolution of the male body size (*lm*, mm) in the phylogeny of *Limnebius* reconstructed in
624 BEAST using a Brownian model of evolution, with the reconstructed origin and lost of
625 secondary sexual characters (SSC) in MESQUITE using parsimony. Numbers in nodes,
626 reconstructed value of *lm*. Arrows mark the appearance of SSCs, crosses mark their loss.

627 **Tables**

628

629 **Table 1.** Type II regression (MRA) between male and female body size. Regressions using a
630 phylogenetic correction (molecular and including all species, Figs 2, 5 and S3) were done using
631 PDAP contrasts.

632

633

	phylogeny	n	slope	95% interval	R2	*p*
Limnebius	molecular	59	1.60	[1.37-1.78]	0.82	<0.0001
Limnebius sstr	molecular	47	1.58	[1.35-1.81]	0.82	<0.0001
Bilimneus	molecular	12	2.38	[0.91-4.30]	0.46	<0.05
Limnebius	all species	89	1.67	[1.43-1.88]	0.52	<0.0001
Limnebius sstr	all species	68	1.67	[1.47-1.85]	0.58	<0.0001
Bilimneus	all species	21	1.02	[0.12-1.28]	0.42	n.s.
Limnebius	raw data	89	1.38	[1.31-1.45]	0.94	<0.0001
Limnebius sstr	raw data	68	1.37	[1.26-1.48]	0.89	<0.0001
Bilimneus	raw data	21	1.20	[0.82-1.45]	0.67	n.s.

634

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638

639 **Table 2.** Correlation (R2) of rSSD with male and female length. *, p <0.05; **, p <0.01; ***, p
640 <0.001

641

	Limnebius		***Limnebius* s.str.**		***Bilimneus***	
phylogeny	**m**	**f**	**m**	**f**	**m**	**f**
molecular tree	0.63***	0.32***	0.64***	0.33***	0.49*	n.s.
all species	0.41***	0.06*	0.42***	n.s.	n.s.	n.s.
raw data	0.72***	0.48***	0.68***	0.43***	0.72***	n.s.

642

643

644 **Table 3.** Correlation (R2) of rSSD with aedeagus length (*lg*), perimeter (*per*) and fractal
645 dimension (*fd*). *, p <0.05; **, p <0.01; ***, p <0.001
646

phylogeny	*lg*	*per*	*fd*
molecular tree	0.13**	0.40***	0.17***
all species	0.06*	0.19***	0.08**
raw data	0.46***	0.46***	0.52***

647

648 **Table 4.** Qualitative changes in male (*lm*) and female (*lf*) body size and in SSD in the individual
649 branches of the phylogeny (see Table S3 for details). In "all changes", all branches are coded as
650 with positive or negative change, irrespective of the amount of change. In "change >5%",
651 changes lower than 5% of the initial value in *lf* or *lm*, and changes in SSD lower than 5% of the
652 total range of SSD changes, are coded as "=".
653

lf	*lm*	SSD	all changes	change >5%
-	-	-	25	8
-	+	-	5	0
+	-	-	7	0
+	+	-	10	1
-	-	+	31	9
-	+	+	6	0
+	-	+	8	2
+	+	+	38	16
-	=	-		1
=	-	-		4
+	=	-		3
=	+	-		3
-	=	+		3
=	-	+		7
+	=	+		0
=	+	+		10
-	=	=		2
-	-	=		9
+	+	=		6
=	+	=		3
=	=	=(+/-)		43

654
655

Table 5. Correlation between the differences in the rates of evolution between males and females in all individual branches of the molecular phylogeny and the change in each sex separately, measured in darwins and in absolute phenotypic change. *, p <0.05; **, p <0.01; ***, p <0.001.

		darwins				**absolute phenotypic change**			
		m		**f**		**m**		**f**	
	n	**R2**	**slope**	**R2**	**slope**	**R2**	**slope**	**R2**	**slope**
Limnebius	130	0.33***	0.34	n.s.	-0.02	0.56***	0.39	0.11***	0.24
Limnebius s.str.	98	0.30***	0.31	n.s.	-0.003	0.59***	0.37	0.17***	0.28
Bilimneus	28	0.87***	0.93	n.s.	-0.45	0.78***	0.95	0.15*	-0.82

Figure 1(on next page)

Figures

Fig. 1. Regression between male (y-axis) and female (x-axis) body size. A, phylogenetic independent contrasts of species in the molecular phylogeny (Figs 2 and 5); B, phylogenetic independent contrasts of the estimated phylogeny with all species (Fig. S3); C, regression with the raw data (without phylogenetic correction). Dotted lines, isometric relationship (slope = 1). See Table 1 for the numerical values of the regressions.

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Fig. 3. Histogram of the differences in darwins of the phenotypic change in males and females in the individual branches of the phylogeny (see Table S3 for the values of the individual branches).

Fig. 4. Evolutionary trajectories of SSD in selected species of *Limnebius*. In the y-axis, reconstructed values of male (filled circles) and female (white circle) body sizes (mm); in the x-axis, nodes in the reconstructed evolution of the species (Figs 2 and 5). Distances in the x-axis are proportional to time (Ma, note that the scale is reversed, i.e. the root of the Limnebiini tree is dated with time 0). Filled arrows, apparition of abdominal secondary sexual characters (SSC); empty arrows, apparition of SSC in the posterior tibiae; crosses, secondary lost of SSC, all according to the reconstruction in Fig. 5. The trajectory represented in F (*L. nitidus* complex) is the same for all the species in this complex, due to the short terminal branches and the uncertain relationships among the species.

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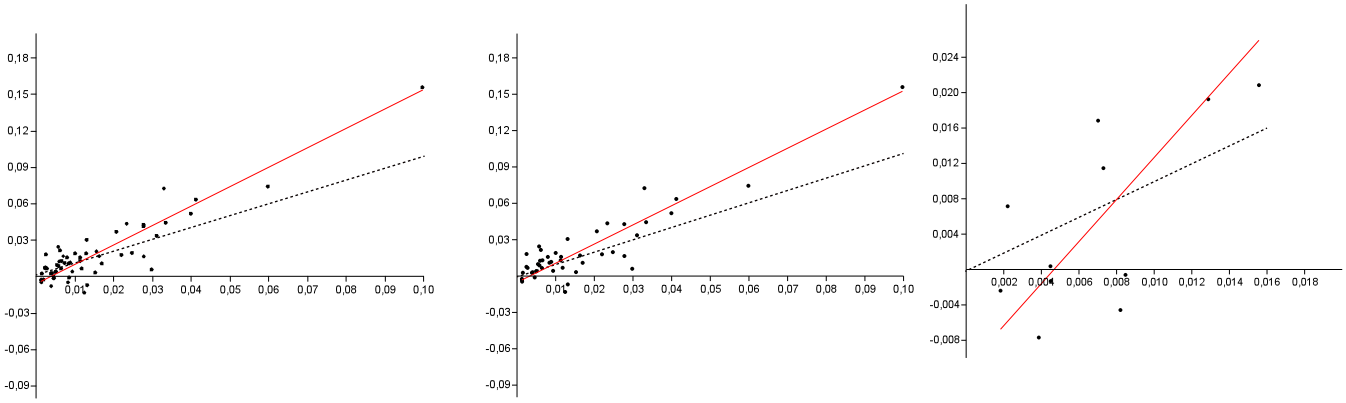
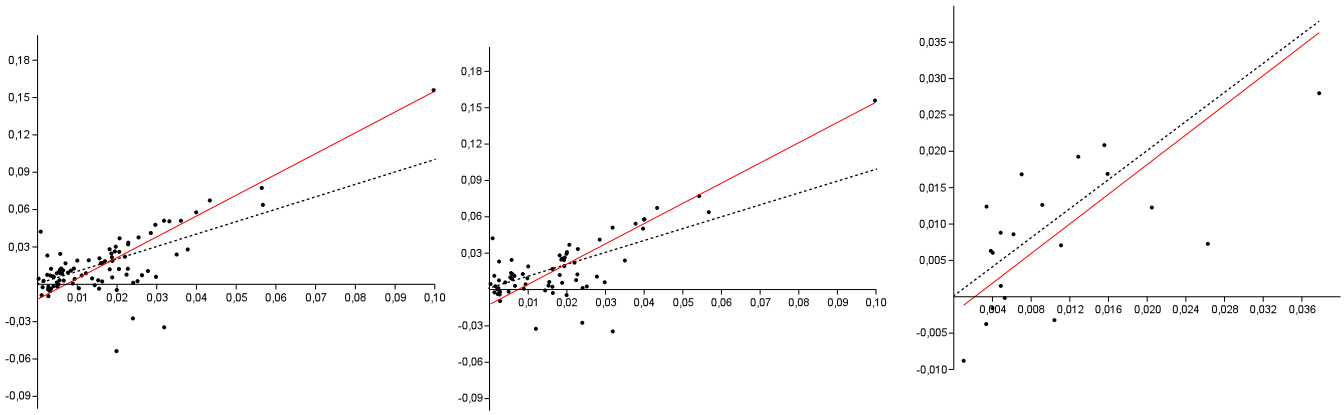
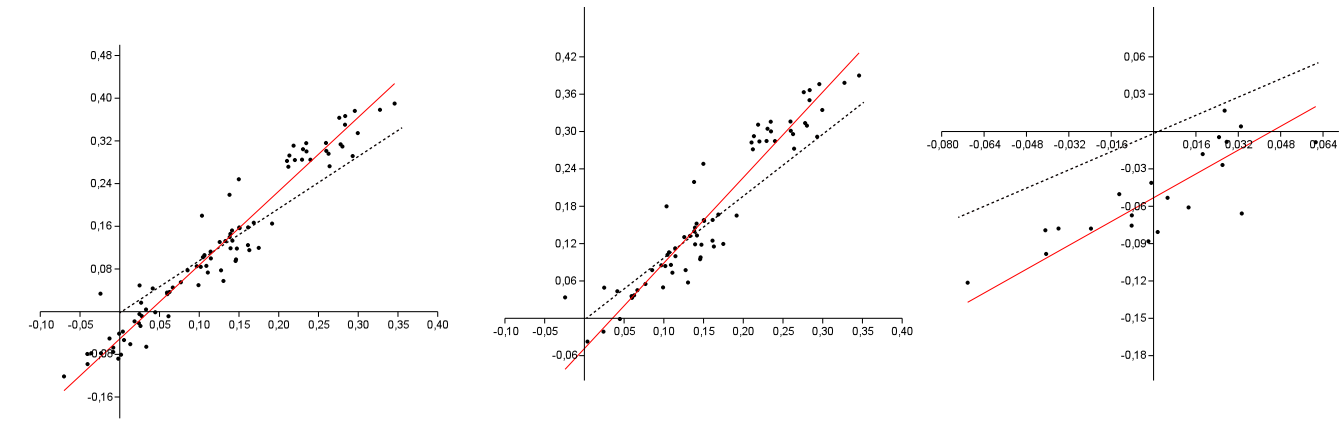
A *Limnebius* *Limnebius* s.str. *Biimnebius*

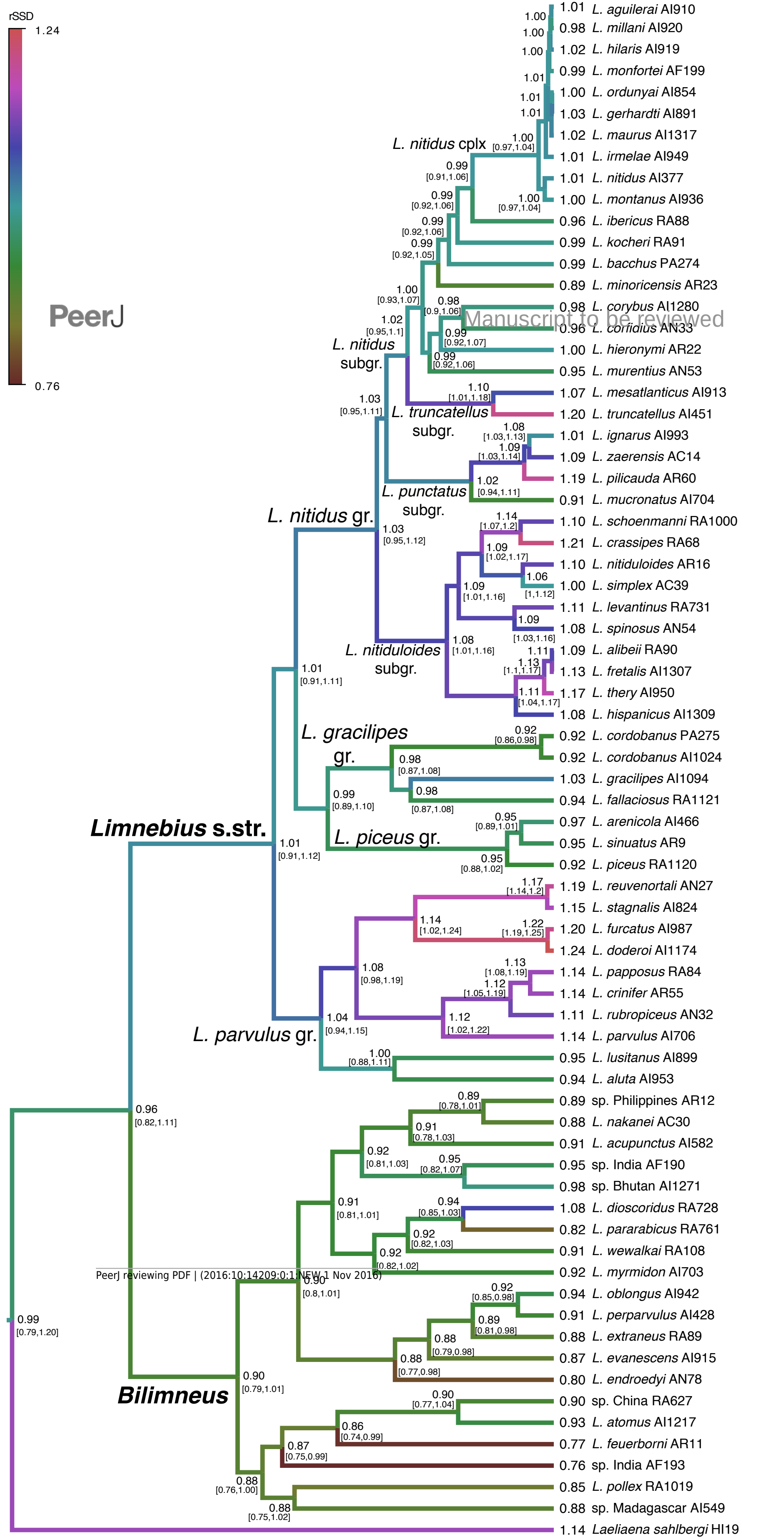


B



C




rSSD
1.24
0.76
PeerJ
Manuscript to be reviewed
Limnebius s.str.
Bilimneus
L. nitidus cplx
L. nitidus subgr.
L. truncatellus subgr.
L. punctatus subgr.
L. nitidus gr.
L. nitiduloides subgr.
L. gracilipes gr.
L. piceus gr.
L. parvulus gr.
1.01 L. aguilerai AI910
1.00 0.98 L. millani AI920
1.00 1.02 L. hilaris AI919
0.99 L. monfortei AF199
1.01 1.00 L. ordunyai AI854
1.01 1.03 L. gerhardti AI891
1.02 L. maurus AI1317
1.00 [0.97,1.04] 1.01 L. irmelae AI949
0.99 [0.91,1.06] 1.01 L. nitidus AI377
0.99 [0.92,1.06] 1.00 [0.97,1.04] 1.00 L. montanus AI936
0.99 [0.92,1.06] 0.96 L. ibericus RA88
0.99 [0.92,1.05] 0.99 L. kocheri RA91
0.99 L. bacchus PA274
0.89 L. minoricensis AR23
1.00 [0.93,1.07] 0.98 [0.9,1.06] 0.98 L. corybus AI1280
1.02 [0.95,1.1] 0.96 L. corfidius AN33
0.99 [0.92,1.07] 1.00 L. hieronymi AR22
0.99 [0.92,1.06] 0.95 L. murentius AN53
1.10 [1.01,1.18] 1.07 L. mesatlanticus AI913
1.03 [0.95,1.11] 1.20 L. truncatellus AI451
1.08 [1.03,1.13] 1.01 L. ignarus AI993
1.09 [1.03,1.14] 1.09 L. zaerensis AC14
1.02 [0.94,1.11] 1.19 L. pilicauda AR60
0.91 L. mucronatus AI704
1.14 [1.07,1.2] 1.10 L. schoenmanni RA1000
1.03 [0.95,1.12] 1.21 L. crassipes RA68
1.09 [1.02,1.17] 1.10 L. nitiduloides AR16
1.06 [1,1.12] 1.00 L. simplex AC39
1.09 [1.01,1.16] 1.11 L. levantinus RA731
1.09 [1.03,1.16] 1.08 L. spinosus AN54
1.08 [1.01,1.16] 1.09 L. alibeii RA90
1.11 1.13 [1.1,1.17] 1.13 L. fretalis AI1307
1.11 [1.04,1.17] 1.17 L. thery AI950
1.08 L. hispanicus AI1309
1.01 [0.91,1.11]
0.92 [0.86,0.98] 0.92 L. cordobanus PA275
0.98 [0.87,1.08] 0.92 L. cordobanus AI1024
1.03 L. gracilipes AI1094
0.99 [0.89,1.10] 0.98 [0.87,1.08] 0.94 L. fallaciosus RA1121
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1.17 [1.14,1.2] 1.19 L. reuvenortali AN27
1.15 L. stagnalis AI824
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1.24 L. doderoi AI1174
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0.91 [0.78,1.03] 0.88 L. nakanei AC30
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0.80 L. endroedyi AN78
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0.86 [0.74,0.99] 0.93 L. atomus AI1217
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0.88 [0.76,1.00] 0.76 sp. India AF193
0.85 L. pollex RA1019
0.88 [0.75,1.02] 0.88 sp. Madagascar AI549
1.14 Laeliaena sahlbergi HI19
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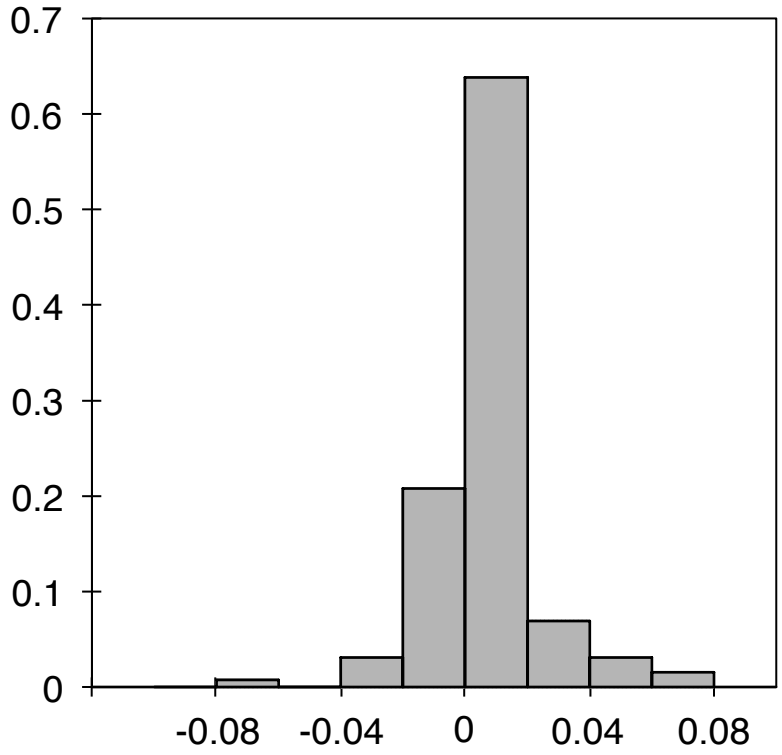
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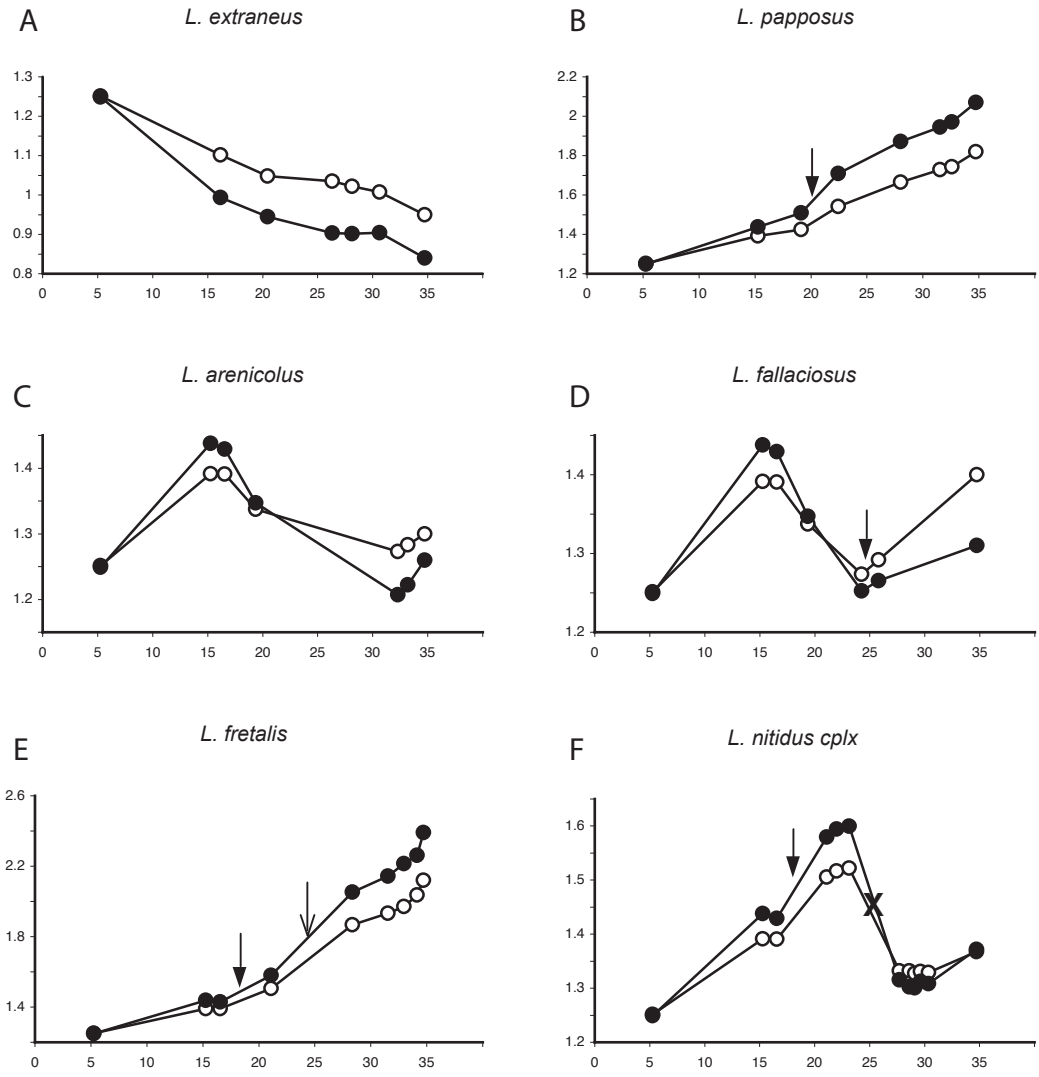
A
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L. fallaciosus
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L. nitidus cplx

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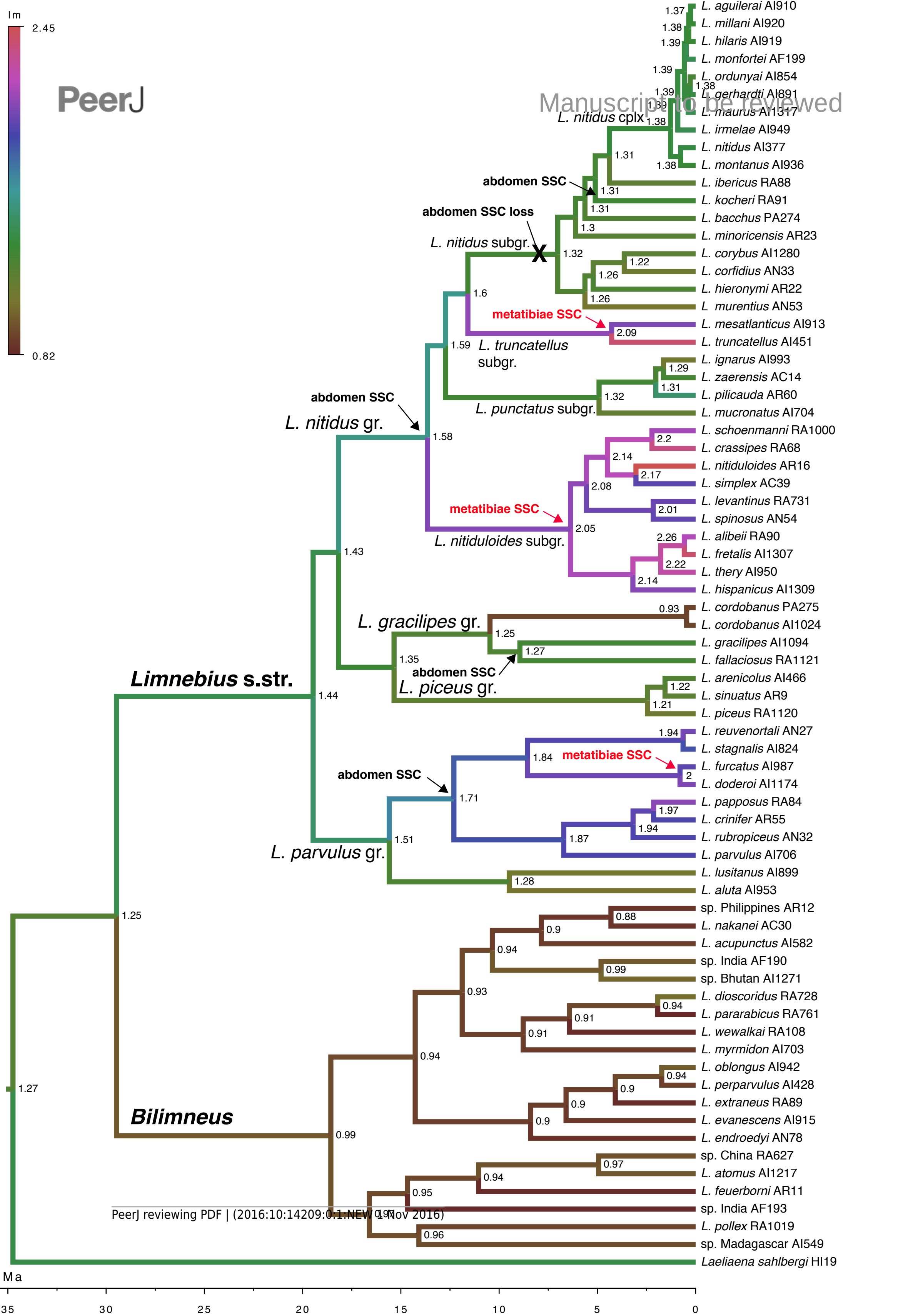
Manuscript to be reviewed