

Mixed Sex Effects on the Second-to-Fourth Digit Ratio of Túngara Frogs (*Engystomops pustulosus*) and Cane Toads (*Rhinella marina*)

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ABSTRACT

Sexual dimorphism in the ratio of digit lengths has been correlated to behavioral, physiological, and morphological traits in a variety of taxa. While sexual dimorphism in the second-to-fourth digit length ratio (2D:4D) is a well-established indicator of prenatal androgen exposure in mammals, investigations into the patterns of 2D:4D and the drivers of such variation in other taxa are lacking. We used linear mixed effects models to gain a mechanistic understanding of the factors that drive variation in the scaling relationship between the lengths of the second and fourth digits in two species of anurans: túngara frogs (*Engystomops pustulosus*) and cane toads (*Rhinella marina*). We found evidence for sexual dimorphism of the 2D:4D scaling relationship on the front feet of túngara frogs, with female frogs having a larger ratio than males resulting from a relatively longer second digit on females. To our knowledge, this mammal-like pattern of sex differences in digit ratio has not yet been reported for anurans. However, given the reduced number of digits on the front feet of anurans, and uncertainty about which digit was lost during evolutionary history, this apparent sexual dimorphism in the front feet of túngara frogs should be treated with caution. In contrast, we found no evidence of sexual dimorphism in 2D:4D on either the front or rear feet of cane toads. This study highlights ambiguities in 2D:4D across taxa and suggests that further research is needed to evaluate the effect of androgens on 2D:4D in animals other than placental mammals. Anat Rec, 299:421–427, 2016. © 2016 Wiley Periodicals, Inc.

Key words: allometry; amphibia; asymmetry; digit ratio (2D:4D); indicator traits; sexual dimorphism

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Sexual dimorphism of the second-to-fourth digit length ratio (2D:4D) has received considerable attention in a variety of taxa (> 450 published studies, reviewed in Voracek, 2011). Once thought to result from differential exposure to sex hormones early in development modulating *Hox* gene expression, it is now accepted that sexual dimorphism in 2D:4D results from the influence of early exposure to androgens on skeletogenic genes, chondrogenic genes, and cell proliferation (Zheng and Cohn, 2011). Therefore, 2D:4D can serve as an indicator trait for attributes that are influenced by exposure to androgens (Voracek and Loibl, 2009). Because of its potential as an indicator trait, a large number of studies have examined correlates of 2D:4D (e.g., visual-spatial memory and numerical states, Bull et al., 2010; cognitive drive, Wakabayashi and Nakazawa, 2010; sexual orientation, Grimbos et al., 2010), and have postulated that it might serve as an indicator of exposure to environmental contaminants in sensitive taxa (Chang, 2008).

The relationship between 2D:4D in males and females varies across taxonomic groups (Fig. 1). Sexual dimorphism in this ratio has been studied most extensively in mammals, particularly humans. In most mammals, males have a smaller ratio than females; however, male Guinea Baboons (*Papio papio*) have larger 2D:4D than conspecific females (Roney et al., 2004). Birds, in contrast, exhibit the opposite pattern to mammals with males having a larger 2D:4D than females (e.g., Lombardo et al. 2008). From there, our understanding of the between-sex variation in 2D:4D within major vertebrate taxa dwindles. The few studies that have investigated digit length ratios in reptiles have had inconsistent results (e.g., Rubolini et al., 2006; Direnzo and Stynoski, 2012; Van Damme et al., 2015) and the pattern across amphibians is also ambiguous. In two species of anurans, males exhibit a larger digit ratio than females (Chang, 2008; Direnzo and Stynoski, 2012), while there is no difference between the sexes in a third species (Germano et al., 2011). Two recent studies on five species of caudates found convincing evidence that females had larger digit ratios than males in one species, but found inconsistent or no sex-biased pattern in the digit ratios in four other species (Balogová et al., 2015; Kaczmarski et al., 2015).

Taxonomic differences in 2D:4D have been attributed to various mechanisms. Differences in chromosomal sex determination or the concentration of sex hormones, for example, have been listed as potential causes of lineage-specific patterns of 2D:4D (Chang et al. 2006; Lombardo et al., 2008; Voracek and Dressler, 2009; Zheng and Cohn 2011). In addition, body size differences between the sexes are suspected to determine the presence and pattern of sexual dimorphism in digit ratios. Sexual dimorphism in 2D:4D may be an artifact of allometric growth given that body size consistently has a significant effect on 2D:4D in a number of species (e.g., Rubolini et al., 2006; Kratochvil and Flegr, 2009; Gomes and Kohlsdorf, 2011; Direnzo and Stynoski, 2012). However, there is some evidence that digit ratio varies independently of body size (Burley and Foster, 2004; Chang et al., 2006). To further understand overarching sex-specific trends in 2D:4D across taxa and evaluate the evolution of this phenomenon, studies that evaluate this ratio in diverse taxonomic groups are necessary. In particular, investigations that address those vertebrate classes that

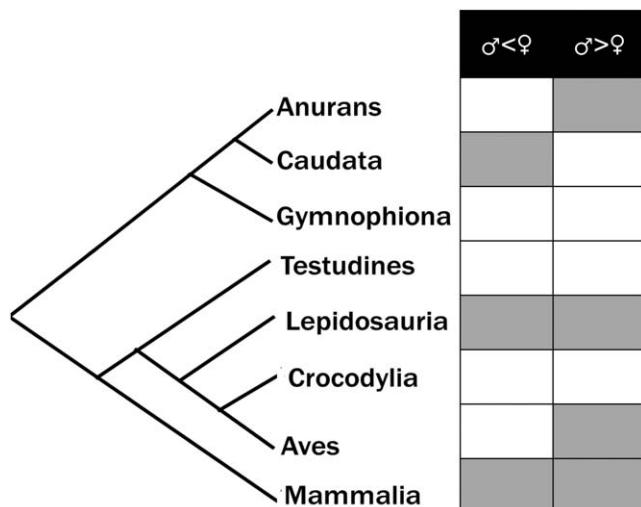


Fig. 1. Phylogenetic patterns of sexual dimorphism in 2D:4D in tetrapod vertebrate taxa. Gray shading indicates that there is evidence for the pattern of sexual dimorphism shown in black and white at the top of the column in that taxonomic group.

have received less attention (reptiles and amphibians) are of particular importance.

With the goal of expanding the current understanding of variation underlying 2D:4D in anurans to gain insight into the evolutionary trajectory of this trait, as well as to evaluate its potential as an indicator trait in anurans, we examined digit lengths in two species: túngara frogs (*Engystomops pustulosus*, formerly *Physalaemus pustulosus*) and cane toads (*Rhinella marina*, formerly *Bufo marinus*). As suggested by Kratochvil and Flegr (2009), we compared linear mixed effects models containing sex, body size, and side of the body as interactive fixed effects on the scaling relationship between the second and fourth digits. While this approach differs from classic null hypothesis testing, model selection is widely used in ecology and allows researchers to make inferences from the weighted support of multiple hypotheses (Johnson and Omland, 2004). A linear model of the lengths of the second to fourth digits is equivalent to using maximum likelihood approaches to estimate the 2D:4D. Using digit lengths instead of the 2D:4D, gives us insight into the mechanism behind differences in ratios and the relationship between digit lengths that could not be observed by simply comparing this ratio between the sexes (Kratochvil and Flegr, 2009).

MATERIALS AND METHODS

Study Site

Túngara frogs and cane toads were collected at and around the facilities of the Smithsonian Tropical Research Institute in Gamboa, Panama ($09^{\circ}07'0''N$, $79^{\circ}41'53''W$). This facility is located in a small town surrounded by tropical rainforest as part of the National Park Soberanía. During the rainy season, túngara frogs aggregate in temporary ponds and ditches. In those aggregations males form a lek mating system, calling to attract mates. Females approach these breeding areas to choose a mate (Ryan, 1985). In contrast, cane toads are

found mainly around buildings and houses. Juveniles and adult toads forage at night usually close to artificial lights where insects accumulate (Zug and Zug, 1979).

Study Species and Digit Measurements

Individual túngara frogs (52 females, 107 males) were collected during July–August 2010. Because individuals were collected during the breeding season, males were recognized by the presence of excess, stretched and darkened skin in the gular region of the frog. Given that males were collected while calling in a lek, they were more abundant than females resulting in a male-biased sample. The frogs were brought to the laboratory where snout-vent length (SVL) was measured three times using a digital caliper accurate to 0.01 mm, and the median value was used for the analysis. In order to accurately measure the small (0.6–8.7 mm) digits of túngara frogs, standardized photos of the front and rear feet of individual frogs were taken while spreading and straightening the digits of each foot. The length of each digit was subsequently measured using ImageJ (Schneider et al., 2012) by two observers unaware of the sex and size of the individual. Fifteen photos were measured by both observers to estimate interobserver variability.

Individual adult cane toads (28 females, 24 males) were collected during June–August, 2011. Cane toads were found foraging and were thus not reproductively active. Although cane toads are commonly seen in this area, this species is not as abundant in their native habitat as they are in their invasive range. Our sample size represents an exhaustive search for adults for about 3 months that resulted in frequent recaptures suggesting we had sampled the majority of active adults. Although our sample size for cane toads is much smaller than our túngara frog sample, it is similar to those of other studies examining sexual differences digit ratios (Balogová et al., 2015). Adults were recognized as individuals larger than 9 cm following Zug and Zug (1979). The sex of each individual was determined by the presence or absence of dorsal mottling and nuptial pads on the medial-most digit on the front feet (Zug and Zug, 1979; McCoy et al., 2008). The SVL of each toad and the length of all digits on both front feet and rear feet were measured three times each using a digital caliper accurate to 0.01 mm, and the median value was used for the analysis.

In both species, digit length was measured from the basal crease of each toe to the tip of the toepad (Fig. 2). Following Chang (2008), digits were numbered medially to laterally on each foot, with 1D being the medial-most digit, 4D being the most lateral digit on the front feet (Fig. 2A), and 5D being the most lateral digit on the rear feet (Fig. 2B). After measurements and photos had been taken of each individual, they were released back at their site of capture. The same procedure was used to measure the digit length and SVL of a laboratory population (5 females and 14 males) of cane toads that had been caught in Florida and purchased from Carolina Biological Supply (Burlington, NC).

Statistical Analyses

We used model selection approaches to determine what factors (e.g., sex, SVL, side of body, etc.) affect the slope of the relationship between the length of the sec-

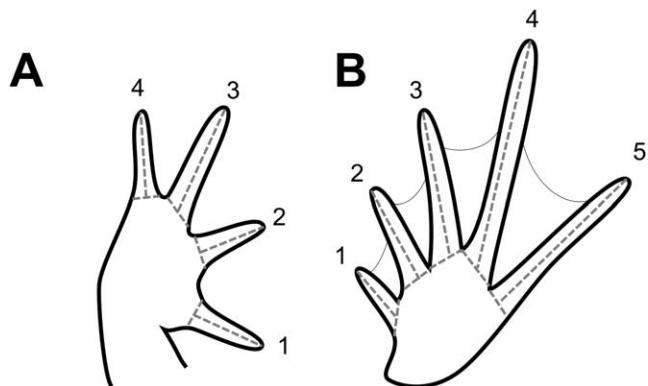


Fig. 2. Diagram demonstrating the measuring and numbering protocol for front (A) and rear (B) feet of both túngara frogs and cane toads.

ond and fourth digit of túngara frogs and cane toads. We used this approach because analyzing raw digit lengths allows for a mechanistic understanding of observed differences in digit ratios. In addition, the slope of a linear regression in which the length of the second digit is on the *y*-axis and the length of the fourth digit is on the *x*-axis (i.e., the scaling relationship between the two digits) is equivalent to using maximum likelihood methods to estimate the population level 2D:4D. Using this approach, any fixed effect that significantly interacts with the length of the fourth digit to predict the length of the second digit has an effect on 2D:4D. After verifying normality assumptions, we constructed competing linear mixed effects models in which the length of the second digit was used as the response variable, individual and geographic population were possible random effects, with varying combinations of factors (i.e., sex, body size, and side of body) potentially interacting with the length of the fourth digit as fixed effects.

We ran separate analyses for the front and rear feet of túngara frogs and cane toads in R Statistical Software (R Core Team, 2015) using packages lme4 (Bates et al., 2015), bbmle (Bolker and R Core Team, 2015), and nlme (Pinheiro et al., 2015). We used Akaike's Information Criterion corrected for small sample sizes (AICc) to select among competing models such that models with lower AICc scores represented models with more support. We kept all models with $\Delta\text{AICc} < 6$ and removed any models with pretending variables following Anderson (2007) and Richards (2008). To look at the relative importance of different fixed effects, we summed the Akaike weights of all models containing each fixed effect.

To estimate inter- and intraobserver variability in digit length measurements, we calculated the single-score intraclass correlation coefficients (ICC; following Voracek et al., 2007) for each digit using package "irr" (Gamer et al., 2012).

RESULTS

Túngara Frogs

The digit length measurements of túngara frogs were moderately precise (ICC = 0.37–0.70). However, because these estimates are based on repeat measures of only 15

TABLE 1. Model selection results for the front and rear feet of túngara frogs and cane toads with degrees of freedom (df), ΔAICc , and Akaike weights (w) for supported alternative models.

Model	df	ΔAICc	w
<i>Túngara frog—front feet</i>			
Fourth digit * sex + individual	6	0	0.91
Fourth digit * SVL + individual	6	4.7	0.09
<i>Túngara frog—rear feet</i>			
Fourth digit * SVL + individual	6	0	0.89
Fourth digit * sex + individual	6	4.1	0.11
<i>Cane toad—front feet</i>			
SVL + individual	4	0	0.96
<i>Cane toad—rear feet</i>			
Fourth digit + individual	4	0	0.97

Only models with ΔAICc less than six are shown.

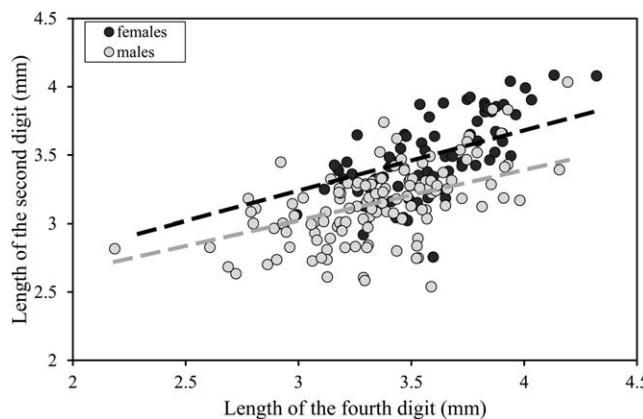


Fig. 3. Scatterplot of the average length of the second and fourth digits on the front feet of individual túngara frogs. Female and male túngara frogs are represented by the dark gray circles and black regression line and the light gray circles and regression line, respectively. The slope of each regression line is equivalent to the average 2D:4D of each sex.

images, they likely underestimate repeatability. In túngara frogs, the best predictors of the length of the second digit differed between the front and rear feet. For the front feet, the length of the second digit was best predicted by an interaction between the length of the fourth digit and the sex of the frog (Table 1; Fig. 3). The estimated coefficient for the length of the fourth digit was positive and less than one, suggesting that the fourth digit is longer than the second digit and that the length of the second digit increases as the length of the fourth digit increases. The sex of the frog influences the length of the second digit such that if the frog is a male, the second digit is smaller than if the frog were a female. The interaction between frog sex and the length of the fourth digit is negative indicating that if a frog is male, the scaling relationship between the lengths of the second and fourth digits, that is, the 2D:4D, is smaller than if the frog were a female (Table 2). In addition, after the length of the fourth digit, the sex of the frog was the most important fixed effect influencing the length of the second digit on the front feet of túngara frogs (Table 3). We found moderate support for the

TABLE 2. Mean snout-vent-length (SVL; \pm SD and second-to-fourth digit length ratios (2D:4D; \pm SD) for the front and rear feet of male and female túngara frogs and cane toads.

	SVL (mm)	Front Feet 2D:4D	Rear Feet 2D:4D
<i>Túngara frogs</i>			
Females	30.67 (\pm 2.0)	0.97 (\pm 0.07)	0.34 (\pm 0.03)
Males	28.2 (\pm 1.73)	0.94 (\pm 0.08)	0.32 (\pm 0.02)
<i>Cane toads</i>			
Females	130.4 (\pm 13.35)	1.09 (\pm 0.07)	0.37 (\pm 0.03)
Males	115.4 (\pm 11.98)	1.08 (\pm 0.07)	0.38 (\pm 0.03)

TABLE 3. Ranked summed Akaike weights (i.e., relative importance values) for each fixed effect for the front and rear feet of túngara frogs and cane toads.

Fixed Effect	Importance
<i>Túngara frog—front feet</i>	
Fourth digit	1.0
Sex	0.91
Body size	0.09
Side	<0.001
<i>Túngara frog—rear feet</i>	
Fourth digit	1.0
Body size	0.89
Sex	0.11
Side	<0.001
<i>Cane toad—front feet</i>	
Body size	0.98
Fourth digit	0.05
Sex	0.01
Side	<0.001
<i>Cane toad—rear feet</i>	
Fourth digit	1.0
Sex	0.03
Side	0.006
Body size	<0.001

importance of body size on the scaling relationship between the second and the fourth digits on the front feet of túngara frogs, but the effect was relatively small compared to the effects of frog sex (Table 3).

In contrast, the length of the second digit on the rear feet of túngara frogs was best predicted by the interaction between body size and the length of the fourth digit with random variation between individuals (Table 1). The estimated coefficient for the length of the fourth digit was positive and less than one, suggesting that the fourth digit is longer than the second digit and that the length of the second digit increases as the length of the fourth digit increases. Body size also had a positive estimated coefficient, suggesting that the length of the second digit increases with frog body size. The interaction between frog body size and the length of the fourth digit was also positive. This coefficient is small, however, suggesting that frogs with a larger SVL have a slightly larger scaling relationship between the lengths of the second and fourth digits than frogs with a smaller body size. Indeed, body size was one of the most important fixed effects influencing the length of the second digit on the rear feet of túngara frogs (Table 3). We also found moderate support for an effect of frog sex on the scaling relationship between the second and fourth

digits, but this effect was relatively small compared to the effect of body size (Table 3).

Cane Toads

Cane toad digit length measurements were highly precise ($ICC = 0.94\text{--}0.98$). In cane toads neither sex nor body size had an effect on the relationship between the length of the second and fourth digits on their front or rear feet. The length of the second digit on the front feet of cane toads was best predicted by body size (Table 1). The effect of body size was positive, suggesting that larger toads have longer second digits regardless of sex or the length of the fourth digit. The length of the second digit on the rear feet of cane toads was best predicted by the length of the fourth digit (Table 1). The estimated coefficient for the effect of the fourth digit was positive, suggesting the presence of isometric growth in the rear feet of cane toads such that as the length of the fourth digit increases, so does the length of the second digit, regardless of toad sex or body size.

DISCUSSION

Our study revealed a lack of consistent sexual differences in 2D:4D across the front and rear feet on the species investigated here. While there was a difference between male and female túngara frogs in the scaling relationship of the second and fourth digits of their front feet, this sex difference was not apparent on the rear feet of túngara frogs or on either the front or rear feet of cane toads. On these other appendages we found evidence for the influence of allometric or isometric growth on 2D:4D, or no scaling relationship at all. While this study is the first to document male frogs with a smaller 2D:4D than females, a pattern similar to that observed in mammals, these results should be viewed with caution.

Prior to this study, evidence for sexual dimorphism of 2D:4D in anurans suggested that females had smaller 2D:4D than males. Previous studies, however, differed as to whether sexual dimorphism was detected on the front or rear feet of frogs and whether or not body size influenced differences in 2D:4D (Chang, 2008; Germano et al., 2011; Direnzo and Stynoski, 2012). We found that male túngara frogs had a smaller 2D:4D than females on their front feet and that body size influenced the 2D:4D on the rear feet and the length of the second digit on the front feet of túngara frogs and cane toads, respectively. Such varied results in the presence, pattern, and location of sexual dimorphism in 2D:4D in anurans suggests that the clear pattern of sexual dimorphism in 2D:4D seen in placental mammals may have evolved after ancestral amniotes split from other tetrapod vertebrates or potentially even later, given the contrasting pattern of sexual dimorphism in 2D:4D seen in birds and placental mammals (Fig. 1).

Methodological differences in measurement techniques may explain the between-species variation observed in our study. Our sample size varied greatly between túngara frogs and cane toads. While this difference is due to the life history and relative abundance of both species, our small cane toad sample size could have been insufficient to detect any patterns between the sexes. In addition, we employed different measurement methods

for each species, using calipers to quantify digit length in cane toads and digital images to measure digit length in túngara frogs. While physical measurements, like calipers, tends to result in higher average 2D:4D estimates (Allaway et al., 2009), this bias would have been consistent within our cane toad sample and likely not responsible for the between-species differences seen in our study. Although the widespread use of calipers to measure digit length in other taxonomic groups also translates well to large anurans such as cane toads, the small size of túngara frogs required the use of digital images. Indeed, the use of different measurement techniques has been proposed to explain varied findings of 2D:4D in *Oophaga pumilio* (see discussion in Durenzo and Stynoski, 2012). It is possible that both our different measurement methodologies as well as phylogenetic inertia are responsible for differences in the pattern and explanatory factors of variation in 2D:4D between the species tested in this study.

Distinctive selective pressures due to different natural history strategies could modulate differences in 2D:4D between species. Given that evolutionary changes in morphology generally result from changes during embryonic development (e.g., Goodman and Coughlin, 2000), it is possible that adaptation to different habitats results in changed expression of traits affected by embryonic condition (e.g., 2D:4D; Gomes and Kohlsdorf, 2011). Perhaps the difference in variables explaining the variation in digit ratios between cane toads and túngara frogs can be attributed to their relatively more terrestrial and aquatic lifestyles, respectively. Additional studies examining more anuran taxa with diverse natural history strategies may provide further insights into this potential relationship.

If differences in 2D:4D are caused by variable exposure to androgens during early development, it is possible that the different egg mass structures of anurans (including cane toads and túngara frogs) are responsible for the reported variation in 2D:4D. In contrast to the condensed, foam-coated egg mass of túngara frogs, the long string-like egg masses of cane toads experience greater microhabitat variability and thus more varied exposure to exogenous sex hormones during embryonic development. Greater variation in androgen exposure in cane toads may generate high variation in 2D:4D preventing detectable differences between the sexes. Studies that investigate the relationship between egg mass structure, androgen exposure and 2D:4D are necessary, however, to determine and understand how variation in breeding strategies may influence digit length ratios.

Given that not all animals have five, homologous digits, comparisons of digit ratio patterns across distant taxonomic groups is problematic. Indeed, studies from disparate taxa often examine different digit ratios. In addition to the most commonly used 2D:4D digit ratio, other digits commonly investigated include 2D:3D, 3D:4D, and 2D:5D, which, in some cases, show stronger differences between sexes than 2D:4D (e.g., McIntyre et al., 2006; Voracek, 2009). While we detected sex differences in 2D:4D in túngara frogs, it was restricted to their front feet, which has a reduced number of digits. Because it is unclear which digit on the anuran forelimb was lost over evolutionary time from their pentadactyl ancestors, the digit numbering scheme used in this and other studies of anuran digit ratios may not be

homologous to digit numbers used in other pentadactyl taxa. Uncertainty about digit homology across vertebrate taxa confounds comparisons of anuran 2D:4D patterns to other groups which could cause misleading results and deserves further attention.

Using linear mixed effects models fit to raw digit lengths instead of comparing digit ratios between sexes, we were able to gain insight into how changes in the relative lengths of digits resulted in different digit ratios. In particular, we were able to determine that observed differences in 2D:4D of túngara frogs was due to a relatively shorter second digit on the front feet of male túngara frogs. While the use of model-fitting approaches on digit lengths was originally promoted by Kratochvil and Flegr (2009), nearly all studies to date, to our knowledge, have conducted ANOVAs on digit ratios to determine if there is a difference between sexes. Our study adds to the effort of implementing statistical methodologies to examine multiple hypotheses underlying variation in digit ratios by incorporating mixed effect models and model selection approaches. We suggest that future studies investigating the differences in, and correlates of, 2D:4D in any taxa use a similar model-fitting approach to digit lengths to better understand variation in 2D:4D.

Given the variation in the relative importance of sex, body size, and the length of the fourth digit in our most supported models for each species, the broader inconsistency across taxa, and the reduced number of digits on the forelimbs of anurans, we suggest that further research is needed to determine the reliability of 2D:4D as an indicator trait of androgen exposure for anurans. A formal investigation into the causal relationship between variation in 2D:4D and exposure to androgens is needed to determine how useful 2D:4D may be as an indicator trait as well as to elucidate the evolutionary history of the patterns of 2D:4D across taxa.

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