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Phenotypic variability in populations of *Phalloceros uai* (Cyprinodontiformes: Poeciliidae) with comments on feeding and reproduction habits

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Phalloceros uai was originally described from the rio das Velhas basin and characterized by a large square lateral spot in larger specimens. Reports of its occurrence in adjacent basins and the presence of alternative phenotypes have raised uncertainties regarding accurate specimen identification and distribution. We assessed the taxonomy, geographic distribution, and natural history of *Phalloceros* populations from south-central Minas Gerais, Brazil. Morphological traits, including standard and total length, body height, and male gonopodium length, were measured and gonopodium anatomical features were examined using clearing and staining. Genetic distances were estimated based on COI gene. Stomach contents were analyzed to characterize diet, and gonads were examined to assess reproductive traits. Specimens showed one to three large, square lateral spots. No anatomical differences were observed in the gonopodium, and no significant differences in gonopodium length were detected among phenotypes or populations. Genetic divergence was below 1%. Diet was consistent across populations and consisted mainly of autochthonous invertebrates. All populations exhibited superfetation. Osteological, meristic and molecular data support the inclusion of all examined populations within *P. uai*. The observed variation in the number of lateral spots reflects intraspecific phenotypic diversity, lacking morphological or molecular divergence sufficient to justify species-level recognition.

Keywords: Asymmetry, Integrative taxonomy, Quadrilátero Ferrífero, Superfetation.

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Phalloceros uai foi originalmente descrito a partir da bacia do rio das Velhas e é caracterizado por uma grande mancha lateral quadrada em espécimes maiores. Relatos de sua ocorrência em bacias adjacentes e a presença de fenótipos alternativos suscitaram incertezas quanto à identificação precisa dos espécimes e à sua distribuição. Avaliamos a taxonomia, a distribuição geográfica e a história natural de populações de *Phalloceros* do centro-sul de Minas Gerais, Brasil. Características morfológicas, incluindo comprimento padrão e total, altura do corpo e comprimento do gonopódio masculino, foram mensuradas, e as características anatômicas do gonopódio foram examinadas por meio de diafanização. As distâncias genéticas foram estimadas com base no gene COI. O conteúdo estomacal foi analisado para caracterizar a dieta, e as gônadas foram examinadas para avaliar características reprodutivas. Os espécimes apresentaram de uma a três grandes manchas laterais quadradas. Não foram observadas diferenças anatômicas no gonopódio, tampouco foram detectadas diferenças significativas no comprimento do gonopódio entre fenótipos ou populações. A divergência genética situou-se abaixo de 1%. A dieta mostrou-se consistente entre as populações, consistindo principalmente de invertebrados autóctones. Todas as populações exibiram superfetação. Dados osteológicos, merísticos e moleculares corroboram a inclusão de todas as populações examinadas em *P. uai*. A variação observada no número de manchas laterais reflete a diversidade fenotípica intraespecífica, carecendo de divergência morfológica ou molecular suficiente para justificar o reconhecimento em nível de espécie.

Palavras-chave: Assimetria, Quadrilátero Ferrífero, Superfetação, Taxonomia integrativa.

INTRODUCTION

The genus *Phalloceros* Eigenmann, 1907, comprising small-sized livebearing fishes, is one of 27 valid genera within the family Poeciliidae, distributed across watersheds in southern and southeastern South America. For approximately a century, the genus remained monotypic until Lucinda (2003, 2008) described 21 new species and redescribed *Phalloceros caudimaculatus* (Hensel, 1868), using exclusively morphological data such as structure of gonopodium and female urogenital papilla, osteology, coloration patterns, ray counts, and morphometry. Subsequently, with the popularization of molecular techniques, challenges in species recognition emerged, including reports of species complexes, as noted for *P. harpagos* Lucinda, 2008 (Oliveira *et al*., 2024; Souto-Santos *et al*., 2025). Furthermore, synonymy in the "sinistral group" (sensu Souto-Santos *et al*., 2023), have been proposed, with *P. aspilos* Lucinda, 2008, and *P. tupinamba* Lucinda, 2008, suggested as synonyms of *P. leptokeras* Lucinda, 2008 (Souto-Santos *et al*., 2022). More recently, three additional species have been described, *P. maldonadoi* Souto-Santos, Lucinda & Buckup, 2023, *P. circummontanus* Souto-Santos, Mejia, Arcila & Buckup, 2025 and *P. mimbi* Serra, Scarabino, Gobel & Laufer, 2025, bringing the total to 23 valid species currently recognized in the genus.

Phalloceros uai Lucinda, 2008, is autapomorphically diagnosed by a wide, square lateral spot in larger specimens and can be further distinguished from most of its congeners by a rightward-curved and laterally positioned female urogenital papilla, the anal opening margin in contact with (or very close to) the first ray of the anal fin, a left hook on the gonopodium, and an asymmetrical gonopodial appendage where the right side is wider than the left (Lucinda, 2008). However, the reliability of the lateral spot pattern as an autapomorphy for *P. uai* has been questioned, as Thomaz *et al.* (2019) reported specimens lacking this character in the rio São Francisco basin. Additionally, Amaral *et al.* (2015) found a small genetic distance (0.8%) between *Phalloceros anisophallos* Lucinda, 2008, from Ilha Grande and Parati (RJ) and *P. uai*, suggesting potential synonymy.

Like other members of Poeciliidae, *Phalloceros uai* is viviparous. Phylogenetic evidence suggest that the common ancestor of this family possessed a placental structure, a trait that was subsequently lost and re-acquired multiple times throughout its lineage (Pollux *et al.*, 2014). All *Phalloceros* species examined to date exhibit placentotrophy (hemotrophic viviparity) (Pollux *et al.*, 2014; Pacheco, 2019). This reproductive mode is generally associated with the absence of bright coloration, reduced male ornamentation, and limited courtship behavior (Pollux *et al.*, 2014). Zandonà *et al.* (2021) investigated maternal provisioning strategies in seven *Phalloceros* species, including the focus species of this work, and found a distinctive pattern in which embryos initially develop under lecithotrophy, losing mass due to metabolic costs (Turner, 1940), followed by a transition to matrotrophy, during which embryos gain mass.

Superfetation has been reported for a few species within *Phalloceros*, such as *P. anisophallos* (Almeida-Silva, Mazzoni, 2014), *P. harpagos* (Gorini-Pacheco *et al.*, 2018) and the populations analyzed by Arias, Reznick (2000), which may represent different species (Zandonà *et al.*, 2021). Although superfetation may confer evolutionary advantages, such as facilitating multiple paternity, it also entails potential costs, including increased risk of physical injury during copulation, reduced foraging efficiency, higher predation risk, and greater susceptibility to sexually transmitted infections in females (Pollux *et al.*, 2014).

Phalloceros uai was originally described from rio das Velhas basin, with subsequent records expanding its range within the same drainage (Alves *et al.*, 2008; Leal *et al.*, 2011). More recently, the species was reported in the rio Doce basin (rio Piracicaba and rio Santo Antônio drainages) (Vieira *et al.*, 2015; dos Santos, Britto, 2021). However, these identifications relied solely on morphological characters and encompass populations that exhibit morphological patterns distinct from those originally described. This study provides the first integrated approach, combining morphological and genetic analyses to more accurately assess these identifications and the species distribution in adjacent basins. Information on feeding habits and reproduction was also evaluated, given the scarcity of data regarding the species' natural history. This multifaceted approach aims to provide a comprehensive understanding of the species identity and ecological characteristics across different drainage systems.

MATERIAL AND METHODS

Sampling. Sampling was conducted at four sites within the Quadrilátero Ferrífero region. Located in the south-central part of Minas Gerais, this region encompasses an area of approximately 7,200 km². Its boundaries are primarily defined by the geological basement, following stratigraphic and structural features (Dorr, 1969). Site 1 - Córrego Congonhas, tributary of the right bank of rio Piracicaba (rio Doce basin), Santa Rita Durão (20°11'28.28"S 43°24'45.82"W); Site 2 - Córrego Caraça, tributary of rio Barão de Cocais ou São João, sub-drainage of rio Santa Bárbara (rio Doce basin), Brumal (19°58'53.40"S 43°27'15.27"W); Site 3 - rio Manso, tributary of the left bank of rio Paraopeba (rio São Francisco basin), Brumadinho (20°09'0.98"S 44°13'53.59"W) and Site 4 - Córrego do Jaque, tributary of the left bank of rio das Velhas (type locality, rio São Francisco basin), Lagoa Santa (19°34'28.58"S 43°55'28.64"W) (Figs. 1, **S1**).

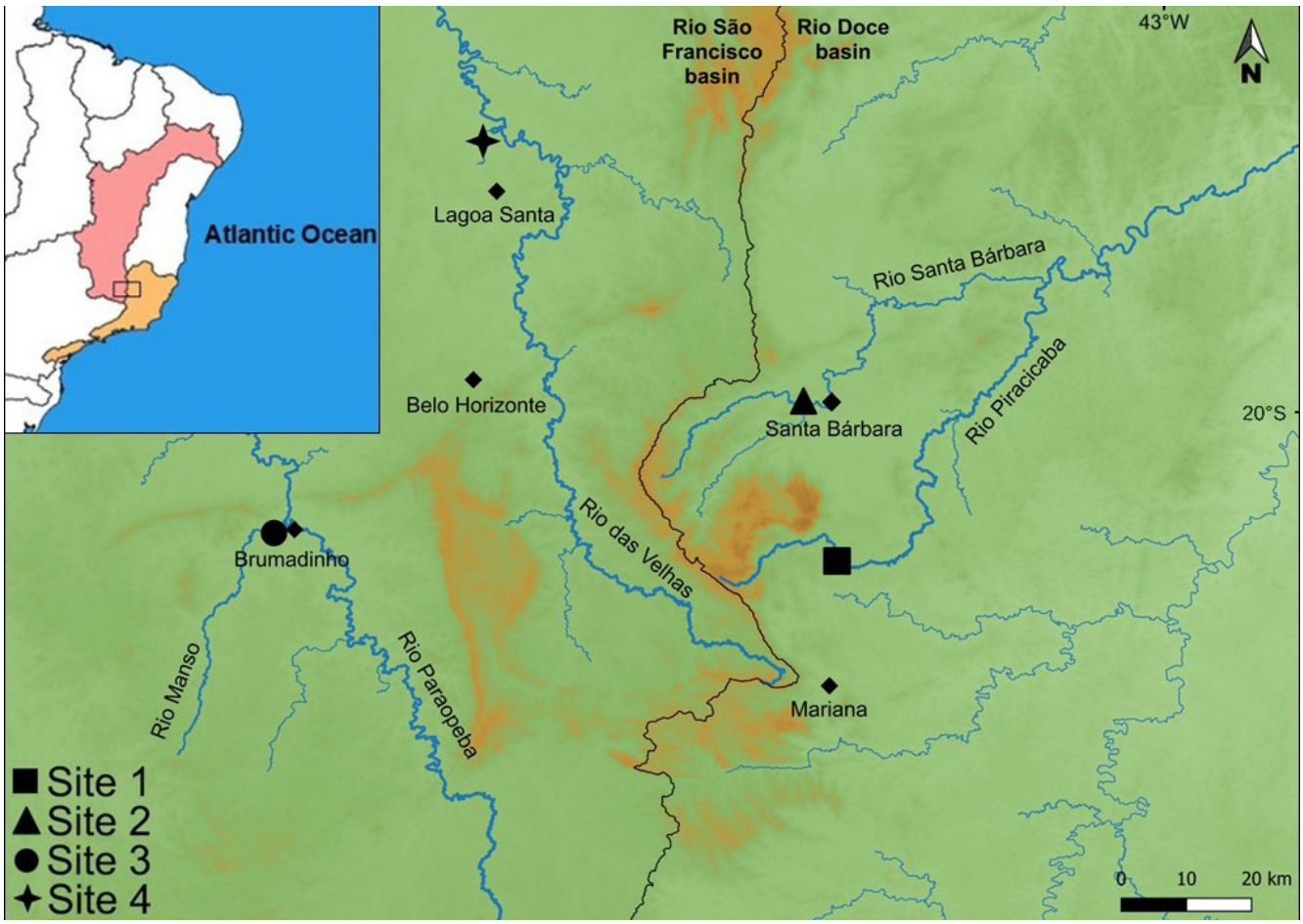

FIGURE 1 | Sampling sites of *Phalloceros* populations in the upper rio Doce and upper São Francisco drainages, MG.

Specimens were collected using half-moon sieves (2 mm mesh). All captured fish were anesthetized and euthanized using a hydroalcoholic solution containing commercial clove oil (Lucena *et al*., 2013). Half of the specimens were fixed in 90° GL ethanol for molecular analyses, while the remaining specimens were fixed in 10% formalin, then washed and transferred to 70° GL ethanol.

All specimens were identified using a Zeiss™ Stemi DV4 stereoscopic microscope and separated into adults (males and females) and juveniles' categories. Post-identification, fishes were packed in labeled plastic bags, separated by collection point, phenotype, and sex. Phenotypes were determined by counting lateral spots on the left and right sides independently and recording them as ordered pairs (*e.g.*, 1x1 indicates one spot on each side, whereas 2x3 and 3x2 represent distinct asymmetric phenotypes). This approach allows for the characterization of both symmetrical and asymmetrical patterns. Voucher specimens are deposited in the ichthyologic collection of Laboratório de Ictiologia do Departamento de Zoologia da Universidade Federal de Minas Gerais, under catalog numbers DZUFMG 159, 160, 161 and 171.

Morphological analysis. Total length, standard length, height (measured at the pelvic fin origin) and gonopodium length were measured using a 150 mm MTX® digital caliper with 0.01 mm resolution. Linear regression models were utilized to assess the relationship between gonopodium length and total length in males, while ANOVA were utilized to test if there were statistically significant differences between the mean gonopodium length of the populations. All statistical analyses were performed in default R (R Development Core Team, 2021).

For osteological examination, 19 males and three females were cleared and stained following Taylor, Van Dike (1985). The gonopodium of males was photographed using a Leica™ M205A stereoscopic microscope. Gonopodium structure nomenclature followed Rosen, Gordon (1953) as modified by Lucinda, Reis (2005).

Molecular analysis. DNA extraction, amplification, and sequencing were performed by EcoMol Consultoria© in Piracicaba, SP, Brazil. DNA sequences of 16 specimens (Tab. **S2**) were analyzed. DNA was extracted from the posterior portion of each fish using the commercial kit DNeasy Blood & Tissue (Qiagen™), following the manufacturer's instructions. Subsequently, DNA quality and concentration (ng/μL) were evaluated using the absorbance ratio at 260/280 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific™) (Tab. **S2**). All samples were normalized to a concentration of 50 ng/μL.

Polymerase chain reaction (PCR) was carried out to amplify cytochrome oxidase subunit I (COI) using the primers H7152 (5' CACCTCAGGGTGTCCGAARAAY-CARAA 3') and L6448–F1 (5' TCAACCAACCACAAAGACATTCGGCAC 3') described by Ivanova *et al*. (2007). Amplifications were performed in both directions in a 15 μL reaction volume containing 2 μL of DNA at 50 ng/μL, 7.5 μL of PCRBio Ultramix (PCRBiosystems), 1 μL of each primer diluted to 10 pmol/μL, and 3.5 μL of ultrapure water. The thermocycle profile consisted of an initial denaturation at 94°C for two min, followed by 35 cycles of annealing at 94°C for 30 sec, another 35 at 52°C for 40 sec, chain extension at 72°C for one min, and finally, the final extension occurred at 72°C for 10 min (Ivanova *et al*., 2007). PCR amplification resulted in

fragments of approximately 750 bp. Amplicons were verified on a 1.5% agarose gel by electrophoresis and purified using Agencourt AMPure XP magnetic beads (BECKMAN COULTER©). Purified products were sequenced using BigDie Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems®). Sequencing reaction products were then purified by precipitation with ethanol/EDTA/sodium acetate, according to the manufacturer's protocol, and analyzed on an ABI 3730xl DNA Analyzer.

Sequences obtained were deposited in Genbank (Tab. **S2**). These sequences plus others available for *Phalloceros* species were manually edited and trimmed in Geneious Prime 2024.0.7 (Kearse *et al*., 2012). Sequences were aligned using MUSCLE algorithm (Edgar, 2004). The final trimmed DNA sequence consisted of a 650 bp fragment. All sequences with less than 600 bp were removed, due to the low sequencing quality, compromising the final alignment. Additionally, *P. harpagos* was excluded in the analysis because, at the time, it showed significant taxonomic uncertainty, and was considered a potential species complex, as later clarified (Oliveira *et al*., 2024; Souto-Santos *et al*., 2025). Genetic distance was estimated based on Kimura 2-parameter model (K2P) using MEGA X (Kimura, 1980; Kumar *et al*., 2018). Phylogenetic reconstructions were performed under maximum likelihood (ML) statistical analysis in IQTree v. 3.0 (Wong *et al*., 2026) under the best-fit model selected (TPM2u + F + R2) by AIC, cAIC and BIC. *Poecilia vivipara* Bloch & Schneider, 1801 was used as the outgroup. For support, we performed a bootstrap test (Felsenstein, 1985) using 10.000 replicates. The resulting tree was visualized and adjusted for presentation in FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Stomach and reproductive analysis. Ninety specimens including all locations and phenotypes, spanning five size categories, were dissected (Tab. **S3**). Dissection involved a horizontal cut on the specimen's side to remove the digestive system and, in females, embryos. In females, reproductive mode and superfetation were assessed following Turner (1937) and Lodé (2012). Stomach contents were analyzed for all dissected specimens using the frequency of occurrence method (Hyslop, 1980). Food items were identified down to order level where possible.

RESULTS

A total of 1622 specimens were sampled. We reported nine different phenotypes (1x1, 1x2, 1x3, 2x1, 2x2, 2x3, 3x1, 3x2 and 3x3 lateral spots) (Figs. 2, 3). Phenotypes differing from the original description of *P. uai* were observed in all populations except those from the type locality in the rio das Velhas (Tab. 1).

Total length (TL) ranged from 8.21–48.60 mm, standard length (SL) from 6.67–42.17 mm, and height/SL from 0.13–0.52. Females generally attained larger sizes and body depth, with greater variation, attributable to sexual dimorphism and abdomen dilation when pregnant (Tab. 2).

Linear regression analysis demonstrated a positive correlation between male gonopodium length and total length ($r = 0.92$; $y = -0.39 + 0.36x$), indicating an expected increase of 0.36 mm in gonopodium length for every 1 mm increase in total length (Figs. 4, **S4**). ANOVA revealed no significant differences in the mean

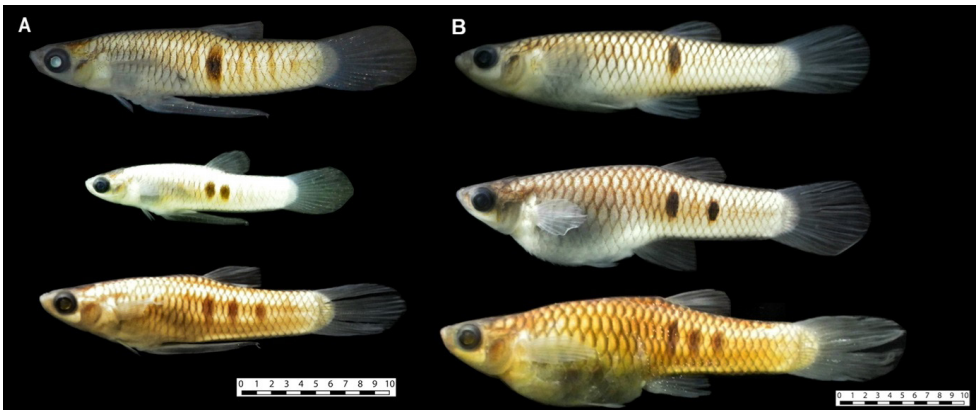

FIGURE 2 | Left lateral view of adult specimens of *Phalloceros uai*, DZUFMG 171, rio Piracicaba, Santa Rita Durão. **A.** Males. **B.** Females. Female with three spots appears to be an anomalous specimen as the urogenital papilla is visible in the left lateral view. Scale in millimeters.

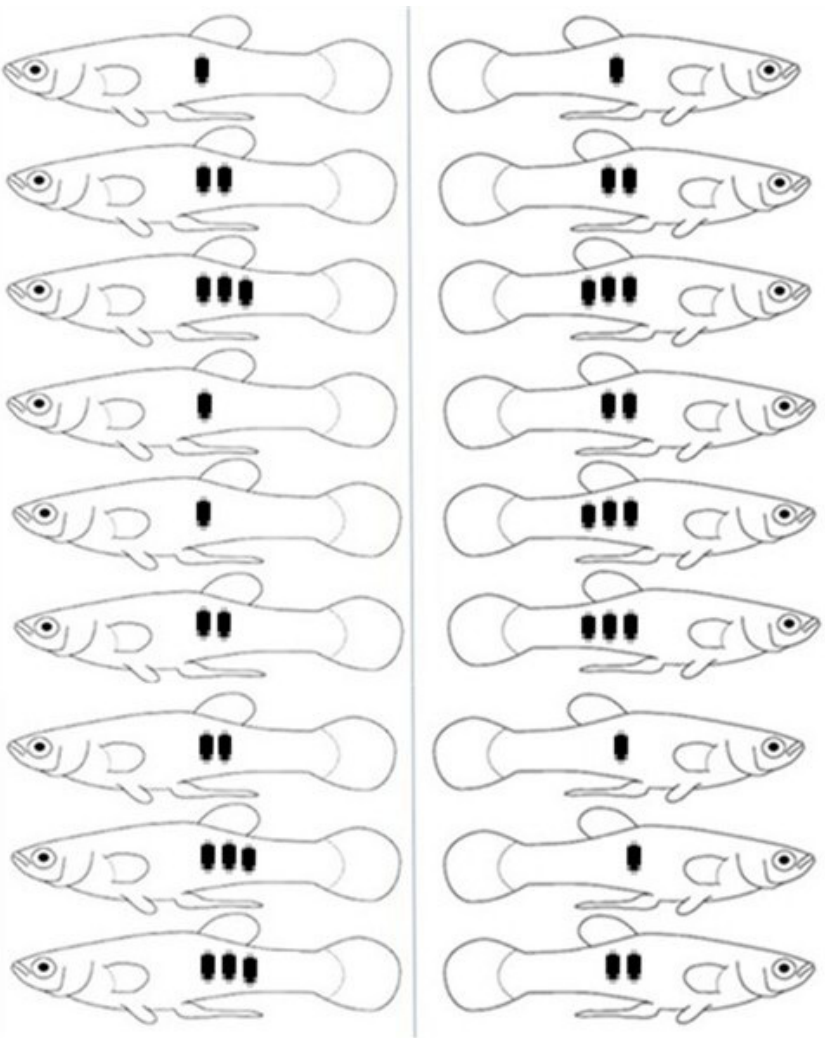

FIGURE 3 | Schematic drawing of *Phalloceros uai* phenotypes showing the variation in the number of lateral spots on each side of the same specimen.

TABLE 1 | Number of *Phalloceros* specimens collected at each locality, categorized by phenotype according to the number of lateral spots, showing total counts and relative frequencies.

Location	Number of spots		Number of specimens	Relative frequency of phenotype by location (%)
	Right	Left		
Santa Rita Durão, Mariana, MG, Brazil	1	1	582	62.85
	2	2	135	14.58
	3	3	1	0.11
	1	2	103	21.49
	2	1	96	
	1	3	1	0.11
	3	1	-	
	2	3	8	0.76
	3	2	-	
Brumal, Santa Bárbara, MG, Brazil	1	1	45	95.74
	2	2	-	-
	3	3	-	-
	1	2	1	4.26
	2	1	1	
	1	3	-	-
	3	1	-	
	2	3	-	-
	3	2	-	
Brumadinho, MG, Brazil	1	1	534	82.53
	2	2	42	6.49
	3	3	-	-
	1	2	37	10.66
	2	1	32	
	1	3	-	-
	3	1	-	
	2	3	-	0.31
	3	2	2	
Lagoa Santa, MG, Brazil	1	1	29	100
	2	2	-	-
	3	3	-	-
	1	2	-	-
	2	1	-	
	1	3	-	-
	3	1	-	
	2	3	-	-
	3	2	-	

TABLE 2 | Morphometric data for male, female and juvenile specimens of *Phalloceros uai* from the upper Doce and upper São Francisco drainages, MG. N = Number of specimens, ẋ = Mean, md = Median, DP = Standard deviation.

Data (mm)	Sex	N	Range	ẋ	md	DP
Total length (TL)	Males	444	15.86 - 30.70	22.83	21.69	3.43
	Females	976	15.67 - 48.60	29.27	29.58	6.99
	Juveniles	179	8.21 - 17.15	13.94	14.43	1.89
Standard length (SL)	Males	453	12.86 - 24.58	18.29	17.35	2.81
	Females	985	12.47 - 42.17	23.71	23.93	5.83
	Juveniles	184	6.67 - 13.91	11.24	11.51	1.46
Height/SL	Males	453	0.14 - 0.29	0.19	0.19	0.02
	Females	977	0.14 - 0.52	0.23	0.22	0.03
	Juveniles	183	0.13 - 0.24	0.17	0.17	0.02
Gonopodium length	Males	453	2.98 - 11.18	7.81	7.34	1.38

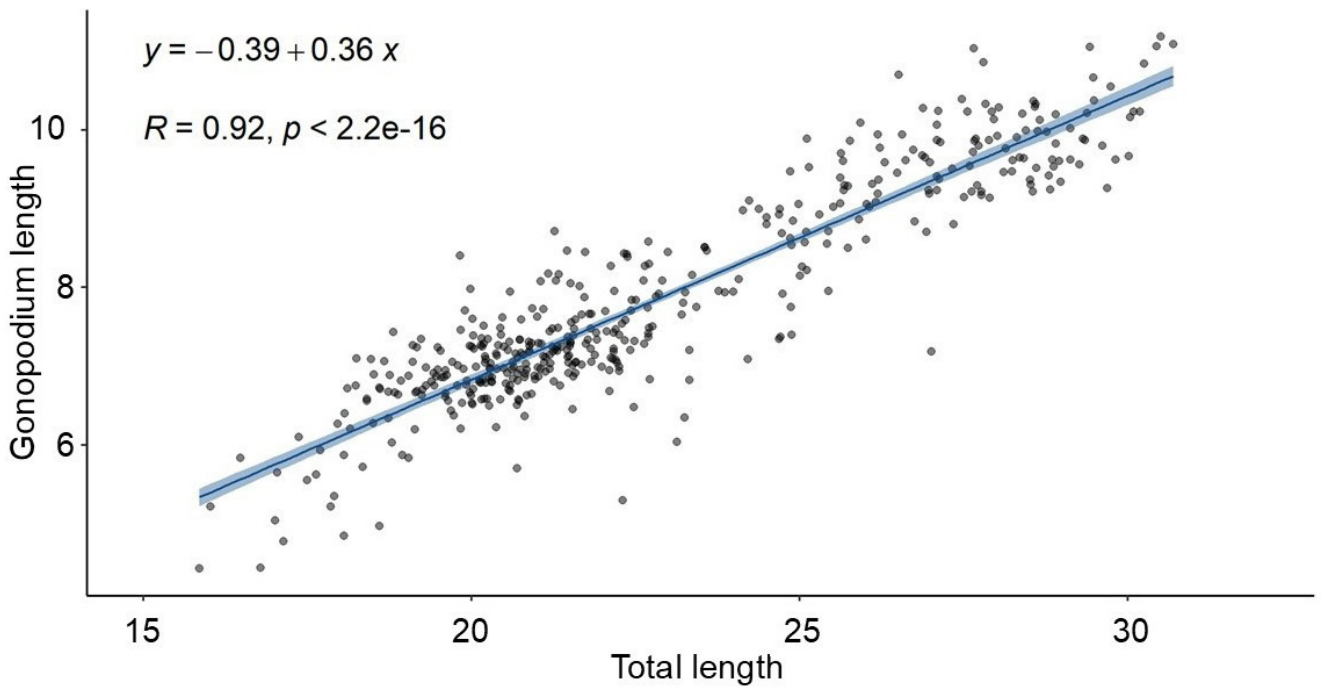

FIGURE 4 | Linear regression of gonopodium length (mm) on total length (mm) in males specimens of *Phalloceros uai* from four locations: rio Piracicaba, rio Santa Barbara, rio Paraopeba and rio das Velhas.

gonopodium length among different populations (F (3, 437) = 0.2407, p = 0.8679). No structural differences were found in the gonopodium among varying morphotypes and populations (Figs. 5, **S5**). All specimens possessed a left hook on the gonopodium, an asymmetrical gonopodial appendage (right wider than the left), a canoe-shaped ray 5, a serrated structure on ray 4p, and radius 3 with spines and extensions, consistent with original description.

Mean genetic distance based on the K2P model were less than 1% among *P. uai* sequences, including those from specimens collected at the type locality, and exceeded 1% between *P. uai* and its congeners (Tab. **S6**), indicating that all analyzed populations belong to *P. uai*. Our maximum–likelihood phylogenetic analyses based on COI recovered all *P. uai* sequences as a monophyletic group, further supporting the conclusion that all analyzed populations belong to *P. uai* (Fig. **S7**).

Approximately half (49%) of the stomachs dissected contained food items, while 51% were empty. Among stomachs with food, the majority contained aquatic invertebrates, predominantly Diptera larvae (18.82%), and debris such as stones and sand grains (28.23%) (Tab. 3). Diet did not differ between females and males, nor among populations in different drainages (Tabs. 3, **S8**).

A total of 2028 embryos were found in 55 females (Tab. 4). Embryos were typically grouped and encased in a thin membrane (Fig. 6). In embryos nearing the final stages of development, eyes, yolk and fins were discernible. Females across all populations carried embryos at different stages of development, confirming superfetation. Embryos in the final development stages exhibited the lateral spots characteristic of the species, with offspring presenting solely three phenotypes (1x1, 1x2 and 2x2). Notably, embryos displayed different phenotypes irrespective of the mother's phenotypes (Fig. 7).

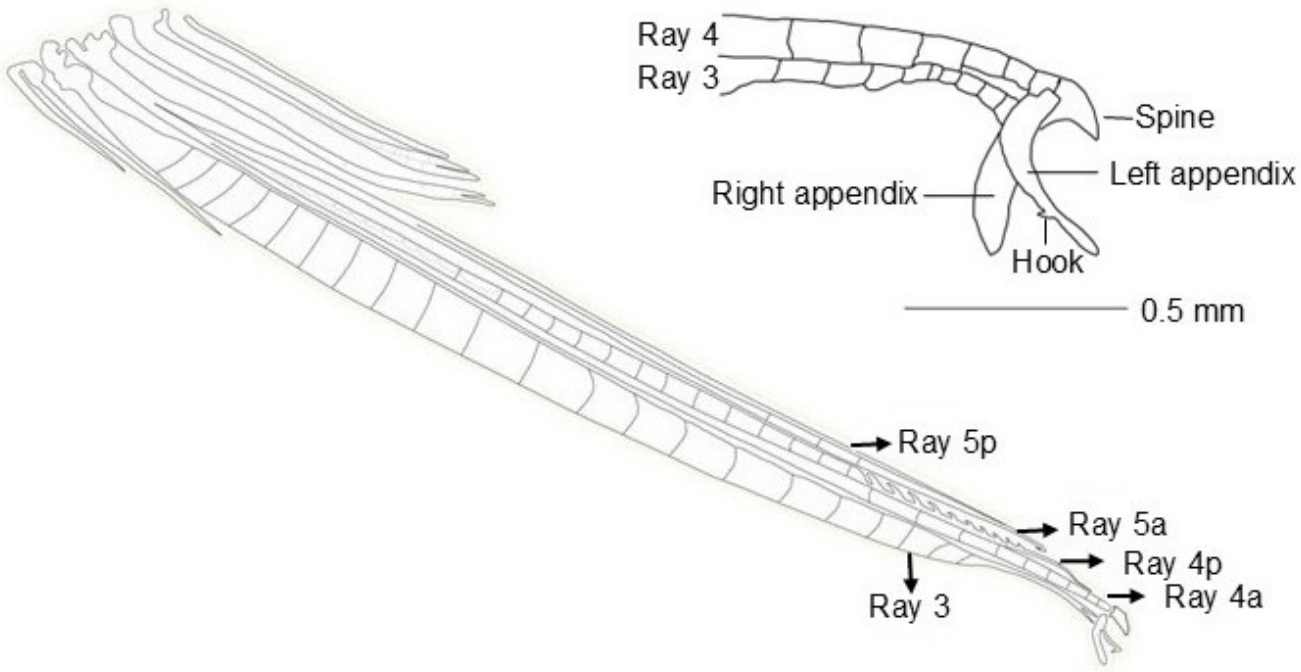

FIGURE 5 | Schematic drawing of the gonopodium of *Phalloceros uai* in lateral view illustrating rays 5, 4 and 3, with the anterior region on the left. Ray 5 canoe-shaped, ray 4p exhibits a serrated structure, and ray 3 possesses spines and extensions.

TABLE 3 | Total number and frequency of stomach contents separated by food item and sex. N = Number of stomachs found with food items.

Food item		Males (n = 35)		Females (n = 55)		Total (n = 90)	
		N	Frequency (%)	N	Frequency (%)	N	Frequency (%)
Aquatic invertebrates	Coleoptera (Adult)	-	-	1	1.82	1	1.18
	Coleoptera (Immature)	-	-	2	3.64	2	2.35
	Diptera (Adult)	1	3.33	2	3.64	3	3.53
	Diptera (Immature)	6	20.00	10	18.18	16	18.82
	Ephemeroptera (Immature)	1	3.33	-	-	1	1.18
	Odonata (Immature)	-	-	1	1.82	1	1.18
	Trichoptera (Immature)	1	3.33	-	-	1	1.18
	Unidentified adult insect	2	6.66	2	3.64	4	4.70
	Unidentified animal material	-	-	6	10.91	6	7.06
Plant material		1	3.33	2	3.64	3	3.53
Debris	Sand/Stone grains	4	13.33	20	36.36	24	28.23
Unidentified material		2	6.66	2	3.64	4	4.70
Empty		16	53.33	27	49.09	43	50.59

TABLE 4 | Number of *Phalloceros uai* embryos by locality.

Area	Range	Average	Median	Standard deviation	Number of embryos
Santa Rita Durão, Mariana, MG, Brazil	4–106	44	28	32.1	1105
Brumal, Santa Bárbara, MG, Brazil	0–34	18	22	13.5	90
Brumadinho, MG, Brazil	0–73	37	34	20.5	744
Lagoa Santa, MG, Brazil	0–46	18	11	18.2	89

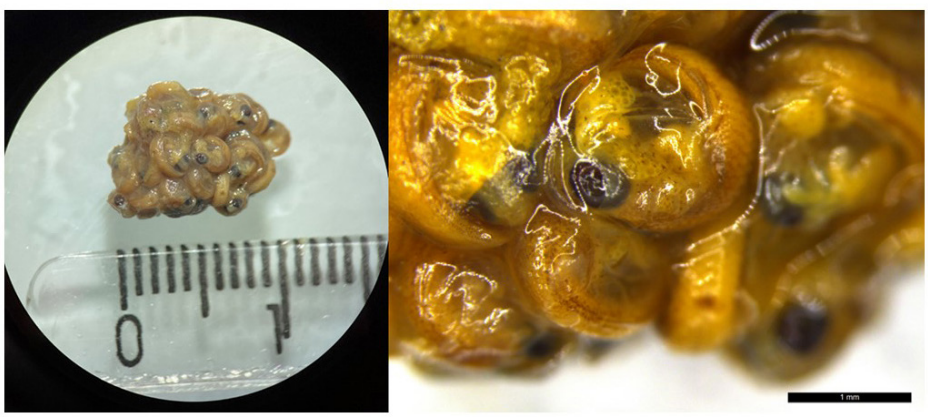

FIGURE 6 | Set of embryos taken from a *Phalloceros uai* female, DZUFMG 159, rio Manso, Brumadinho, 35 mm SL, surrounded by a thin membrane. Scale bar = 1 mm.

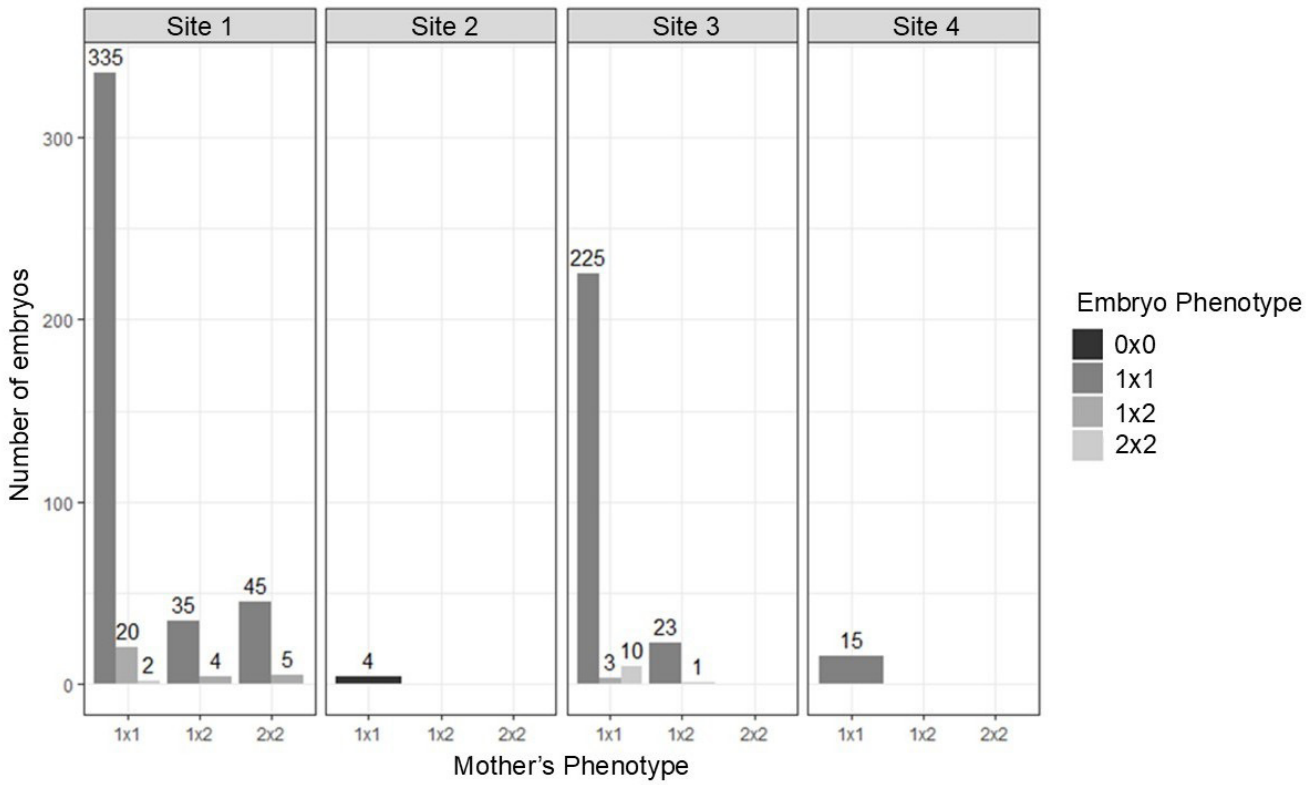

FIGURE 7 | Distribution of late-stage embryos by phenotype category in 55 *Phalloceros uai* females, separated by maternal phenotype and locality. Bars indicate the number of embryos in each phenotype category within each mother's brood.

DISCUSSION

Phalloceros uai was originally described based on specimens from córrego do Jaque, a tributary of the left bank of rio das Velhas (Lucinda, 2008). Subsequent studies expanded its documented distribution within the rio das Velhas basin to include the rio Cipó (Alves *et al*., 2008), and the main channel of the rio das Velhas (Leal *et al*., 2011). Our surveys further extend its known distribution to the rio Paraopeba basin and confirm its identification in the rio Piracicaba drainage (rio Doce basin), as initially reported by Vieira *et al*. (2015). Additionally, we consider the occurrence in the rio Santo Antônio drainages (rio Doce basin) as valid, as indicated by dos Santos, Britto (2021). Consequently, current data indicate that *P. uai* is widely distributed and common throughout the Quadrilátero Ferrífero region.

In all analyzed populations most specimens were identified as *Phalloceros uai* based on the diagnostic characteristics of the gonopodium and urogenital papilla described by Lucinda (2008), even though some individuals exhibited phenotypic variation relative to the original description in the number and arrangement of lateral spots, which has not been previously reported. Thomaz *et al.* (2019) reported specimens without lateral spots in the rio das Velhas. In contrast, all individuals in our study possessed lateral spots, and this discrepancy warrants further investigation.

Although the phenotype described by Lucinda (2008) was the most abundant in our dataset, individuals bearing asymmetric spot patterns were also common, as well as those with two or three lateral spots. Specimens with three lateral spots were found exclusively in the upper Piracicaba population, whereas only individuals exhibiting the original diagnostic phenotype were observed at the type locality. The absence of variation in lateral spot number at the type locality may indicate limited intraspecific diversity within that population. This observation could be associated with evolutionary processes such as natural selection and genetic drift, or with local environmental heterogeneity. In this context, experimental evidence from *Poecilia wingei* shows that variation in thyroid hormone (TH) levels can directly affect the development of color patterns (Prazdnikov, 2021), suggesting that physiological regulation may contribute to the differences observed among populations. Alternatively, the apparent uniformity at the type locality may reflect the limited number of specimens analyzed, which restricts our ability to fully assess the phenotypic diversity of that population.

In poecilids, coloration patterns are influenced by predation (Endler, 1978), female mate choice (Endler 1984; Endler, 1987; Jönck, 2004), environmental factors such as background color (Jönck, 2004) and luminosity (Endler, 1987), and intrinsic factors including stress, age, genetics (Houde, 1992), and thyroid hormones (Prazdnikov, 2021). Taken together, these factors suggest that, these patterns vary not only among species and genera, but also among populations of the same species, which may account for the variation observed in this study.

Most studies on color patterns in *Phalloceros* were conducted prior to 2008, when the genus was considered monotypic. Although these studies provide valuable insights into the genus, caution is warranted when using their data for interspecific comparisons. Endler (1982, 1984, 1987) highlighted three key aspects of *P. caudimaculatus* color patterns: (1) cryptic coloration, often matching sand or sediment, to evade predators; (2) a decrease in spot size and number with increasing local predation pressure (negative selection); and (3) female preference for more conspicuous males (positive selection). We did not find any visible differences in background among sampling sites, and our data supports the hypothesis that fishes with fewer spots may be less attractive prey, as they are more frequent across all populations. Other color patterns such as conspicuous or well-developed vertical bars (Thayer, 1909; Cott, 1940; Endler, 1978) and reddish catecholamines spots (Jönck, 2004), were not recorded in either live or fixed specimens.

Thyroid hormones (THs) are important regulators of fish ontogeny, linking environmental changes to phenotypic expression, and are associated with the speed of appearance, intensity, and asymmetry of color patterns. Experimental studies show that high TH levels favor asymmetry in fish (Prazdnikov, 2021), which might explain the different phenotypes and asymmetry observed in *P. uai*. Asymmetry could provide an advantage during guppy courtship, as males can present both body sides to the female, showcasing different phenotypes and expanding mate choice possibilities (Řežucha, Reichard, 2015), thus constituting an evolutionary advantage for phenotypically asymmetrical animals. However, if asymmetry conferred such an advantage, a higher frequency of asymmetrical individuals would be expected, a condition not supported by our data.

Poeciliidae exhibit a range of reproductive behaviors, including sneaky mating or gonopodial thrusting (coercion), courtship (cutting), nibbling (oral or nasal contact of

the male in the female urogenital opening), postcopulatory jerking (post-copulatory masturbation) and forced reproduction. These behaviors imply competition, where females tend to maximize partner quality to avoid energy loss, while males are often more promiscuous, aiming to maximize the number of partners (Baerends *et al*., 1955; Farr, 1977, 1980, 1984; Endler, 1984; Pollux *et al*., 2014; Pacheco, 2019). Coercion involves males inserting the gonopodium directly into the female's urogenital papilla without her permission or perception (Rosen, Tucker, 1961). In populations where this act is common, smaller males with longer genitalia are positive selected, limiting females mate choice (Pollux *et al*., 2014). We hypothesize that *P. uai* may engage in coercive acts, thereby facilitating the persistence of different phenotypes within populations.

All analyzed populations of *Phalloceros* showed genetic distances of less than 1% relative to other *Phalloceros uai* sequences available in GenBank and to the population from the type locality, supporting their conspecificity (Hebert *et al*., 2003; Lara *et al*., 2010; Pereira *et al*., 2011, 2013). Furthermore, all sequences clustered into a single monophyletic group with 92 bootstrap support. Amaral *et al.* (2015) reported a genetic divergence of 0.8% between *P. uai* and *P. anisophallos*, suggesting a possible synonymy and the need for taxonomic reassessment. In contrast, our analysis recovered a genetic divergence greater than 1% between these two species, and each was placed in a distinct, well-supported monophyletic clade. Additionally, *P. anisophallos* can be morphologically distinguished from *P. uai* by autapomorphies, including a hook on the middle portion of the left side of the gonopodial appendage (approximately one-quarter to one-third from the distal end) and an elongated medial spot forming a vertical bar (Lucinda, 2008).

Based on our dietary analysis, *Phalloceros uai* is considered a carnivorous species, primarily feeding on aquatic invertebrates, a trophic habit consistent with observations reported for other species of *Phalloceros* (Aranha, Caramaschi, 1999). Conversely, other *Phalloceros* species have been described as omnivorous (Casatti, 2002; Mazzoni *et al*., 2010), detritivorous (Wolff *et al*., 2022), and even strictly herbivorous in some populations (Costa, 1987; Teixeira, 1989).

Based on our reproductive analyses, females from all populations of *P. uai* carried multiple broods of embryos at different developmental stages, confirming the occurrence of superfetation. As suggested by Pollux *et al.* (2014), species exhibiting superfetation typically lack conspicuous coloration and ornamentation, possess simple sexual traits, and show little or no courtship behavior. *Phalloceros uai* shares the first two characteristics, and although courtship behavior has not yet been studied in this species, it is likely consistent with this pattern.

Our integrative analyses combining distributional data, morphology, coloration patterns, diet, reproduction, and molecular evidence support the recognition of *Phalloceros uai* as a widely distributed and common species in the Quadrilátero Ferrífero region. Despite exhibiting phenotypic variation in lateral spot number and symmetry among populations, most examined specimens share the diagnostic gonopodial and urogenital characters of *P. uai*, and genetic distances below 1% coupled with strong monophyly corroborate their conspecificity. Variation in coloration patterns likely reflects local environmental conditions, intrinsic physiological factors, and selective pressures rather than taxonomic differentiation. Further studies are necessary to ascertain the origin of spot pattern variation in *P. uai* and its role in the allopatric divergence observed among different populations.

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AUTHORS' CONTRIBUTION

Eric Bruschi Haddad: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing-original draft, Writing-review and editing.

Tatiana Moura Barroca: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Writing-original draft, Writing-review and editing.

Fábio Vieira: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing-original draft, Writing-review and editing.

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ETHICAL STATEMENT

The authors declare no conflicts of interest. The specimens were obtained during studies for environmental licensing issued by Instituto Estadual de Florestas (IEF–MG).

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and the accompanying supplementary material.

AI STATEMENT

Portions of the manuscript text were reviewed using ChatGPT (OpenAI) solely to improve grammar, clarity, and wording. No scientific content, data analysis, interpretation, or conclusions were generated by artificial intelligence, and all final text was written, reviewed, and approved by the authors.

COMPETING INTERESTS

The authors declare no competing interests.

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